

Particulate adjuvants for use in cancer vaccination

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Declaration of authorship page & Permissions

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Ross Ward

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Abstract

It is estimated that 1 in 2 people will develop cancer in their lifetime, highlighting the urgent need for improved preventative and therapeutic strategies. Cancer immunotherapy has emerged as a key pillar of cancer treatment, and in this context, both prophylactic and therapeutic cancer vaccines have shown significant promise as standalone therapies or in combinatorial approaches with checkpoint blockade therapy. However, the development of such vaccines has been hindered by a lack of adjuvants that effectively drive protective cell mediated immunity (CMI), coupled to poorly defined adjuvant mechanisms of action (MOA), arising from empirical adjuvant development instead of rational design. The size of particulate adjuvants has emerged as a key parameter influencing adjuvanticity, and polymeric nanoparticles (NP) of approximately 50nm in diameter are promising adjuvants capable of eliciting CMI. However, there are outstanding questions relating to nanoparticle adjuvant MOA and the functionality of adjuvant induced T cell responses.

Research in the Lavelle Lab has identified highly deacetylated chitin-derived polymers as potent CMI driving adjuvants, with adjuvanticity associated with potent activation of STING and IFNAR signalling. As a result, both 50nm NPs and highly deacetylated chitin-derived polymers are promising adjuvant candidates for use in cancer vaccine approaches and this project aims to characterise their efficacy and further their rational design through providing insights into their MOA.

This work has identified components of the canonical and non-canonical inflammasomes, particularly caspase 11 as essential to 50nm NP adjuvanticity, providing crucial mechanistic insights into how size is instructive in mediating particle induced CMI. Crucially, intramuscular vaccination with 50nm NP was shown to promote protective anti-tumour immunity in prophylactic and therapeutic tumour challenge settings, largely dependent on the induction of CD8⁺ T cells. In addition, this work has characterised the C100 (100% deacetylated chitin) polymer as a potent adjuvant for use in intratumoral vaccination approaches. This anti-tumour therapeutic efficacy of C100 was dependent on STING and IFNAR signalling, with CD8⁺ T cells characterised as cellular mediators of protection. Vaccination with either C100 or 50nm polystyrene NP adjuvants was shown to synergise with PD-1 checkpoint blockade in eliciting anti-tumour immunity, highlighting the possibility of future combinatorial therapeutic strategies. Altogether this project has furthered the rational design of two vaccine adjuvants with potential application in cancer vaccine approaches.

Publications

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*=Equal contribution

List of Abbreviations

ACD	Accidental cell death
ADCC	Antibody dependant cellular cytotoxicity
ADCP	Antibody dependant cellular phagocytosis
AHHS	Amorphous aluminium hydroxyphosphate sulfate
ANXA1	Annexin A1
APC	Antigen presenting cell
ASC	Apoptosis associated spec-like protein containing a CARD
ATP	Adenosine triphosphate
BATF3	Basic leucine zipper ATF-like transcription factor 3
BCC	Basal cell carcinoma
BCG	Bacillus Calmette-Guérin
BMDC	Bone marrow derived dendritic cell
BRCA1/2	Breast cancer gene 1/ 2
CALR	Calreticulin
CAR	Chimeric antigen receptor
CARD	Caspase-activation and recruitment domain
CCL	C-C motif ligand
CD	Cluster of differentiation
CDCC	Complement dependant cellular cytotoxicity
CDN	Cyclic dinucleotide
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CLR	C-type lectin receptor
CMI	Cell mediated immunity
CpG ODN	Cytosine-phosphor-Guanine oligodeoxynucleotide
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
DAMP	Danger associated molecular pattern
DC/cDC	Dendritic cell/ Classical dendritic cell
DD	Degree of deacetylation
DDA	Dimethyldioctadecylammonium bromide

DMF	Dimethyl fumarate
DMSO	Dimethyl sulfoxide
DMXAA	5,6-Dimethylxanthenone-4-acetic acid
DNA	Deoxyribonucleic acid
DOPS	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-L-serine
EBV	Epstein-Barr virus
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal-transition
EOMES	Eomesodermin
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FLT3L	FMS-like tyrosine kinase 3 ligand
FSC-A	Forward scatter area
GM-CSF	Granulocyte macrophage – colony stimulating factor
GSDMD	Gasdermin D
GSDME	Gasdermin E
GSK	GlaxoSmithKline
GTP	Guanosine triphosphate
GZMA/B	Granzyme A/B
HAMPs	Homeostasis altering molecular processes
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HHV8	Human herpesvirus-8
HMGB1	High mobility group box 1
HPV	Human papillomavirus
HSV	Herpes simplex virus
HTLV1	Human T-cell leukaemia virus 1
IARC	International Agency for Cancer Research
IAVI	International AIDS Vaccine Initiative
ICANS	Immune effector cell-associated neurotoxicity syndrome
ICB	Immune checkpoint blockade

ICD	Immunogenic cell death
IFN	Interferon
IFNAR	Interferon alpha and beta receptor
IgG	Immunoglobulin
IKK	IkappaB kinase
IL	Interleukin
ILC	Innate lymphoid cell
IRF	Interferon-regulatory factor
ISGs	Interferon stimulated genes
ISV	<i>In situ</i> vaccination
KIR	Killer-cell immunoglobulin-like receptor
KSHV	Kaposi's sarcoma-associated herpesvirus
LAG3	Lymphocyte activation gene-3
LDH	Lactate dehydrogenase
LN	Lymph node
LNP	Lipid nanoparticle
LPS	Lipopolysaccharide
M.tb	<i>Mycobacterium tuberculosis</i>
mAB	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MDA5	Melanoma differentiation-associated protein 5
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain like pseudokinase
MMPs	Matrix metalloproteinases
MOA	Mechanism of action
MoDCs	Monocyte derived dendritic cells
MPL	Monophosphoryl lipid A
NBD	Nacht binding domain
NFkB	Nuclear factor kappa light chain enhancer of activated B cells
NHP	Non-human primates
NIH	National Institute of Health
NINJ1	Ninjurin 1

NK	Natural killer cell
NLRP3	Nucleotide-binding domain (NOD)-like receptor protein 3
NMIBC	Non-muscle invasive bladder cancer
NP	Nanoparticles
NSA	Necrosulfonamide
NSCLC	Non-small cell lung cancer
OVA	Ovalbumin
oxPAPCs	Oxidised phospholipids
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PDGF	Platelet derived growth factor
PhS	Phosphatidylserine
PLGA	Poly-lactic co-glycolic acid
PRF	Perforin
PRR	Pattern recognition receptor
PS	Polystyrene
PYD	Pyrin domain
RCC	Renal cell carcinoma
RCD	Regulated cell death
RIPK1/3	Receptor-interacting serine/threonine protein kinase 1/3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SASP	Senescence associated secretory phenotype
SD	Standard deviation
SEM	Standard error of the mean
SLPs	Synthetic long peptides
SSC-A	Side scatter area
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAA	Tumour associated antigen
TAM	Tumour associated macrophage

TBK1	TANK-binding kinase 1
TCR	T cell receptor
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
Tfh	T follicular helper cell
TGF- β	Transforming growth factor beta
Th	T helper cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIL	Tumour infiltrating lymphocyte
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TSA	Tumour specific antigen
T-Vec	Talimogene Laherparepvec
VEGF	Vascular-endothelial growth factor
VISTA	V-domain Ig suppressor of T cell activation
VLP	Virus-like particle

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Chapter One - Introduction

1.1 Cancer

Cancer is the name given to a group of diseases arising from the loss of control of cellular proliferation, differentiation, and death, leading to uncontrollable cellular growth with resultant disruption to tissue homeostasis. There are 8 distinctive hallmarks of cancer in cells: constitutive proliferative signalling, evasion of growth suppression, resistance to cell death pathways, acquisition of stem-cell like replicative immortality, angiogenesis/vascularisation, tissue invasion and ability to metastasise, dysregulated metabolism, and evasion of immunosurveillance¹ with two more hallmarks proposed: high phenotypic plasticity and induction of cellular senescence (in particular activation of senescence-associated secretory phenotype (SASP))². The acquisition of these hallmarks is facilitated by the presence of DNA damage and dysregulated repair mechanisms which gives rise to random mutations³ in conjunction with epigenetic modifications². This results in fitness selection of tumour cell clones. The presence of chronic inflammation and the regulatory and tissue remodelling capabilities of tumour recruited immune cells are also associated with the development of these hallmarks of cancer, creating a favourable microenvironment niche for establishment and further cancer progression^{4,5}.

Cancer incidences are increasing globally, it is now estimated that 1 in 2 people will develop cancer in their lifetime⁶, and in 2020, cancer accounted for at least 10 million deaths worldwide⁷. Aside from impacts on mortality, the economic costs of cancer are substantial, in 2017 it was estimated that in the EU, €57.3 billion was spent on healthcare associated with cancer treatment, and €58.5 billion was lost due to loss of productivity associated to cancer illnesses⁸. Therefore, it is essential to dissect the factors contributing to this increased incidence rate globally to develop preventative/combative strategies. Hereditary genetics are estimated to play a major role in 5-10% of cancers⁹, through the passing down of oncogenic genes or oncogenes e.g. BRCA1 and BRCA2. While increasing life expectancy contributes to the rising incidence rates¹⁰, both environmental and lifestyle factors are key determinants in the risk of developing cancer. It was estimated that in the US in 2014, 42% of all cancer incidences were associated with preventable risk factors¹¹, this is in line with data from the UK indicating that preventable risk factors contributed to 38% of cancers incidences in 2015¹². Tobacco smoking and obesity are the leading contributing risk factors, with alcohol intake, certain bacterial and viral infections, low fruit/vegetable intake, and UV light exposure constituting other risk factors¹¹⁻¹³.

However, there has been a notable improvement in the prognosis of cancer patients, reflected in significant increases in 5-year relative survival rates^{6,14} with a caveat being these improvements are largely restricted to high/middle income countries and not observed in low income-countries where healthcare systems are often ill-equipped for effective detection and treatment of cancer¹⁵. Improvements in the prognoses and overall survival rates of cancer can be largely attributed to improved diagnostics, leading to earlier detection of cancer in patients and the development and implementation of novel cancer therapeutic regimens. For example, Irish data indicates a significant increase in survival rates for patients diagnosed with breast, cervical and colorectal cancer, associated with earlier detection associated with screening programmes¹⁶. The advent of immunotherapy, which will be discussed in some detail below, has had stellar impacts on certain cohorts of patients, drastically improving patient outcomes^{17,18}. This dynamic field of study is paving the way for the development of novel immunotherapeutic treatment regimens for many different cancers.

1.1.1 Anti-tumour innate immunity

Oncogenic mutations and subsequent pre-cancerous/ cancerous cells commonly arise in our bodies, but these cells mostly die through intrinsic pathways or are killed by immune cells via immunosurveillance. This process of tumour cell detection and elimination can be achieved by components of the innate and adaptive arms of the immune system.

The innate immune response can play a crucial role in both tumour control and tumour progression. Innate immune cells can contribute to anti-tumour immunity through direct tumour cell killing, tumour antigen sampling with subsequent presentation to prime adaptive immune responses, and through the production of chemokines and cytokines that potentiate anti-tumour responses and modulate tumour cells themselves.

NK cells

NK cells, a subset of innate lymphoid cells (ILCs), are the best characterised innate immune cells in mediating direct tumour cell killing and innate tumour immunosurveillance, exerting cytotoxic effects mainly via the secretion of lytic granules containing perforin and granzyme proteins¹⁹, and via the engagement of cell death receptors on the tumour cell surface such as FAS and TRAIL-R1/2²⁰. NK cells express a range of activatory and inhibitory cell surface receptor molecules e.g., killer cell immunoglobulin receptors (KIRs), NKG2D and NKp46. These receptors detect MHC Class I and MHC like proteins alongside cell surface molecules expressed during perturbations in cellular homeostasis^{21,22}. NK cells also express co-inhibitory ligands and receptors including TIGIT and PD-1²³. NK cell activation is dependent on the complex balance of activatory and inhibitory signalling pathways downstream of such receptors and cytokine signalling, constituting a 'rheostat' model of activation. NK cells and CD8⁺ T cells often act in tandem to eliminate tumour cells, with evasion of detection by CD8⁺ T cells often rendering the tumour sensitive to NK mediated detection²⁴ and vice versa. Activation of the cGAS-STING pathway and subsequent arming of the type I IFN signalling pathway in the TME has emerged as an important inducer of NK cell activity in the tumour^{24–27}.

In addition, NK cells can kill tumour cells via antibody dependant cellular cytotoxicity (ADCC), whereby FcγRs such as CD16 and CD32 are engaged via IgG antibodies bound to tumour cell surface antigens, with important implications for monoclonal antibody (mAB) cancer therapies^{22,28} (Fig. 1.1). In the tumour microenvironment (TME), NK cells are a prominent source of anti-tumourigenic cytokines including IFNγ and TNFα, and chemokines which mediate

intratumoral homing of immune cells including CCL5 and XCR1²⁹ (Fig. 1.1). The importance of NK cells in the detection and elimination of cancer cells is illustrated in spontaneous tumour mouse models where NK cell depletion leads to an increase in tumour burden³⁰ and there is some evidence that patients with NK cell deficiencies have an increased susceptibility to certain cancers, especially those with viral origins³¹. Similarly, there is clinical evidence demonstrating that patients with tumours displaying a higher infiltration of NK cells have a better prognosis³⁰ and better response to immunotherapeutic interventions³².

Dendritic Cells (DCs)

Another key role of the anti-tumour innate immune response is the activation of the adaptive arm of immunity. This can be achieved through sampling of antigens by antigen presenting cells (APCs), of which dendritic cells (DCs) and macrophages are the most prominent. Tumour antigens are sampled, and adaptive immune cells can either be activated locally, or distally in the draining lymph nodes. DCs can be broadly classified into distinct subsets: plasmacytoid DCs, cDC1s, cDC2s³³ and a novel proposed subset mregDCs ('mature DCs enriched in immunoregulatory molecules') which originate from cDC1 and cDC2s³⁴. While cDC2s can cross-prime both CD4⁺ and CD8⁺ T cells, cDC1s, characterised via expression of BATF3 and IRF8 transcription factors³⁵, are functionally adapted to the cross-priming of CD8⁺ T cells³⁶ and induction of Th1 responses. Migratory DCs can transfer tumour antigen to lymph node resident DC1 via synaptic transfer of antigen containing vesicles³⁷. CD103⁺ cDC1 (analogous to the human CD141⁺ cDC1 population) are proficient at tumour antigen sampling and trafficking to the tumour draining lymph nodes (tdLN)^{38,39}, and their relative abundance intratumorally has prognostic value for responses to checkpoint immunotherapeutic strategies^{39,40}. Indeed, NK cells and cDC1s have a close relationship in terms of tumour recruitment and infiltration, as NK cells have been shown to mediate the recruitment of cDC1s via the secretion of CCL5 and XCL1²⁹ and can further encourage DC tumour infiltration and cDC1 polarisation via secretion of FLT3L⁴¹ (Fig. 1.1 and 1.8). The presence and co-localisation of these cell types intratumorally are associated with improved patient prognoses and response to immunotherapy^{29,41}. Indeed, depletion of DCs before systemic α -PD-L1 therapy abrogates therapeutic efficacy⁴². Clinically, systemic administration of FLT3L has been shown to improve response to a DC targeted cancer vaccine, with efficacy attributed to the expansion of cDC1 subsets⁴³. Impaired recruitment of cDC1s is noted in tumours displaying active β -catenin signalling, which results in impaired priming of anti-tumour T cell responses and lower tumour infiltrating T cells⁴⁴.

In addition to their role in antigen presentation, DCs provide a crucial source of cytokines and chemokines in the TME and draining lymph nodes that promote and potentiate anti-tumour immunity, particularly important in the early stages of tumour immune encounter. DCs can be an early source of Type I IFNs, IL-12, IL-2 and chemokines such as CXCL10 and CXCL9 which promote the recruitment of CD8⁺ T cells and NK cells into the tumour^{45,46} (Fig.1.1 and 1.9), promoting anti-tumour immune responses.

However, despite their central role in initiating and sustaining effective anti-tumour immune responses, tumour cells can impair the activity of tumour infiltrating DCs and convert them to a more tolerogenic phenotype previously mentioned – mregDCs. This shift in phenotype has been noted in cDCs following uptake of tumour antigens associated with apoptotic tumour cells³⁴. These cDCs upregulate the expression of migration, maturation and co-inhibitory receptors while downregulating expression of genes associated with TLR signalling shifting towards a more immune regulatory phenotype³⁴. mregDCs are enriched in tumours and tumour draining lymph nodes and these tumour infiltrating subsets are generally conserved between patients and different tumour types⁴⁷.

Macrophages

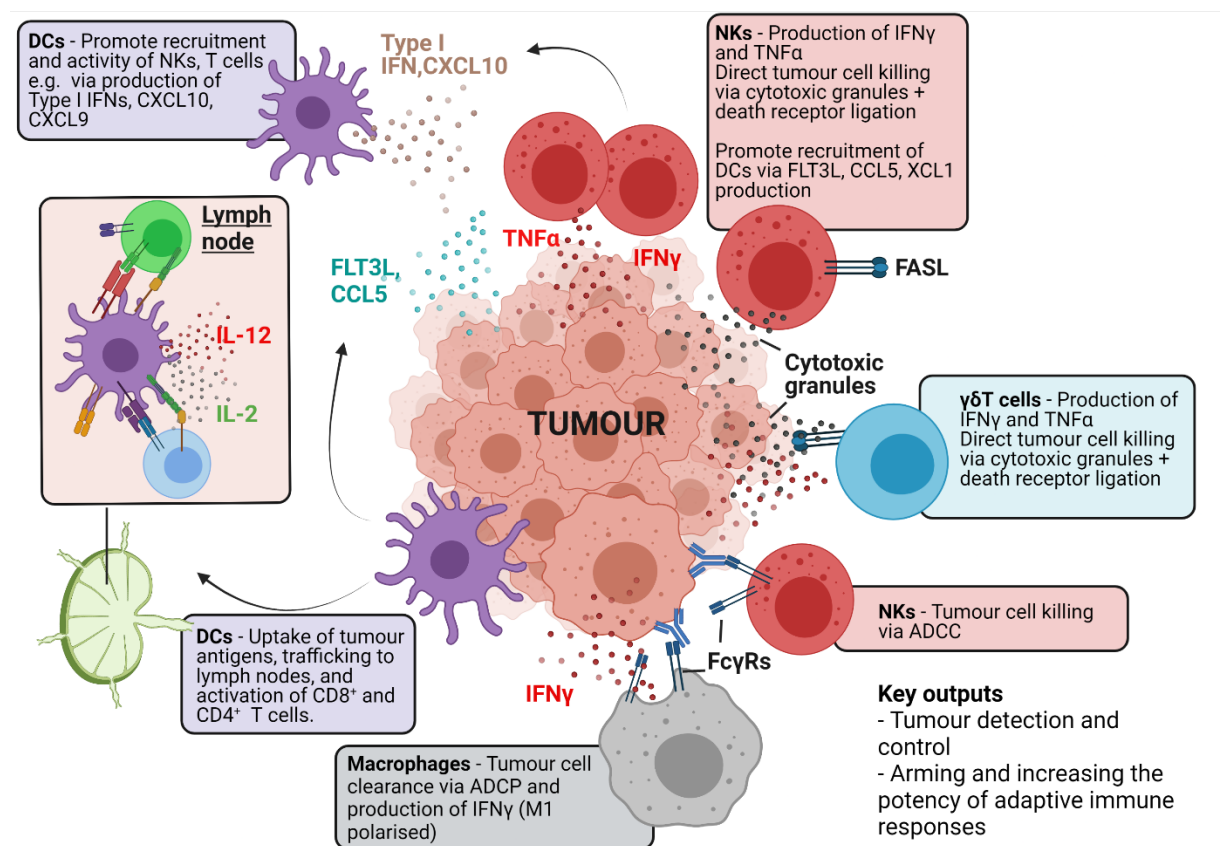
Macrophages display phenotypic plasticity, with M1 polarised macrophages possessing anti-tumour capabilities and M2 macrophages possessing more anti-inflammatory pro-tumourigenic properties⁴⁸. This polarisation forms a gradient of macrophage phenotypes, with M1 and M2 macrophages representing opposing phenotypes on the plasticity spectrum. In general, M1 macrophages are polarised downstream of IFN γ and IL-12 signalling, while M2 macrophages are polarised by IL-4 and IL-13⁴⁹. M1 macrophages can function as an APC in the context of eliciting adaptive anti-tumour immune responses and can contribute to the direct killing of tumour cells via ADCC and additionally antibody dependant cellular phagocytosis (ADCP), dependant on Fc γ Rs binding to IgG antibodies bound to tumour cell surface antigens. As with NK cells, macrophages are key mediators of the therapeutic effects of mAB cancer therapies²⁸.

However, tumours often exploit the phenotypic plasticity of macrophages, polarising macrophages to a more immunosuppressive tissue remodelling phenotype⁴⁸, akin to an M2 phenotype, and as a result tumour associated macrophages (TAMs) often favour tumour progression. TAMs can suppress anti-tumour immune responses via production of anti-inflammatory mediators (e.g IL-10⁵⁰ and arginase), encourage tissue remodelling (through the release of TGF- β and matrix metalloproteases (MMP)) and neovascularisation (through release

of growth factors and chemokines e.g VEGF, PDGF) which can promote tumour development and metastasis⁵¹. Owing to their plasticity and dichotomous roles in anti-tumour immunity, therapies are being designed to modulate macrophage recruitment to the tumour and to reprogramme polarisation in the TME, as reviewed in⁵¹.

$\gamma\delta$ T cells

$\gamma\delta$ T cells belong to the group of ‘unconventional’ T cells, possessing characteristics of NK and T cells. These cell types can play an important role in anti-tumour immunity through direct cytotoxic effects on tumour cells themselves or in shaping existing anti-tumour immune responses through cytokine production. $\gamma\delta$ T cells can constitute prominent early sources of IFN γ production⁵², express cell death ligands including FASL and TRAIL, and can release cytotoxic granules consisting of granzyme A + B and perforin⁵³. $\gamma\delta$ T cells express NKG2D which can bind to cell stress ligands often expressed on tumour cells including MICA and MICB. In addition, there is some evidence that $\gamma\delta$ T cells may be able to mediate ADCC⁵³. The activation of $\gamma\delta$ T cells is also influenced through cytokine signalling with IL-2, IL-12, IL-15, IL-18, IL-21 and IL-36 promoting the activation of IFN γ ⁺ $\gamma\delta$ T cells with anti-tumour functionality^{53,54}.



1.1.2 Anti-tumour adaptive immunity

Like the innate immune system, the adaptive arm of immunity can exert pro-tumourigenic and anti-tumourigenic effects, but components of the ideal adaptive anti-tumourigenic response are of more relevance to this project and will be discussed below.

CD8⁺ T cells

Cytotoxic T lymphocytes (CTLs) play a crucial role in the elimination of tumour cells and are key orchestrators of tumour immunosurveillance, and while CD4⁺ T cells can be cytotoxic, CD8⁺ CTLs are most common cytotoxic cells. CD8⁺ T cells require extensive activation by specialised APCs, predominantly the cDC1 populations mentioned previously^{36,38}. Successful activation of anti-tumour CD8⁺ T cells relies on the engagement of MHC-I molecules bearing tumour antigens on the APC with the TCR and CD8⁵⁵ molecules expressed on the T cell surface, providing activation signal 1. The activation of CD8⁺ T cells further requires costimulatory interactions such as CD28:CD80/86⁵⁶ and CD27:CD70⁵⁷ providing activation signal 2, and cytokine signalling (e.g. IL-21 and IL-2^{58,59}) providing activation signal 3. To successfully prime a naïve CD8⁺ T cell, all 3 activation signals are required, with inadequate activation resulting in induction of T cell anergy/tolerance. On the other hand, T cell activation is also negatively regulated through co-inhibitory interactions such as PD-1:PD-L1 and CD80/86:CTLA-4⁵⁶ or through immunosuppressive cytokine signalling e.g. TGF- β /TGF- β R⁶⁰. Upon successful activation naïve T cells undergo clonal expansion, undergoing multiple rounds of cell division, with the resulting phenotype of the CD8⁺ T cells dependent on the milieu of surrounding cytokines. To capture this phenotypic diversity, subsets of CD8⁺ T cells have been proposed, akin to those documented for their CD4⁺ counterparts. Only Tc1 (T-bet⁺, EOMES⁺, STAT4⁺), Tc2 (GATA3⁺, STAT6⁺) and TC22 (AhR⁺) have been documented to possess cytotoxic capabilities, while the Tc1 phenotype is the most desirable subset in the context of anti-tumour immunity due to their additional capacity to produce IFN γ and TNF α ⁶¹. Tc1 differentiation is governed by IL-12 signalling during T cell activation⁶².

Following successful activation and expansion in the lymphatics (lymph nodes), effector CD8⁺ T cells must migrate to the tumour site, relying on the expression of chemokine receptors including CXCR3, CCR4 and CCR5. CXCR3 binds to CXCL9, CXCL10 and CXCL11, and these chemokines can be expressed by a variety of cells in the TME, but predominantly rely on cDC1 mediated production^{45,46}. Tumours expressing high levels of these chemokines generally have an improved prognosis, correlating with a high degree of infiltration of CD8⁺ T cells⁶³. The

expression of CXCR3 has been shown to promote DC:CD8⁺ T cell interactions in the tumour, resulting in increased efficacy of αPD-1 therapy⁶⁴. The presence of intratumoral CD8⁺ CTLs is associated with improved prognoses in many cancer types and better responsiveness to checkpoint immunotherapies^{65–67}. Infiltration of CD8⁺ T cells into the tumour is inhibited by the presence of stroma surrounding the tumour and in addition TAMs have been demonstrated to inhibit the infiltration of CD8⁺ T cells through sequestering them at the stroma-tumour interface, which could be combatted through macrophage depletion strategies⁶⁸. There is an inverse correlation between the expression of DNA repair enzymes and β-catenin, and the degree of tumour immune infiltrate, and interestingly this has been observed not to be dependent on expression of tumour neoantigens⁶⁹.

Once in the tumour, activation of the cytolytic effector function of CD8⁺ CTLs relies on binding to tumour antigens expressed on MHC Class I molecules on the tumour surface. Like NK cells, CD8⁺ CTL dependant tumour cell lysis can be mediated through the secretion of lytic granules and subsequent uptake by target cells⁷⁰, alongside the physical exertion of force on target cell membranes⁷¹, or through ligation of death receptors on the tumour cell surface. CD8⁺ T cells can also produce an array of anti-tumourigenic cytokines including IFNγ and TNFα, which can arrest tumour growth, mediate tumour cell death, potentiate existing anti-tumour immune responses and alter the secretome of tumour cells themselves. However, tumour infiltrating T cells often become dysfunctional, acquiring an exhausted phenotype, characterised with upregulation of checkpoint molecules on the cell surface (PD-1, LAG3, TIM3 etc), loss of proliferative capacity, and blunting of their anti-tumour effector mechanisms⁷². This phenotype shift is induced by chronic TCR stimulation and an array of mediators in the TME, including TGF-β and IL-10. Fortunately, T cell exhaustion can be reversed, and functionality reinvigorated before transition to a terminally exhausted phenotype⁷³, using targeted immunotherapeutics discussed below.

The presence of CD8⁺ T cells possessing a ‘stem-like’ phenotype, indicated through elevated expression of stem markers like *Tcf7* (TCF1), *Sell* (CD62L), *Lef1* (LEF1/TCF10), and *Tox* (TOX) has been shown to correlate with an improved response to checkpoint inhibitor therapies^{74,75}, identifying these cells as critical mediators of induced anti-tumour immunity. Similarly, the efficacy of adoptive cell transfer strategies has been shown to hinge on the presence of cells possessing this phenotype also⁷⁶. These cells have the capacity for self-renewal, facilitating expansion and waves of anti-tumour T cell responses. Therefore, it is not only essential to assess

a vaccines capacity to drive CD8⁺ T cell responses but also to determine the phenotype of these cells to infer functionality and potential therapeutic combinations.

CD4⁺ T cells

Certain CD4⁺ T cells subsets can also exert anti-tumourigenic effects, with T helper 1 cells (Th1s) constituting the subset with the best characterised anti-tumour roles. Th1 cells, characterised by the expression of the transcription factor T-bet⁷⁷, are prominent producers of IFN γ and TNF α and play a crucial role in the initiation and maintenance of anti-tumour immune responses. T helper cells play an important role in CD8⁺ T cell activation and expansion to cognate antigen via DC licencing. DC licencing involves CD40:CD40L and MHC-II:TCR interactions, coupled to cytokine production (IL-21⁷⁸, IL-2), whereby the ability of DCs to cross-prime CD8⁺ T cell responses and furthermore promote the induction of CD8⁺ T cell memory responses is potentiated⁵⁷ (Fig.1.2). CD8⁺ T cells activated with CD4⁺ T cell help have improved anti-tumour functionality through enhanced cytolytic and cytokine production capacity alongside an increased capacity to infiltrate into tumours⁵⁷. The presence of Th1 cells intratumorally is also correlated with improved prognoses in multiple cancer types^{65–67}, in particular a PD-1⁺ ICOS⁺ Th1 subset was demonstrated to be tumour reactive in human solid tumour samples⁷⁹.

Aside from their role in promoting CD8⁺ T cell responses and in effector cytokine production, CD4⁺ CTLs have been documented in murine^{80–82} and human tumours⁸³, exerting their cytotoxic function upon sensing of tumour antigen presentation through MHC Class II molecules on the tumour cell surface⁸². While MHC Class I expression is more ubiquitously expressed in cancers compared to MHC Class II and therefore CD4⁺ mediated tumour recognition and killing is less prevalent, this nonetheless challenges the paradigm of CD8⁺ T cells being the sole mediators of T cell elicited cytotoxicity. The importance of CD4⁺ T cell mediated recognition of tumours is also indicated through the clinical observation that immunogenic tumour neoantigens are more likely to elicit CD4⁺ rather than CD8⁺ T cells⁸⁴.

Like their CD8⁺ counterparts, CD4⁺ T cells can become phenotypically exhausted in the TME resulting in upregulation of the checkpoint molecules PD-1, TIM-3 and CTLA-4, and downregulation of effector cytokine production, which can also be reversed through targeted immunotherapeutic strategies⁷³.

B cells

B cells and plasma cells also can exert anti-tumourigenic effects and their presence intratumorally or in tumour associated tertiary lymphoid structures (TLS) has been shown to have positive prognostic implications in multiple tumour types⁸⁵. B cells can function as APCs and may support intratumoral T cell responses via antigen presentation and costimulatory interactions^{86,87}. Furthermore, B cells themselves may exert cytotoxic effects on tumour cells via expression of granzyme B and TRAIL, and in a study conducted by Shi *et al*, IFN γ ⁺ IL-12⁺ Granzyme B⁺ TRAIL expressing B cells were shown to colocalise with CD8⁺ T cells intratumorally, associated with improved clinical outcomes⁸⁸. The production of intratumoral antibodies mediated by plasma cells may potentiate anti-tumour immunity via the opsonisation of tumour antigens, and promotion of ADCP and ADCC of tumour cells⁸⁵ principally mediated by tumour antigen bound IgG1 and Fc γ R_s expressed on NK cells and macrophages.

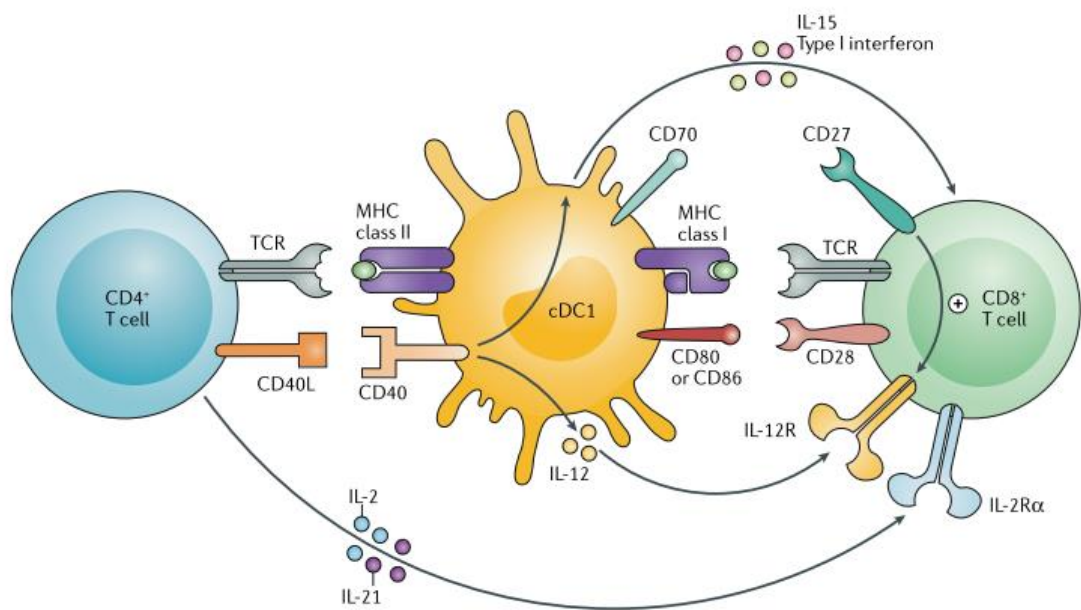


Figure 1.1 Brief overview of innate anti-tumour immunity

The innate immune system is essential in the initial detection and control of tumours, cell types like NKs, $\gamma\delta$ T cells and macrophages can all exert cytotoxic effects on tumour cells. Additionally, the innate immune system is essential to prime adaptive immunity, with DC functionally adapted to sample tumour antigen and activate T and B cells in the draining lymph nodes. Mediators produced by the innate immune system also serve to increase the magnitude and potentiate both innate and adaptive anti-tumour immune responses e.g., cytokines like IFN γ , IL-12 and Type I IFNs and chemokines like CXCL10 and CCL5.

Figure 1.2 CD4⁺ T cell help in CD8⁺ T cell priming

cDC1s can present different epitopes of same cognate antigen to CD4⁺ and CD8⁺ T cells. CD40 ligation on the cDC1 by CD4⁺ T cells promotes upregulation of antigen presentation, costimulatory molecule expression (CD70, CD80/86) and production of cytokines (IL-12, IL-15, and Type I IFNs). This enhances the capacity of cDC1s to activate CD8⁺ T cells. Additionally, the CD4⁺ T cell produces cytokines (IL-2 and IL-21) which further enhance CD8⁺ T cell activation. Figure taken from Borst et al⁵⁷.

1.1.3 Immunotherapy – a key pillar of cancer treatment

Cancer treatment has been revolutionised multiple times in history, firstly with the advent of surgical resection in the late 19th century, then with the implementation of radiotherapy in the early mid-20th century and then with the discovery and use of chemotherapeutics in the mid-late 20th century. Crucially cancer immunotherapy has emerged as another revolutionary pillar of cancer therapy, with the flagship approvals of *in situ* BCG therapy for bladder cancer in 1990⁸⁹, recombinant IL-2 (rIL-2) therapy in 1992⁹⁰, the DC/T cell based vaccine Sipuleucel T in 2010⁹¹, the first checkpoint inhibitor therapy – the CTLA4 targeting mAB Ipilimumab in 2011⁹², and the first chimeric antigen receptor (CAR) T cell therapy in 2017⁹³. Arguably the first clinical implementation of immunotherapy was long before this at the end of the 19th century, instructed by Dr William Coley. Tumours were injected with crude preparations of *Streptococcus* bacteria and associated products – now termed Coley's Toxin, and remarkably tumour regressions were observed in some patients⁹⁴. However it wasn't until the key concepts of tumour immunology involving tumour immunosurveillance and elimination were characterised, alongside the identification of the cellular components and regulatory mechanisms governing their activity, that the rational design of immunotherapeutics could be carried out⁶⁷.

Adoptive cell therapy & CAR T NK therapy

Adoptive cell therapy utilising *ex vivo* expansion of TILs has had some success clinically, here TILs are isolated from patients, expanded and enriched for neo-antigen specific T cells, and are reinfused post lymphodepletion (e.g using cyclophosphamide)⁹⁵. These are highly personalised therapies as these neoantigens are unique to each patient.

Another cell therapy involves the use of CAR-T cells which have had stellar impacts clinically in some haematological malignancies. Currently, there are 6 FDA approved CAR T cell products for clinical use⁹⁶. CAR T cell therapies are engineered to express a modified receptor, composed of short chain variable fragment (scfv) of a monoclonal antibody, joined to the transmembrane

zeta chain of CD3 joined to the intracellular domain of CD28 or 41BBz. These variable chains are designed to recognise specific tumour antigens and binding results in activation of the T cell and unleashing of effector mechanisms. In addition, the plasmids encoding the CAR can be engineered to induce expression of cytokines like IL-2, IL-15 and IL-18 which can favour CAR T cell proliferation and survival *in vivo*, these modified cells are termed Armoured CAR-T cells^{97,98}. CAR expression is long lived in patients and has been detected up to 10 years after treatment in patients with leukaemia remission, with retention of functional proliferation and effector function⁹⁹.

While effective, these therapies are challenged by issues including antigen escape, CAR-T cell persistence, as an inverse correlation between TCR affinity and T cell persistence has been observed, and additionally in overcoming the immunosuppressive mechanisms present in the TME¹⁰⁰. Adverse effects in recipients remain problematic and these can include cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS)¹⁰⁰.

NK cells can also be engineered to express CARs and resulting CAR NKs have been trialled both preclinically and clinically. CAR NKs have some distinct advantages over CAR-T cells in that CRS and ICANS are not observed in recipients, while still facing challenges surrounding immune suppression in the TME¹⁰¹.

While CAR therapies have shown promise in haematological cancers, they have performed underwhelmingly in solid tumours, attributed to issues including poor tumour infiltration and increased difficulty in antigen selection.

Checkpoint inhibitor therapy

Following the discovery of CTLA-4 and PD-1/PD-L1, it was shown that blockade of these coinhibitory or checkpoint molecules utilising targeted monoclonal antibodies (mAB) could potentiate and restore anti-tumour immunity preclinically^{102,103}. A number of these checkpoint inhibitors have subsequently undergone or are still undergoing clinical evaluation, culminating in the current FDA approval of 9 checkpoint inhibitors:

- Ipilimumab targeting CTLA4
- Pembrolizumab, Dostarlimab, Cemiplimab & Nivolumab targeting PD-1
- Atezolizumab, Durvalumab & Avelumab targeting PD-L1
- Relatlimab targeting LAG-3

These immunotherapies have received approval for use in a wide range of cancer types and have had success not only as monotherapies, but also in combination due to their non-redundancy of action (e.g anti-PD-1 and anti-CTLA4 combination^{66,104}, and α -PD1 and α -LAG3 combination¹⁰⁵) with associated improved response rates¹⁰⁶. Further checkpoint molecules such as TIGIT and VISTA have been characterised and targeted for cancer immunotherapy, with potential combinatorial approaches suggested⁶⁶.

α -CTLA4 immunotherapy primarily mediates its effects at the stage of T cell priming, associated with the expansion of an ICOS⁺ CD4⁺ T-bet⁺ intratumoral T cell population that mediates anti-tumour effects alongside expansion of intratumoural CD8⁺ T cells with an exhausted phenotype^{107,108}. In addition, α -CTLA4 therapy results in the expansion of circulating anti-tumour T cells, while PD1 therapy does not¹⁰⁹. These observations may indicate that α -CTLA4 therapy is more suitable than α -PD-1 for driving T cell responses into an immunologically cold tumour e.g., prostate, and renal tumours. However adverse effects are more severe with α -CTLA4 than α -PD1 therapy¹¹⁰, and as a result patients can only receive α -CTLA4 therapy for a relatively short time compared to α -PD1 therapy (months vs years).

α -PD1 therapy mediates its anti-tumour effect mainly through acting on peripheral/intratumoral CD8⁺ T cells, restoring proliferation, activation, and effector function¹⁰⁸, however there is some evidence that T cell priming is additionally targeted in the tumour draining lymphatics¹¹¹ facilitating waves of anti-tumour CD8⁺ T cell responses. Therapeutic efficacy appears to be reliant on the presence and expansion of 'stem-like' CD8⁺ T cells in the tumour⁷⁴ as these cells have an increased capacity for self-renewal. DCs are crucial to the efficacy of α -PD1/PD-L1 therapy and in particular intratumoral CD103⁺ DCs are required for resulting CD8⁺ T cell activation³⁹. In addition, antigen spreading is crucial for the optimal efficacy of these therapies, along with induction of bystander tumour cell killing which can be mediated by innate and adaptive immune cells in the TME.

While immunotherapeutics have been traditionally used in adjuvant therapeutic regimens (therapy following surgical resection), they are increasingly being trialled in neoadjuvant therapy regimes (prior to surgery). Neoadjuvant therapy is designed to improve tumour resectability, and often entails the use of chemotherapy or radiotherapy. While neoadjuvant immunotherapy can aid surgical resection, it additionally aims to establish systemic anti-tumour immunity, taking advantage of the existing contact between tumour and T cells. These treatments involve a short course of therapy prior to surgical resection, resuming after recovery from surgery. Overall, patients receiving neoadjuvant immunotherapy have had promising responses in terms of progression free survival which has correlated with induction of tumour neoantigen specific T

cell responses^{84,112,113}. These trials additionally allow for large tissue samples to be taken during surgery which provide insights into the mechanism of action of the therapeutic and identification of biomarkers of successful therapeutic response¹¹².

Although checkpoint therapies have had stellar impacts clinically, this is often restricted to certain cohorts of patients or certain tumour subtypes. A study conducted in 2018 determined that although there has been a substantial increase in the percentage of patients eligible (Table 1.1) for any checkpoint therapy in the US owing to an increase in FDA approvals, the response rates in these cancers generally remain low at around 30%¹⁸. However, this estimated response rate is further skewed positively, as response rates in non-small cell lung cancer (NSCLC) to checkpoint therapies was much higher than other cancers examined in the study. These phenomena of low response rates can correlate with the immune landscape of tumours, where immunologically ‘hot’ tumours with extensive immune infiltrates, have better prognoses than those that are immunologically ‘cold’^{65,66}.

To combat these, sometimes-disappointing, clinical response rates, combination approaches with cancer vaccines have been proposed. These therapeutic cancer vaccines would aim to promote immune infiltration intratumorally and predispose patients to respond to checkpoint immunotherapy approaches. In addition, cancer vaccines could be used to improve the response rates to CAR therapies and may address difficulties in their use in treating solid tumours. These therapeutic cancer vaccines may provide another important component of cancer immunotherapy and will be discussed below.

Table 1.1 Eligibility and clinical benefit of checkpoint inhibitor drugs for specific cancers from 2011-2018 (Data from Haslam et al)¹⁸

Year	2011	2012	2013	2014	2015	2016	2017	2018
Eligibility	1.54	1.59	1.63	1.66	26.86	31.95	39.54	43.63
%	(1.51-	(1.56-	(1.60-	(1.62-	(26.75-	(31.84-	(39.41-	(43.51-
(95% CI)	1.57)	1.62)	1.67)	1.69)	26.98)	32.07)	39.66)	43.75)
Benefit	0.14	0.15	0.15	0.53	5.86	8.36	11.04	12.46
%	(0.13-	(0.14-	(0.14-	(0.51-	(5.80-	(8.29-	(10.96-	(12.37-
(95% CI)	0.15)	0.16)	0.16)	0.55)	5.92)	8.43)	11.12)	12.54)

*CI = Confidence Interval

**Specific cancers examined: Melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), urothelial carcinoma, Hodgkin lymphoma, head and neck squamous cell carcinoma, Merkel cell carcinoma (MCC), microsatellite instability-high noncolorectal cancer, gastric cancer, primary mediastinal large B-cell lymphoma, cervical cancer, small cell lung cancer (SCLC).

***Checkpoint inhibitor drugs examined: ipilimumab (α -CTLA-4) (first approval 2011), nivolumab (α -PD-1) (first approval 2014), pembrolizumab (α -PD-1) (first approval 2014), atezolizumab (α -PD-L1) (first approval 2016), avelumab (α -PD-L1) (first approval 2017), and durvalumab (α -PD-L1) (first approval 2017)

1.2 Vaccines

Vaccines are one of the most cost-effective and successful medical interventions in history, their introduction revolutionised the prevention of infectious disease and has resulted in extraordinary reductions in related mortality and morbidity worldwide^{114,115}. Vaccination against infectious diseases aims to induce robust, long-lasting immunity to pathogens, through the elicitation of pathogen specific adaptive immune responses, in particular targeting essential antigens that mediate colonisation and the development of pathogen associated disease¹¹⁶. Recently the global race to develop and implement a vaccine for SARS-CoV-2 highlighted the importance of vaccine research, with vaccines approved and rolled out in record time¹¹⁷. In addition, a wide range of vaccine platforms have subsequently been approved against SARS-CoV-2 including inactivated virus (e.g., BBIBP-CorV (Sinopharm)), viral vectored vaccines (e.g., ChAdOx1-nCoV19 (Oxford/AstraZeneca)), protein subunit (e.g. Nuvaxovid (Novavax)), and mRNA vaccines (e.g., BNT162b2 (Pfizer)). This highlights the importance of having a wide repertoire of vaccine platforms and designs which can be implemented to design vaccines for novel pathogens and for diseases with unmet vaccine needs.

Traditionally, vaccines were composed of whole pathogen preparations, where the pathogen is either inactivated or attenuated, and had stellar impacts such as the contribution to the global eradication of smallpox¹¹⁸ and near eradication of polio¹¹⁹. However, safety concerns surrounding the use of whole pathogen killed and attenuated vaccines arose. In rare cases attenuated vaccines can revert to their virulent form and cause disease, and for whole pathogen killed vaccines reactogenicity concerns arose when administered via parenteral vaccination routes (e.g with the whole cell killed pertussis vaccine). These issues, coupled with the advancement of technology that facilitated identification of vaccine correlates of protection and developments in antigen discovery and expression led to a shift toward using subunit vaccination approaches. These subunit antigens, however, possess lower immunogenicity than their whole pathogen counterparts, and as a result require the incorporation of vaccine components called adjuvants to promote the induction and propagation of vaccine-specific immune responses.

It is worth highlighting that the use of vaccine adjuvants is not restricted to subunit vaccine approaches. Successful mRNA vaccine approaches, including both Pfizer and Moderna's mRNA vaccines for SARS-CoV-2, utilise a lipid nanoparticle (LNP) that encapsulates the mRNA and can act as an adjuvant, promoting follicular helper T cell (Tfh) and germinal centre B cell activation¹²⁰.

Furthermore, this adjuvanticity is retained when empty LNPs are administered with free protein antigen¹²⁰. Adjuvants have also been trialled with viral vector approaches, for example, intracellular delivery of the cyclic-di nucleotide (CDN) STING agonist 2'3'-cGAMP following vaccination with a human adenoviral vectored vaccine was shown to enhance immune responses¹²¹, albeit with adjuvanticity dependant on the kinetics of cGAMP delivery, with early delivery impairing adjuvanticity via type I IFN dependent antiviral mechanisms. Vaccine adjuvants also have an application in whole pathogen inactivated/live attenuated vaccine approaches, for example α -GalCeramide has been shown to promote induction of antigen specific humoral and cellular immune responses to orally administered whole cell killed *Helicobacter pylori*¹²², *Vibrio cholera*¹²³ and Enterotoxigenic *Escherichia coli* (ETEC)¹²⁴ vaccines.

Adjuvanted protein subunit vaccines have been very successful clinically with vaccines against human papilloma virus (HPV), hepatitis B virus (HBV) and *Haemophilus influenzae* type B (Hib) providing examples. However, there are still many transmissible diseases where highly effective vaccines have not yet been developed, particularly in the case of intracellular pathogens and diseases where correlates of protective immunity are poorly defined, examples including malaria and *Mycobacterium tuberculosis* (M.Tb). Intracellular pathogens are problematic, as even though neutralising antibodies can prevent initial infection, elicitation of cell mediated immunity (CMI) is required for subsequent control. This has traditionally been harder to achieve through subunit vaccination approaches than vaccination strategies eliciting predominantly humoral immunity, as traditional vaccine adjuvants like alum do not optimally promote CMI. Despite not being an infectious disease, cancer too can be targeted through vaccination, but the successful development of cancer vaccines has similarly been hampered through a lack of vaccine approaches that strongly enhance CMI.

1.2.1 Cancer vaccines

The reinvigorated research into novel and existing vaccine adjuvants and platforms following the emergence of COVID-19, has resulted in a renewed focus on research into cancer vaccination approaches. Cancer vaccines can be broadly categorised as prophylactic or therapeutic, and research into their design and development will be discussed below

Prophylactic

Prophylactic cancer vaccines (illustrated in Fig 1.3) are generated through a classical vaccine approach, aiming to prevent cancer development and establishment in the first place, for example, human papillomavirus virus (HPV) and Hepatitis B virus (HBV) vaccines (Table 1.2). These vaccination strategies aim to prevent infection/colonisation by these pathogens through targeting of surface antigens used for intracellular entry. Implementation of these vaccination strategies have had stellar impacts in reducing the burden of cancer in regions with robust vaccination programmes. Despite the identification of additional oncogenic pathogens: Epstein-Bar Virus (EBV), *Helicobacter pylori*, Human herpesvirus-8 (HHV8)/ Kaposi's sarcoma herpesvirus (KSHV), Human T-cell leukaemia virus 1 (HTLV1), Hepatitis C virus (HCV) and Merkel cell polyomavirus (MCPyV)¹²⁵, there are no approved vaccines for these pathogens. Furthermore, except for two ongoing vaccine clinical trials for EBV (Table 1.3) there are no vaccine candidates for these oncogenic pathogens in ongoing/recruiting clinical trials. Apart from EBV, where infection is somewhat ubiquitous, many of these oncogenic pathogens are more endemic in vulnerable populations and lower income countries. As a result, vaccine research into these pathogens has been neglected and remains an area of significant unmet need.

For certain cancers of viral origin, vaccination theoretically can not only prophylactically prevent infection and colonisation upstream of associated carcinogenesis but could also therapeutically target tumour cells where viral protein expression is retained such as HPV associated cervical/head & neck tumours retaining E6 and E7 protein expression and similarly some EBV associated tumours, as these viral antigens can be effectively targeted. However, successful utilisation of this approach may require the use of alternative vaccine adjuvants, as none of the adjuvants utilised in these approved HPV and HBV vaccines are effective drivers of antigen specific CD8⁺ T cells in humans, which are required for effective cytolytic control of tumour establishment.

While issues surrounding a lack of suitable vaccine adjuvants is relevant to prophylactic cancer vaccine design, vaccine development is further compounded by challenges relating to the choice of antigen in malignancies not associated with oncogenic pathogens. Theoretically, tumour associated antigens (TAA) (expressed by some tumour and somatic cells) and tumour specific antigens (TSA) such as neoantigens arising from cumulative mutations can be targeted. Targeting tumour neoantigens is an attractive concept yet it is unlikely to ever be a 'one size fits all approach', the degree and composition of mutational burden is a tumour-specific

phenomenon arising through random mutation alongside fitness selection. As such there can be a distinct lack of shared neoantigens between patients with the same tumour types¹²⁶ and this neoantigen heterogeneity can be seen between metastatic and primary tumours in the same patient¹²⁷. Prophylactic cancer vaccines, outside of viral associated tumours, are therefore unlikely to be a broadly applicable approach.

Table 1.2. Licenced prophylactic cancer vaccines

<u>Vaccine Name</u>	<u>Target</u>	<u>Description</u>	<u>Immune response</u>
Gardasil (Merck)	HPV (Type 6, 11, 16, 18, 21, 33, 45, 52, and 58)	Virus like particles (VLPs) of major capsid proteins (L1), adsorbed onto amorphous aluminium hydroxyphosphate sulfate (AHHS)	High titres of serum neutralising antibodies (predominantly IgG) directed at capsid proteins, and antigen specific Th1 responses. Memory B cell induction ¹²⁸
Cervarix (GSK)	HPV (Type 16 and 18)	VLPs of major capsid proteins (L1) adjuvanted with GSKs AS04 adjuvant (Aluminium hydroxide + MPL)	High titres of serum neutralising antibodies (predominantly IgG) directed at capsid proteins, and antigen specific Th1 responses. Memory B cell induction (Increased immunogenicity compared to Gardasil) ¹²⁸
HBVaxPro (Merck)	HBV	Recombinant Hepatitis B surface antigen (HBsAg), adsorbed onto AHHS	Serum neutralising antibodies and low antigen specific Th1 responses ¹²⁹
Engerix-B (GSK)	HBV	HBsAg adsorbed onto aluminium hydroxide	Serum neutralising antibodies and low antigen specific Th1 response ¹³⁰
Hepilisav B (Dynavax)	HBV	HBsAg, with CpG 1018 adjuvant	High titres of serum neutralising antibodies and antigen specific Th1 responses. Generation of memory B and T cell responses.

			(Superior immune response when compared to Engerix-B ¹³⁰)

Table 1.3. Prophylactic cancer vaccines undergoing clinical trials

<u>Vaccine Name</u>	<u>Target</u>	<u>Description</u>	<u>Immune response</u>	<u>Clinical trial phase</u>
mRNA-1189 (Moderna)	EBV	LNP encapsulating mRNAs encoding gH, gL, gp350, and gp42 (EBV envelope proteins)	No data available for mRNA-1189 specifically but Moderna's mRNA-LNPs promotes antigen specific antibodies alongside CD4 ⁺ and CD8 ⁺ T cell responses ¹³¹	Phase I (NCT05164094)
EBV gp350-Ferritin vaccine (NIH)	EBV	Self-assembling ferritin nanoparticles displaying surface gp350, adjuvanted with Matrix M	Ferritin nanoparticles have been shown to promote gp350 specific neutralising antibodies in mice ¹³² . Matrix M has been shown to enhance antibody titres and induction of polyfunctional Th1 cells ¹³³	Phase I (NCT04645147)

Therapeutic

Therapeutic cancer vaccination approaches (illustrated in Fig.1.3), aim to target established tumours in patients. These therapeutic strategies can be categorised into two main types: 1) Personalised vaccination, and 2) Off-the-shelf *in situ* vaccination (ISV) approaches.

Personalised approaches comprise cancer vaccination given via more canonical routes, with a reliance on the identification of patient/tumour specific tumour antigens. This can involve sampling of tumour antigens via surgical resection of tumours or through taking tumours/tumour biopsies from a patient alongside normal tissue, with subsequent neoantigen

identification and sequencing carried out using DNA and RNA sequencing techniques. Crucially, the immunogenicity of neoantigen candidates is determined through computational MHC binding modelling techniques and these neoantigens can then be synthesised and incorporated into vaccines^{134,135}. An example of such a neoantigen identification pipeline is OpenVax, which has been utilised to design a personalised multi-neoantigen cancer vaccine (PGV-001) trialled in Mount Sinai Hospital, New York¹³⁶. PGV-001 utilises synthetic long peptides (SLPs) containing the identified neoantigens sequences, adjuvanted with poly:ICLC and has been shown to elicit both neoantigen specific CD4⁺ and CD8⁺ T cell responses when trialled in vaccine approaches in patients with HNSCC (NCT02721043)¹³⁷. However Poly:ICLC has been previously identified as a suboptimal adjuvant to drive CD8⁺ T cell responses in cancer vaccination trials using peptide antigens¹³⁸, with induction of tumour antigen specific CD8⁺ T cells only observed in some patients vaccinated with formulations including both Poly:ICLC and the water in oil emulsion adjuvant Montanide ISA™ 51. This highlights the need for novel vaccine adjuvants that can drive both antigen specific CD4⁺ and CD8⁺ T cells responses which will be discussed later in section 1.2.2.

Utilising peptide-based tumour antigens in cancer vaccination approaches may prove less effective than using mRNAs encoding tumour neoantigens due to the intrinsic immunogenicity possessed by mRNA and the capacity of mRNA vaccines to drive robust humoral and cellular immunity. Indeed, pharmaceutical, and biotech companies like Moderna and BioNTech are attempting to take advantage of their successful use of mRNA technology in the design of vaccines for COVID-19 and apply it to therapeutic cancer vaccination approaches. These mRNA vaccines are being investigated as monotherapies or in combination with existing chemo/immunotherapies¹³⁹. Moderna is trialling LNP formulated neoantigen specific mRNAs with their vaccine mRNA-4157 (NCT03313778 & NCT03897881) building on promising results when used in combination with pembrolizumab¹⁴⁰. LNP formulations utilise a lipid shell to encapsulate the mRNA, with ionisable lipids incorporated into the LNPs that provide adjuvanticity and facilitate endosomal disruption and escape^{120,141}. In a recent press release, Moderna disclosed that mRNA-4157 displayed synergistic effects with Keytruda, leading to a reduction in recurrence/death by 44% in patients when compared to Keytruda alone treated patients (<https://investors.modernatx.com/news/news-details/2022/Moderna-and-Merck-Announce-mRNA-4157V940-an-Investigational-Personalized-mRNA-Cancer-Vaccine-in-Combination-with-KEYTRUDAR-pembrolizumab-Met-Primary-Efficacy-Endpoint-in-Phase-2b-KEYNOTE-942-Trial/default.aspx>).

BioNTech is utilising Lipoplex formulations in their cancer vaccine trials, with multiple vaccines in clinical trials including the neoantigen based BNT-122 vaccine (NCT04486378, NCT038515058 & NCT04161755). These formulations involve complexing RNA with cationic components (e.g., lipids, polymers) with further options for encapsulation sometimes utilised using liposomes. While there have been promising data arising from mRNA cancer vaccine trials, much of the success has been restricted to use with checkpoint blockade. It remains to be determined if a successful mRNA based neoantigen therapeutic cancer vaccine that can be used as a monotherapy is feasible.

Other personalised cancer vaccination approaches involve the use of autologous DCs loaded with tumour antigens, and these have had some success clinically with Sipuleucel-T providing a prime example (see Table 1.4). Issues remain however, surrounding the choice of tumour antigen but also crucially the phenotype of DCs utilised. To date, DCs have been derived from PBMCs and have a phenotype akin to moDCs, which have an inferior capacity to prime anti-tumour immune responses when compared to cDC1 counterparts¹⁴², and this may contribute to sub optimal therapeutic efficacy. Furthermore, these vaccines are further challenged by the relatively low yield of DCs obtained from PBMCs. To address these challenges, a culture protocol generating large numbers of DCs (comprising pDCs, cDC1s, cDC2s) from CD34⁺ stem progenitors from umbilical cord blood¹⁴³, may provide a basis for improved DC based cancer vaccines.

Alternatively, a patients tumour cells themselves can be utilised to form personalised cancer vaccines. A prime example of this is GVAX, which is composed of irradiated autologous tumour cells and/or allogenic tumour cell lines which have additionally been genetically modified to express GM-CSF. GVAX has been investigated in multiple malignancies alone or combination with existing therapies. A phase I trial investigating a combination of GVAX and ipilimumab showed synergistic effects in promoting overall survival in patients with metastatic pancreatic cancer¹⁴⁴, while other studies have demonstrated a lack of response in other malignancies following treatment¹⁴⁵. At the time of writing there are 11 ongoing/recruiting clinical trials utilising GVAX. ERC1671 (Gliovac™) is another example of a personalised tumour cell-based vaccine for recurrent glioblastoma (GBM) using both autologous and allogenic tumour cells, with allorecognition proposed as underlying optimal efficacy¹⁴⁶. This vaccine involves mixing whole inactivated tumour cells with tumour cell lysates, both autologous and from 3 allogenic tumour cells adjuvanted with GM-CSF (see Table 1.5) and used in conjunction with cyclophosphamide treatment. ERC1671 has been demonstrated to synergise with bevacizumab (α -VEGF therapy, standard treatment in recurrent GBM) in terms of overall survival, with CD4⁺ T cells identified as potential mediators of this anti-tumour immunity¹⁴⁷. Furthermore combinations of ERC1671,

bevacizumab and PD-1 targeted immunotherapies yielded promising results clinically¹⁴⁸, and pave the way for further combinations with immunotherapeutics or the use of neoadjuvant treatment strategies.

These approaches utilising patient specific tumour antigens/neoantigens are ultimately highly personalised to each patient as there is a distinct lack of shared neoantigen expression between different patients with the same cancers^{126,149}. This higher degree of personalisation can result in a labour intensive and time & resource consuming processes, which is reflected in the cost of these personalised vaccines.

Less personalised, off-the-shelf, approaches promoting neoantigen release from tumour cells are therefore an attractive, potentially broadly applicable alternative. This can be achieved using *in situ* vaccination (ISV)¹⁵⁰ aiming to elicit ICD or potentiate immune cell infiltration into tumours. These *in situ* vaccination approaches can also be combined with cytotoxic chemo/radiotherapies, aiming to potentiate the release of tumour antigens. The principle underlying ISV is that the tumour itself becomes the vaccine formulation, with the release of antigen and DAMPs promoting induction of anti-tumour immunity. Crucially this negates the need for the intensive process of antigen identification prior to vaccination. ISV is not a new concept and as mentioned previously Coley's toxin was utilised as an intratumoral therapeutic late in the 19th century⁹⁴, albeit without the mechanistic insights underlying therapeutic efficacy that we possess today. There are currently 3 approved cancer vaccines used for ISV detailed in Table 1.4. BCG is a standard therapy for non-muscle invasive bladder cancer following surgical resection, T-Vec is routinely used in recurrent metastatic melanoma where surgery is not an option, and imiquimod is used in some instances when surgical resection of basal cell carcinoma is not an option. While imiquimod and T-VEC may not be canonical vaccines, they provide the prerequisites for vaccination, acting as vaccine adjuvants that promote recruitment and activation of APCs to the tumours and subsequent induction of adaptive tumour antigen specific immune responses.

Moving forward, the characterisation of effective vaccine adjuvants is a quintessential aspect of developing successful ISV approaches, and many vaccine adjuvants are being trialled in these approaches as detailed in Table 1.6.

PRR agonists constitute many of the trialled adjuvants, including TLR3, TLR9 and STING agonists. Engagement of PRRs in the tumour can promote the release of cytokines and chemokines that promote the anti-tumour activities of innate and adaptive immune cells, leading to local and systemic anti-tumour immunity⁸⁴.

ISV with the synthetic TLR3 agonist Poly:ICLC has been demonstrated to have some clinical benefit associated with an influx of intratumoral T cells in a small pilot clinical trial involving advanced solid tumours (NCT02423863)¹⁵¹, and preliminary clinical data suggest that ISV with poly:ICLC can synergise with a therapeutic combination strategy involving DC vaccination and radiotherapy¹⁵². ISV with Poly:ICLC is now being investigated in combination with Flt3L and CD40 targeted therapies (Table 1.6), and results are awaited from two recently completed trials combining Poly:ICLC with α -PD1 and α -CTLA4 therapies (NCT02423863 & NCT02643303).

SD-101, a class C CpG ODN TLR 9 agonist, has yielded positive therapeutic results in patients with advanced melanoma¹⁵³ and in a subset of HPV⁺ HNSCC patients¹⁵⁴ when combined with α -PD1 therapy. Despite some therapeutic efficacy, in both trials response rates were restricted to patient cohorts with either more immunogenic tumours or with less pre-existing resistance to PD-1 blockade. This may be indicative of issues surrounding mouse-human translation of TLR9 targeted adjuvants, as TLR9 expression is more restrictive to the myeloid cell compartment in humans compared to mice. This may underly the poor efficacy of the CpG ODN adjuvant Tilsotolimod in a recent phase 3 clinical trial, which ultimately led to trial termination (NCT03445533). The use of SD-101 in ISV approaches is still undergoing extensive clinical evaluation alone or in combination with other immunotherapeutic interventions (Table 1.6).

STING agonists have garnered much attention for their potential as adjuvants in ISV approaches¹⁵⁵ and despite suboptimal performance in early trials, next generation agonists have been developed and have entered clinical trials (Table 1.6). These will be discussed in more detail in Section 1.4.

In addition to triggering local anti-tumour immunity, it is critical that the ISV results in the activation of systemic anti-tumour immunity and abscopal therapeutic effects. Another advantage of ISV approaches is the opportunity to reduce systemic toxicity associated with treatments, facilitating the use of lower dosages, and restricting much of the immunomodulatory effects locally to the tumour. However, the therapeutic efficacy of cancer vaccination can be blunted by immunosuppressive conditions in the tumour, and as a result cancer vaccination and immune checkpoint blockade have the potential for synergistic effects. Similarly cancer vaccination approaches can aim to predispose tumours for response to checkpoint therapies and improve therapeutic response rates⁶⁶. Therefore, this potential synergy is an intense area of focus in ongoing clinical trials.

Table 1.4. Licenced therapeutic cancer vaccines

<u>Vaccine Name</u>	<u>Cancer type/ Approval</u>	<u>Description</u>	<u>Immune response/ Mechanism of action</u>	<u>Efficacy</u>
Sipuleucel- T/Provenge (Dendreon)	FDA/EMA approval (2010) for treatment of metastatic asymptomatic/ minimally symptomatic prostate cancer (castration resistant)	Intravenously administered autologous PBMCs cultured for 3 days with fusion protein of prostatic acid phosphatase (PAP) and GM- CSF. Minimum of 50 x 10 ⁶ CD54 ⁺ APCs (Once every 2 weeks, total 3 doses)	Predominantly on the promotion of PAP specific CD8 ⁺ T cell responses via transferred autologous CD54 ⁺ APCs (predominantly DCs)	Median survival extension approx. 4-4.5 months when compared to placebo ^{156,157}
BCG (Merck) (TICE substrain)	Approved (1990) for treatment of non-muscle invasive bladder cancer (NMIBC)	Intravesicular (<i>in situ</i>) delivery of BCG (live attenuated <i>M.bovis</i> strain now predominantly TICE® substrain used) following surgical resection. Initial weekly treatment for 3-6 weeks, with maintenance	Stimulation of local innate immune responses via release of cytokines and chemokines, including IL-1 β , IL- 15, GM-CSF and CXCL10 following internalisation by bladder urothelial cells. Tumour killing via activation of	55-75% response rates following initial therapy ^{158,159} . Maintenance/ Ongoing therapy required for optimum efficacy, 83% 5-year survival ¹⁶⁰ .

		treatment every 3-6 months ($1-8 \times 10^8$ CFU)	neutrophils, macrophages, and NK cells. Priming of adaptive anti-tumour immunity, predominantly Th1 responses. ¹⁵⁸	
Imiquimod/ Aldara (3M)	Approved (2004) for treatment of basal cell carcinoma	Topical application of 5% imiquimod (TLR7/8 agonist) cream daily, 5 days a week for duration of 6 weeks.	Stimulation of local innate immune responses via release of type I IFN, IL-6 and TNF-alpha. Likely recruitment of cytotoxic NK cells and activation of adaptive immune responses ¹⁶¹	Clearance observed in 77-80% of patients ¹⁶²
T-VEC (Talimogene Laherparepvec) (Amgen)	Approval (2015) for non-resectable metastatic melanoma	Intratumoral delivery of oncolytic virus (modified HSV-1) which additionally encodes GM-CSF. Volume of injection dependant on tumour size (0.1ml – 4ml). Initial injection 10^6 PFU/ml with subsequent treatments 10^8 PFU/ml given	Replication in tumour cells resulting in subsequent induction of melanoma tumour cell lysis. Recruitment and activation of local innate immune cells including DCs. Subsequent drainage of tumour antigens leads to priming of anti-tumour T cell	Overall response rate of 26% ¹⁶⁵ .

		every 2 weeks, 3 weeks after initial injection.	responses leading to abscopal effects ^{163,164}	

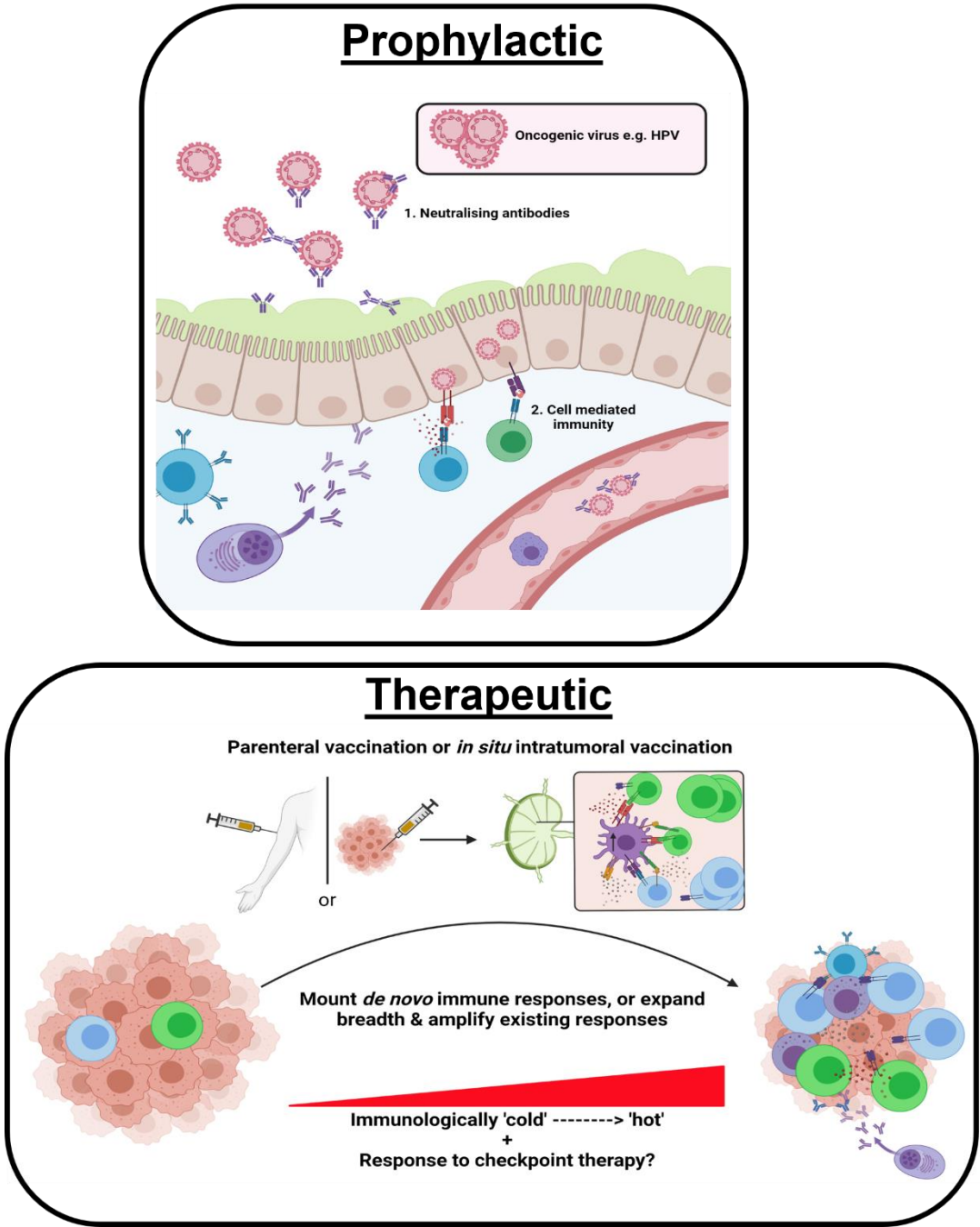


Figure 1.3 Prophylactic and therapeutic cancer vaccines.

Cancer vaccines can be utilised as prophylactic or therapeutic interventions. Prophylactic cancer vaccines aim to prevent infection and/or colonisation with an oncogenic pathogen. This can be primarily achieved through production of antigen specific neutralising antibodies both locally at the injection site and

systemically. Additionally, induction of antigen specific cellular immune responses is crucial for the control of viral replication within infected cells. Therapeutic cancer vaccines can also be administered via distal injection routes, once the tumour antigen has been identified, facilitating induction of tumour antigen specific adaptive immunity. Due to the reliance on prior tumour antigen characterisation, this is a highly personalised approach. Alternatively, *in situ* vaccination strategies can be utilised to promote local intratumoral immune responses. This can lead to release of tumour antigens and subsequent drainage to the lymphatics, and subsequent priming of adaptive immunity. Both therapeutic strategies aim to promote *de novo* anti-tumour immune responses or expand the breadth, diversity and potency of existing anti-tumour immunity.

1.2.2 Cancer vaccine adjuvants

Vaccine adjuvant research began as early as 1924 with observations by Gaston Ramon that antibody responses to diphtheria toxoid in horses could be improved by co-administering substances like breadcrumbs or tapioca with resultant abscess formation¹⁶⁶, describing these substances as ‘adjuvants’ for the first time. These findings coupled to the discovery by Alexander Glenny that aluminium salts could enhance antibody responses to injected diphtheria toxoid¹⁶⁷ were the birth of the field of vaccine adjuvant research. Insoluble aluminium salts (Alum) would go on to be developed and approved for use in a multitude of vaccines. Alum remained the only licenced vaccine adjuvant for decades, and still today there remains only 9 vaccine adjuvants incorporated in vaccines approved for use in humans – Alum, MF59, AS01, AS03, AS04, Matrix M, CpG 1018, GM-CSF and Imiquimod^{168,84}. In the context of licenced cancer vaccination approaches, alum, AS04 and CpG 1018 are used in prophylactic vaccines, while GM-CSF and imiquimod are used in therapeutic approaches.

Vaccine adjuvants can modulate the magnitude and type of adaptive immunity, facilitate dose sparing¹⁶⁹ and improve vaccine responses in challenging cohorts e.g. elderly, neonates¹⁶⁹. In theory, different vaccine adjuvants eliciting varied immune responses could be used and tailored for different vaccination approaches, informed through established pathogen-specific correlates of protection¹¹⁶. However, vaccine adjuvants have traditionally been identified through empirical approaches and as a result mechanisms of action have been poorly understood, and development has been slow¹⁶⁸. As an alternative method of vaccine adjuvant development Pulendran et al¹⁶⁸ recently proposed a systems vaccinology model for the rational development of novel adjuvanted vaccines displayed in Figure 1.4, where a divergence from linear development is suggested. This would retain the extensive use of *in vitro* experiments and *in*

vivo mouse and non-human primate (NHP) models but would incorporate multiple small-scale experimental human trials before progressing into Phase II/III trials. Following the completion of each small-scale study, systems biology approaches would be used to obtain insights into vaccine efficacy and immunological readouts. This would allow analysis of multiparametric data at early stages which can inform modifications to vaccine/adjuvant design, allowing optimisation through further preclinical research before entering larger clinical trials. Uncovering an adjuvants mechanism of action is a key principle of rational vaccine adjuvant development¹⁷⁰ and is centred on key questions surrounding adjuvanticity. Namely, how an adjuvants physiochemical properties relate to adjuvanticity and how these properties influence activation of immune cells and signalling pathways. These insights allow for a more rational development of an adjuvant following basic principles of design. Apart from the TLR agonist adjuvants CpG 1018 and Imiquimod, and the cytokine/growth factor adjuvant GM-CSF, we have relatively poor insights into the mechanism of action of approved vaccine adjuvants (Table 1.5).

Traditionally vaccines have aimed to induce a potent humoral responses with the generation of antigen-specific antibody titres in the recipient, and the most widely used vaccine adjuvant, alum, is particularly suited to elicit this effect^{167,171}. The generation of neutralising antibodies is an important aspect of vaccination against oncogenic viruses and as a result, Alum, AS04 and CpG 1018 have had success in prophylactic vaccines against HPV & HBV (Table 1.5). However, for optimal vaccination approaches targeting cancer cells, the generation of potent CMI is required. Optimally this cellular immunity would comprise antigen specific CD8⁺ CTLs and Th1 cells. The adjuvants AS01¹⁷², AS04¹⁷³, CpG 1018¹⁷⁴, Matrix M^{175,176}, Poly ICLC^{138,177} and CAF09^{178–180} have been shown to promote the induction of IFN γ ⁺ Th1 cells in mice and human trial participants, and these adjuvants are being investigated in ongoing cancer vaccine clinical trials (Table 1.6). The emulsion adjuvants MF59 and its mimetic Addavax™, and Montanide ISA™ 51 have been shown to promote the induction of CD8⁺ T cells in mice and humans^{138,168,181,182}. However to date, no adjuvant has been shown to induce a magnitude of antigen-specific CD8⁺ T cells and memory CD8⁺ T cells in humans similar to that elicited through live attenuated viral vaccines, which provide a gold standard in vaccination strategies aiming to elicit CMI¹⁶⁸. Apart from Montanide ISA™ 51 no vaccine adjuvant included in licenced prophylactic cancer vaccines (Table 1.5) or included in prophylactic cancer vaccines in clinical trials (Table 1.6) has been shown to induce CD8⁺ T cell responses in humans when injected parenterally. Despite the induction of antigen specific CD8⁺ T cells by the emulsion adjuvants MF59 and Montanide ISA™ 51 there remains outstanding questions relating to their functionality, as water in oil emulsion adjuvants

have been shown to induce dysfunctional CD8⁺ T cells which can be retained and die at the injection site¹⁸³, attributed to an antigen depot effect.

In light of these findings, it is clear that novel adjuvants capable of eliciting robust CMI with defined mechanism of action are required, paving the way for novel vaccine formulations that can be tailored for specific applications.

As mentioned previously, a wide range of vaccine adjuvants are being trialled in ISV approaches, mainly comprising PRR agonists and cytokines (Table 1.6). However, to date no PRR agonist adjuvant has progressed beyond phase III clinical trials in these approaches. Suboptimal efficacy in some instances has been attributed to poor human-mouse translatability, for example TLR9 agonists (Tilsotolimod and SD-101) and the STING agonists (DMXAA and ADUS100). This highlights again the need for a rational design of adjuvants, which should provide better insights into their clinical translatability.

Table 1.5. Adjuvants in use in licenced cancer vaccines

<u>Adjuvant</u>	<u>Licenced use</u>	<u>Description</u>	<u>Immune response</u>	<u>Mechanism of action</u>
Alum	Prophylactic vaccines: Gardasil, HBVaxPro, Engerix B.	Aluminium hydroxide or amorphous aluminium hydroxyphosphate sulfate (AHHS)	Induction of robust antigen specific antibody responses	Not fully defined. Roles suggested for NLRP3 ¹⁸⁴ and associated activation of IL-1 family cytokines ¹⁸⁵ but importance in adjuvanticity contested ¹⁸⁶ . Enhanced antigen bioavailability suggested as important for optimal induction of antibody responses ¹⁸⁷ .

AS04	Prophylactic vaccine: Cervarix	Aluminium hydroxide with adsorbed MPL	Induction of neutralising antibodies. Relatively low induction of Th1 responses ¹⁷³	Synergistic effect proposed between alum and MPL which signals via TLR4 to promote immune responses ¹⁸⁸
CpG 1018	Prophylactic vaccine: Hepisav B	TLR 9 oligonucleotide agonist	Induction of neutralising antibodies and Th1 immune responses ¹⁷⁴	Activation of TLR9 signalling pathway
GM-CSF	Therapeutic cancer vaccine: Sipuleucel T	Cytokine influencing differentiation of myeloid cell populations, with a focus on DCs	Promotes differentiation, expansion, and activation of APC subsets	Promotes recruitment and differentiation of APC subsets, predominantly DCs, and subsequent APC:T cell interactions ¹⁸⁹
Imiquimod	Used in therapeutic <i>in situ</i> vaccination for basal cell carcinoma	Synthetic imidazoquinoline TLR 7/8 agonist	Promotes activation and recruitment of innate APCs and effector immune cells ¹⁶¹	Induction of type I IFN, IL-6 and TNF release. Subsequent activation of APCs and priming of adaptive immunity ¹⁶¹

Table 1.6. Adjuvants in ongoing/recruiting cancer vaccine clinical trials

<u>Adjuvant</u>	<u>Description</u>	<u>Immune response</u>	<u>Mechanism of action</u>	<u>Ongoing trial details</u>
Prophylactic cancer vaccines				
Self-assembling ferritin nanoparticles	Self-assembling nanoparticles composed of the natural iron storage protein, ferritin. Nanoparticles are engineered to express target antigen on surface.	Ferritin nanoparticles have been shown to induce neutralising antibody titres in vaccinated mice ^{132,190} .	Proposed preferential targeting of DC populations following vaccination	Phase I (NCT05164094)
Matrix M™	Nanoparticles composed of <i>Quillaja saponins</i> , cholesterol and phospholipids	Induction of antigen specific antibody and Th1/Th2 responses ^{175,176} in human recipients.	Ill-defined mechanism of action. Local recruitment and activation of APC populations.	Phase I (NCT05164094)
<u>Adjuvant</u>	<u>Description</u>	<u>Immune response</u>	<u>Mechanism of action</u>	<u>Ongoing trial details</u>
Therapeutic cancer vaccines				
Poly:ICLC (Including Hiltonol™)	Synthetic doubled stranded RNA complexed with poly-L-lysine for stability. A TLR3 and MDA5 agonist.	Induction of antigen specific antibody and Th1 responses ^{138,177}	Activation of TLR3 and MDA5 signalling pathways and subsequent induction of type I IFN signalling and NF-κB	Phase I (NCT02924038) (NCT01130077) (NCT05111353) (NCT02549833) (NCT03956056) (NCT04799431) (NCT05010200)

			activation ¹⁹¹ . Polarisation of cDCs to promote Th1 responses ¹⁹¹	(NCT02826434) (NCT02737787) Phase I/II (NCT01720836) (NCT04364230) (NCT03665545) (NCT03617328) (NCT03206047) (NCT02960230) (NCT03789097) Phase II (NCT02358187) (NCT03606967)
Montanide ISA™ 51	Water in oil emulsion adjuvant (Mineral oil and mannide monooleate surfactant)	Demonstrated to promote antigen specific antibody titres, CD4 ⁺ and CD8 ⁺ T cells ^{138,192,193} .	Depot effect of antigen proposed	Phase I (NCT03929029) (NCT01176474) (NCT01176461) (NCT04298606) (NCT04270149) (NCT02193347) (NCT02737787) (NCT03893903) Phase I/II (NCT03617328) (NCT05642195) (NCT02229084) (NCT02938442) (NCT04106115) (NCT02955290) (NCT02960230) (NCT02818426) (NCT03047928) Phase II

				(NCT05243862) (NCT02455557)
Montanide ISA™ 720	Water in oil emulsion adjuvant (Squalene and mannide monooleate surfactant)	Less induction of antigen specific antibody titres and IFN γ responses than Montanide™ 51 ¹⁹⁴	Depot effect of antigen proposed	Phase I (NCT01376505) (NCT04432207)
GM-CSF	Cytokine influencing differentiation of myeloid cell populations, with a focus on DCs	Promotes differentiation, expansion, and activation of APC subsets	Promotes recruitment and differentiation of APC subsets, predominantly DCs, and subsequent APC:T cell interactions ¹⁸⁹	Phase I (NCT04270149) (NCT02193347) (NCT00809588) (NCT05101356) NCT05269381 NCT05641545 Phase I/II (NCT01784913) (NCT00458536) (NCT03382977) Phase II (NCT03376477) (NCT026365827)) (NCT03012100) (NCT00799110) (NCT05242965) (NCT02465268) (NCT05455658) (NCT05075122) (NCT04382664) (NCT00101101)

CAF09	Liposomal adjuvant composed of cationic DDA and monomycoloyl glycerol analogue (CLR ligand) lipids, and Poly-IC	Induction of Th1 and some CD8 ⁺ T cells (better cellular responses induced following mucosal administration) alongside antibody responses ^{178–180} .	Depot at injection site. Targeting of migratory CD103 ⁺ and CD8α ⁺ DCs to facilitate cross-priming ¹⁷⁹ .	Phase I/II (NCT03715985)
IL-2	Pleiotropic cytokine which promotes the activation, differentiation, and expansion of T cells	Promotes the activation and expansion of both CD4 ⁺ and CD8 ⁺ T cells. Can promote expansion of both anti and pro-tumourigenic T cell subsets ⁹⁰	Many of the strategies utilise autologous APCs modified to express elevated IL-2 levels, promoting more efficient expansion of activated anti-tumour T cells.	Phase I (NCT04318379 vaccine for chronic HCV) Phase I/II (NCT00703222) Phase II (NCT00101101) (NCR02487550) (NCT02919644) (NCT01697527)
CD40 agonist (Including CDX-1140)	Agonists of CD40 constituting recombinant CD40L or targeted monoclonal antibodies.	Enhanced activation of T cell responses by APCs	CD40 signalling upregulates costimulatory molecule expression and cytokine production in APCs ¹⁹⁵ .	Phase I/II (NCT04364230) Phase II (NCT00101101)

Imiquimod	Synthetic imidazoquinoline TLR 7/8 agonist	Promotes activation and recruitment of innate APCs and effector immune cells ¹⁶¹	Induction of type I IFN, IL-6 and TNF release. Subsequent activation of APCs and priming of adaptive immunity ¹⁶¹	Phase I (NCT05641545) Phase II NCT00799110
Resiquimod	Synthetic imidazoquinoline TLR 7/8 agonist	Higher induction of cytokine production by APCs when compared to imiquimod ¹⁹⁶	Induction of type I IFN, IL-6 and TNF α release. Subsequent activation of APCs and priming of adaptive immunity ¹⁶¹	Phase II (NCT01204684)
Flt3L (Including CDX-301)	Hematopoietic cytokine promoting differentiation of progenitor cells into myeloid and lymphoid cell populations. Key role in DC and NK cell differentiation	Systemically administered Flt3L increases the numbers of circulating and resident APCs, thereby promoting response to vaccination ⁴³ .	Promotes differentiation of progenitor cells into myeloid and lymphoid cell populations that mediate effects of vaccination.	Phase I/II (NCT03206047) (NCT03789097)
AS01	Liposomal with cholesterol, QS-21 saponin and MPL	Induction of Th1 and antibody responses	Not fully defined. Roles suggested for subcapsular sinus (SCS) LN macrophages, with caspase 1 activation,	Phase I/II (NCT03382977)

			DAMP release, and IL-12/18 in these cells. This promotes production of IFN γ in the LN and downstream activation of cDC1 populations ^{197,172} .	
<u>Adjuvant</u>	<u>Description</u>	<u>Immune response</u>	<u>Mechanism of action</u>	<u>Ongoing trial details</u>
Therapeutic <i>in situ</i> cancer vaccines				
Poly:ICLC	Synthetic doubled stranded RNA complexed with poly-L-lysine for stability. A TLR3 and MDA5 agonist.	Upregulation of IFN signalling pathways in tumour, increased APC trafficking and NK/T cell activation profiles in patients ¹⁵¹	Proposed activation of NK cells and APCs promoting cytokine production and antigen presentation. Resulting activation and recruitment of CTLs to tumour ¹⁵¹	Phase I (NCT04525859) (NCT04616248) Phase I/II (NCT03789097)
Glucopyranosyl lipid A (G100)	Synthetic TLR4 agonist (GLA) administered in an oil-in-water emulsion	Still unclear clinically but abscopal anti-tumour T cell recruitment noted indicating	Promotes maturation of APCs and production of type I IFNs, TNF α and IL-6. Priming of adaptive	Phase II (NCT02406781)

		induction of adaptive immune responses ¹⁹⁸	immunity indicated by abscopal therapeutic benefit ¹⁹⁸	
SD-101	Synthetic CpG ODN (Class C) TLR9 agonist	Increased tumour infiltration of active CD8 ⁺ T cells, Th1, NK and B cells ^{153,154} .	Activation of pDCs via TLR9 signalling, subsequent type I IFN production and promotion of APC function	Phase I (NCT05607953) (NCT04935229) (NCT03410901) (NCT03831295) (NCT04050085) Phase I/II (NCT05220722) (NCT02927964) (NCT03007732) Phase II (NCT01042379)
IMO-2125/ Tilsotolimod	Synthetic CpG ODN TLR9 agonist	Intratumoral IFN signatures, APC maturation and T cell priming ¹⁹⁹	Type I IFN signalling in tumour promotes function of APCs and subsequent priming of anti-tumour T cells ¹⁹⁹	Phase I (NCT04270864) Phase II (NCT04126876) (NCT03865082) **Phase III trial terminated due to lack of efficacy (NCT03445533)
BMS-986301	Synthetic CDN STING agonist	No clinical data on immune response induced. Impressive tumour clearance in	No mechanistic data published. Mechanism likely dependant on STING activation, subsequent type I IFN production,	Phase I (NCT03956680)

		preclinical models reported with increased anti-tumour T cell activity ²⁰⁰	APC maturation and promotion of anti-tumour adaptive immunity.	
GSK 3745417	Non-CDN STING agonist. Utilises dimeric aminobenzimidazole scaffold	No preclinical/clinical data published	No preclinical/clinical data published	Phase I (NCT03843359) ((NCT05424380)
exoSTING	Exosomes loaded with CDN STING agonist	Local activation and trafficking of APCs, with elevated ISG pathways in patients. Intratumoral recruitment of CD8 ⁺ T cells. ²⁰¹	Retention of agonist and enhanced uptake in tumour facilitated by exosomal delivery. APC function enhanced through type I IFN signalling and subsequent induction of CD8 ⁺ CTLs and Th1 cells ²⁰² .	Phase I/II (NCT04592484)
IMSA-101	Synthetic CDN STING agonist	No preclinical/clinical data published	No preclinical/clinical data published	Phase I/II (NCT04020185)
BI 1387446	Synthetic CDN STING agonist	No preclinical/clinical data published	No preclinical/clinical data published	Phase I (NCT04147234)

IP-001 (N-dihydrogalactose-chitosan)	Glucosamine polymer with galactose molecules attached to amine groups	Company reports that preclinical data suggests activation of intratumoral APCs via type I IFN signalling, and generation of anti-tumour CD8 ⁺ and Th1 responses	No data published. Induction of type I IFN signalling likely drives APC maturation, facilitating T cell priming.	Phase I/II (NCT03993678)
IL-2	Pleiotropic cytokine which promotes the activation, differentiation, and expansion of T cells	Intratumoral IL-2 therapy drives expansion of tumour infiltrating T cells. A caveat being that this can be both effector and regulatory cells.	IL-2 is a growth factor for T cells. IL-2 can preferentially activate Tregs if not targeted to effector subsets ²⁰³ .	Phase II/III (NCT03928275)
Flt3L (Including CDX-301)	Hematopoietic cytokine promoting differentiation of progenitor cells into myeloid and lymphoid cell populations. Key role in DC and	Intratumoral delivery of Flt3L can increase the infiltration of intratumoral DCs.	Promotes differentiation of progenitor cells into DC populations in the tumour.	Phase I (NCT04616248) Phase I/II (NCT03789097)

	NK cell differentiation			
CD40 agonist (CDX-1140)	CD40 targeted monoclonal antibody	Enhanced activation of T cell responses by APCs locally	CD40 signalling upregulates costimulatory molecule expression and cytokine production in APCs ¹⁹⁵ .	Phase I (NCT04616248)

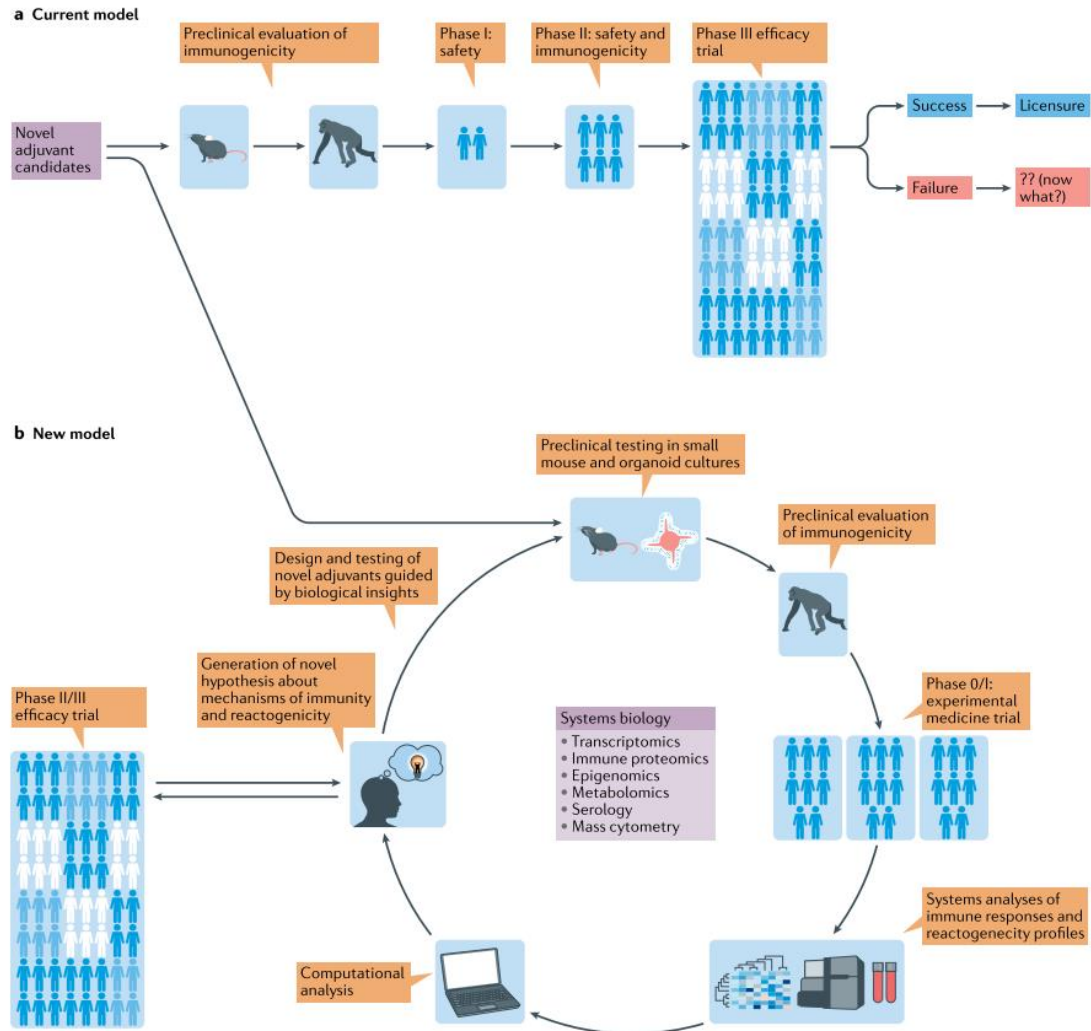


Figure 1.4 Alternative model of adjuvanted vaccine development

A) Current vaccine design follows a linear progression from preclinical to clinical research, with little retrospective analysis. This model is inefficient and leaves little opportunity to gain insights into how the vaccine and/or adjuvant can be improved. B) A new model is proposed maintaining robust pre-clinical

testing coupled to subsequent small-scale clinical trials. Systems biology is used to obtain immunological readouts that can be used inform vaccine and/or adjuvant optimisation and improvement before further trialling. Figure taken from Pulendran et al¹⁶⁸.

1.2.1 Polymeric nanoparticle adjuvants

Polymeric particulate adjuvants have been extensively studied in preclinical models and many microparticle and nanoparticle formulations have been shown to possess adjuvant properties. Several physicochemical parameters have been suggested to contribute to particulate adjuvanticity, including surface charge, shape, and most importantly for this project – size^{204,205}. Deciphering the impact that these parameters have on adjuvanticity and characterising the molecular and cellular mechanisms underlying their impact is a key aspect of rational polymeric particulate adjuvant design. This is crucial as despite decades of research, there is neither a licenced polymeric particulate adjuvant for use in vaccines, nor any polymeric particulate adjuvant currently in clinical trials.

Nanoparticle size has been shown to modulate uptake by responding cell types, with smaller particles in a viral size range, <200nm, taken up by endocytosis and those >500nm taken up through phagocytosis²⁰⁶. Smaller size can mediate more efficient drainage to lymph nodes, with smaller nanoparticles (<100nm) able to efficiently drain to lymph nodes independent of APC uptake, reaching high proportions of lymph node resident DCs within 24-48hours²⁰⁷, far quicker than larger counterparts which often interact with DCs locally at the injection site²⁰⁸. It is worth noting that nanoparticles larger than 5-10nm are not capable of entering the bloodstream from healthy tissues or indeed vice versa but can enter lymphatic vessels. Once inside the lymph nodes, different localisations according to size can be observed with small nanoparticles often preferentially associated with CD8 α ⁺ DCs²⁰⁸.

There are conflicting reports on the impact that polymeric nanoparticle adjuvant size exerts on resultant immune responses²⁰⁵. OVA antigen and CpG loaded 300nm poly-lactic co-glycolic acid (PLGA) particles have been previously shown to induce more robust antigen-specific CD8⁺ T cell and IgG responses when compared to 1 μ m, 7 μ m and 17 μ m sized counterparts in a murine vaccination model²⁰⁹. Similarly two studies have shown that 230nm sized (measured following antigen conjugation) nanoparticles, made from polystyrene²¹⁰ or lecithin/glyceryl monostearate emulsions²¹¹ were identified as potent drivers of antigen-specific antibody and CD8⁺ T cell

responses with anti-tumour functionality^{210,211}. However in other studies utilising polymeric nanoparticles, larger particles have outperformed smaller sizes in eliciting cellular and humoral immune responses, or exerted comparable effects, leading to a lack of consensus in the field²⁰⁵. Some of these contradictory results likely stem from different methods of antigen addition (ultimately affecting particle size), different vaccination routes utilised, and different metrics used to measure induction of CMI (e.g., the use of IgG1/IgG2a ratios to measure Th1/Th2 responses, no measurement of CD8⁺ T cell responses).

Size has also been shown to alter immune responses induced by other adjuvants, alum nanoparticles loaded with CpG and OVA in the 90nm size range were shown to be able to induce cross priming of CD8⁺ T cells and mount anti-tumour immune responses in mouse models²¹², although this response was dependent on the inclusion of CpG in the formulation. The emulsion adjuvants, MF59 and AS03, are composed of droplet sizes that are on average 155-160nm in diameter²⁰⁴. Using a similar oil-in-water emulsion it was shown that droplets in this 160nm size range were more effective than those 20nm or 90nm in size, in inducing antigen-specific antibody titres and TNF α ⁺ CD4⁺ T cell responses, although induction of IFN γ ⁺ CD4⁺ was comparable between groups²¹³.

Throughout this thesis polystyrene particles are principally used due to cost effectiveness, excellent stability, and uniformity in size, but questions over biocompatibility due to an inability to be degraded *in vivo* hinder translatability. Validation of responses seen when polystyrene particles are used using biodegradable PLGA is therefore an essential step for clinical translatability. PLGA is readily broken down via hydrolysis into lactic and glycolic acid and as a result has excellent biocompatibility²¹⁴ leading to approval from the FDA and EMA for use in humans in drug delivery approaches. Through close collaborations with Prof Yvonne Perrie, University of Strathclyde, and now with the Vaccines Formulation Institute, Geneva, 50nm PLGA nanoparticles with different charges and excellent uniformity in size have been obtained, a breakthrough as prior to this, obtaining 50nm PLGA nanoparticles with good uniformity in size had been a substantial hurdle. Cutting edge continuous flow microfluidics technology (NanoassemblrTM)²¹⁵ is used to prepare these PLGA nanoparticles. Additionally, these particles can now be freeze dried, retaining size and adjuvanticity upon resuspension, combatting logistical issues surrounding stability and storage.

This thesis sought to investigate the mechanisms underlying this polymeric particle size dependant adjuvanticity and investigate the suitability of 50nm NPs as adjuvants in prophylactic and therapeutic cancer vaccine approaches.

1.2.2 Highly deacetylated chitin derived adjuvants

Chitosan is a cationic polysaccharide derived through the deacetylation of chitin, one of the most abundant polymers on earth, found in the shells of insects and crustaceans and the cell walls of fungi. This deacetylation can be achieved chemically, predominantly using alkaline reagents, or enzymatically using chitin deacetylases, to convert acetyl groups to amine groups²¹⁶ with chitosan denoting polymers with $\geq 50\%$ of acetyl groups removed (Fig 1.5). Chitosan has been explored for use as a delivery agent and vaccine adjuvant owing to its biocompatibility, biodegradability/excellent safety profile, its adjuvanticity^{217–219}, and additionally its mucoadhesive properties – suggesting suitability in mucosal vaccination/delivery approaches²²⁰. Chitosan has already received FDA approval for use in bandages and in food supplements, signalling promising clinical translatability.

The Lavelle Lab has previously characterised Protasan CL213, a commercially available chitosan salt with an 86% degree of deacetylation (DD), as an effective vaccine adjuvant when delivered via the intraperitoneal and intranasal routes, eliciting antigen-specific Th1 and antibody responses (in particular IgG2c)²¹⁸. This adjuvanticity was lost in mice deficient in IFNAR, cGAS or STING, and *in vitro* the presence of mitochondrial ROS (mtROS) and cytoplasmic DNA was required for Protasan mediated activation of BMDCs through type I IFN and IFNAR signalling²¹⁸. Chitosan mediated activation of the cGAS-STING pathway results in the secretion of type I IFNs, CXCL10, and the maturation of APCs as measured by upregulation of CD40, and CD80/86^{218–220}.

To further develop these chitin-derived adjuvants utilising rational design principles, the impact of polymer charge on adjuvanticity was previously investigated in our lab group. As mentioned, chitosan can be enzymatically or chemically deacetylated, removing negatively charged acetyl groups and generating amine groups which are protonated and bear a positive charge under physiological conditions. Therefore, the higher the DD, the higher the net positive charge of the chitosan polymer will be. These positively charged amine groups on chitosan have been shown to have a stronger interaction and disruptive effect on lipid bilayers²²¹.

Through close collaborations with Dr Mats Andersen, Flexichem AB, chitosan polymers with varying levels of deacetylation have been obtained, of particular relevance to this project are the highly deacetylated polymers C90 and C100 – with 90% and 100% DD respectively. Both C100 and C90, have yielded promising results both *in vitro* and *in vivo* when injected with antigen via the s.c route^{219,222}, both proving to possess more potent adjuvant properties than Protasan CL213²¹⁹. Recently our lab, through the work of Dr Joanna Turley have further dissected chitin-derived polymer adjuvanticity. These polymers were shown to exert endoplasmic reticulum (ER) stress in BMDCs with resultant release of ER Ca²⁺, leading to Ca²⁺ mediated mitochondrial fission and release of mtROS, with subsequent activation of STING and release of type I IFNs, inducing the maturation of BMDCs²²³. Increasing polymer deacetylation was shown to increase mitochondrial stress and mtROS release, and promote more effective maturation of BMDCs^{223,224}. These *in vitro* results correlated with *in vivo* data following subcutaneous vaccination whereby the degree of deacetylation was instructive in a chitosan polymers ability to induce antigen specific Th1 responses²²⁴ with C100 identified as the most potent candidate adjuvant. This research critically identified the induction of mitochondrial stress *in vitro* as an effective metric for measuring chitosan polymer adjuvanticity. In addition to the induction of mitochondrial stress and DNA release, nuclear DNA damage and release was also implicated in the adjuvanticity of C100^{223,224}. This research culminates in identifying highly deacetylated chitosan polymers, in particular C100 as novel and potent vaccine adjuvants. Furthermore, STING has recently been identified as a potential target for cancer therapies, owing to its immunomodulatory properties, and as a result there has been an increase in STING activating molecules being trialled as cancer therapeutics, which Le Naour et al¹⁵⁵ have recently reviewed, and will be discussed in more detail later.

This thesis sought to determine the suitability of highly deacetylated chitosan polymers as adjuvants in both prophylactic and therapeutic cancer vaccine approaches.

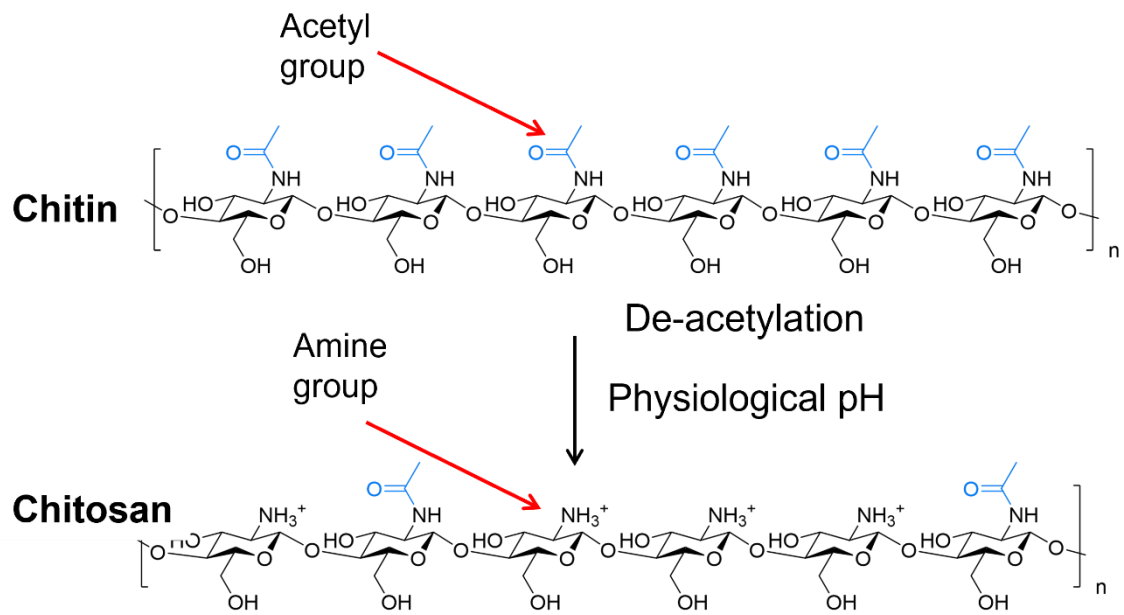


Figure 1.5 Chemical/Enzymatic generation of chitosan (Chemical drawings by Dr Mimmi Lundahl)

1.3 Immunogenic cell death pathways

Research into cell death has exploded over the last few decades, bringing with it a deeper understanding of the essential role that cell death plays in homeostasis and its intricate ties to the immune system. Cell death modalities can be broadly broken down into two distinct types: accidental cell death (ACD) and regulated cell death (RCD)²²⁵ (Fig.1.6). Necrosis is a prime example of ACD; lysis of the plasma membrane occurs, and the intracellular milieu is released. *In vitro* necrotic cell death can be recapitulated through exposure of cells to stressful stimuli e.g. extreme temperatures, pH or mechanical forces, and necrotic cell death frequently arises in our bodies, for example during trauma, infections, and tumour growth²²⁶. While necrotic cell death is inflammatory it crucially lacks immunogenicity, and as a result we see a poor capacity for necrotic cell death to stimulate antigen-specific adaptive immune responses²²⁷, providing a safeguard against the development of autoimmunity.

There have been a wide range of RCD modalities described, the most prominent forms being apoptosis, necroptosis, and pyroptosis (Fig.1.6), these RCD forms may also fall into the bracket of immunogenic cell death (ICD), if certain criteria are fulfilled, providing antigenicity and adjuvanticity, resulting in the capacity to mount adaptive immune responses to antigens expressed by the dying cell. This is fundamentally centred on the production and release of damage associated molecular patterns (DAMPs) which will be discussed in more detail later. ICD is now appreciated as the backbone on which many cancer therapies involving radiotherapy and chemotherapy rely^{228,229}. The gold standard for determining ICD is through the vaccination of mice with killed tumour cells, with subsequent tumour challenge with the same cell line, as described in Løve Aaes et al²²⁷. If cells die through ICD, then mice will be protected from the tumour challenge as tumour specific adaptive immune responses will have been mounted following vaccination.

Apoptosis

Apoptosis is a form of RCD stimulated either intrinsically, in response to perturbations in cellular homeostasis or extrinsically, principally mediated through ligation of death receptors²²⁵. Apoptotic cell death arises mainly downstream of initiator caspase 9 or 8-mediated activation of the executioner caspases 3 and 7. Apoptotic cell death is predominantly an immunologically quiet form of cell death, with the formation of apoptotic bodies which are subsequently cleared by phagocytes, promoted through the sensing of flipped phosphatidylserine (PhS) on the cell

surface, without the induction of inflammation and sometimes promoting the induction of immunological tolerance instead²³⁰. There are variations of apoptotic cell death which can shift towards ICD, termed immunogenic apoptosis, examples being the apoptotic cell death induced by some chemotherapeutic agents including doxorubicin, oxaliplatin and mitoxantrone²²⁹. This immunogenicity is due to the expression and release of DAMPs in dying cells that can promote adaptive immune responses.

Necroptosis

Necroptosis is another RCD pathway, initiated downstream of death receptor signalling, with TNF α and FAS ligand binding two prominent drivers via their cognate receptors. RIPK3 is activated, predominantly via RIPK1 and subsequently activates MLKL via phosphorylation. Phosphorylated MLKL translocates and binds to phospholipids in the cellular membrane to form pores which destabilise membrane integrity²³¹. Necroptotic and apoptotic cell death pathways are intricately tied, as RIPK1 and RIPK3 activity can also promote apoptotic cell death. Caspase 8 plays a crucial role in this interplay, directing cells towards apoptosis and suppressing necroptosis^{232,233}. Necroptosis has been shown to be an ICD pathway, eliciting potent anti-tumour immunity in vaccination models^{227,234}.

Pyroptosis

Of particular relevance to this project however is pyroptosis, a RCD pathway with a key role in host immunity to intracellular pathogens²³⁵ (Fig.1.7). Pyroptosis can be elicited via a canonical or non-canonical pathway, through PRR mediated sensing of PAMPs, DAMPs and HAMPs (Homeostasis altering molecular processes)²³⁶. The canonical pathway involves the assembly of canonical inflammasome complexes, of which the NLRP3 inflammasome is perhaps the best characterised in the pyroptotic pathway. Inflammasome components assemble in response to cognate PRR stimuli in the cytosol or cellular stress, with recruitment and assembly facilitated via nucleotide binding domains (NBD), and some inflammasomes additionally recruit the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) via pyrin domains (PYD) interactions. Pro-caspase 1 is then recruited via caspase-activation and recruitment domain (CARD) domains in the recruited ASC molecules e.g. NLRP3, or through CARD domains on the PRR molecules themselves e.g. NLRC4 inflammasome²³⁶. Pro-caspase 1 monomers can trans-activate via enzymatic cleavage and activated caspase 1 can then cleave IL-1 β , IL-18 and gasdermin D (GSDMD) molecules into their mature active form. Activated GSDMD

monomers dimerise and insert into the cellular membrane via binding to phosphoinositides (PIPs), with subsequent oligomerisation to form pores, and if sufficient pores are formed, membrane integrity is lost. GSDMD has been recently characterised as a potential cellular redox sensor, as cellular reactive oxygen species (ROS) have been shown to mediate the oxidation of the Cys191/192 residue of GSDMD which sensitises the protein for subsequent activation²³⁷. This provides a crucial link between the induction of cellular stress and the elicitation of pyroptotic cell death.

Alternatively, pyroptosis can be elicited via the non-canonical route via caspase 11^{238,239} (Human caspase 4 and 5 ortholog) mediated activation of GSDMD. Caspase 11 possesses an N-terminal CARD domain and a C-terminal catalytic domain, and upon ligand binding to the CARD domain, caspase 11 undergoes dimerisation via the CARD domain followed by oligomerisation and then proximity induced autoproteolysis. This results in the cleavage of the CARD domain and the remaining activated catalytic subunit of caspase 11²⁴⁰. Activated caspase 11 can then mediate the cleavage of GSDMD, IL-18 and possibly IL-1²⁴¹. Caspase 11 acts as an intracellular sensor for LPS, however oxidised phospholipids (oxPAPCs) have been identified as endogenous ligands. While oxPAPCs have been shown to activate the non-canonical inflammasome, they have not been identified to induce pyroptotic cell death, instead inducing a state of cellular hyperactivation, whereby IL-1 family cytokines are released from GSDMD pores without progression to pyroptotic cell death²⁴². This may be attributed to a lower magnitude of GSDMD activation, and resultant lower disruption to membrane integrity. Cells possess membrane repair machinery, and endosomal sorting complexes required for transport (ESCRT) proteins have been shown to inhibit the induction of pyroptosis following GSDMD pore formation²⁴³. Successful induction of membrane lysis in these settings likely hinges on NINJ1, a cell surface protein with transmembrane domains, that has been recently identified as a crucial determinant of plasma membrane rupture. NINJ1 was found to play a key role in the release of DAMPs too large for pore escape²⁴⁴. These findings were demonstrated following induction of necrosis, secondary necrosis and pyroptosis. The role of NINJ1 in determining whether cells undergo pyroptosis or hyperactivation has yet to be investigated, but a threshold of activation through loss of membrane fluidity may be a crucial determinant.

The canonical and non-canonical pyroptotic pathways are intricately linked to each other, and activation of the non-canonical pathway often leads to subsequent activation of its canonical counterpart via perturbations of cellular homeostasis and NLRP3 activation²³⁸. Pyroptosis has been demonstrated to be a form of ICD that can initiate potent adaptive immune responses against tumours^{245,246}. In addition another gasdermin protein, gasdermin E (GSDME) can

mediate secondary pyroptosis downstream of caspase 3 activation²⁴⁷, with activation of GSDME additionally noted in GSDMD KO cell lines upon stimulation with pyroptotic stimuli²⁴⁸.

It is worth noting however that while some of the RCD pathways listed above have been demonstrated to be forms of ICD, there is some context dependency, and this arises from the unique release of and response to DAMPs following death stimuli.

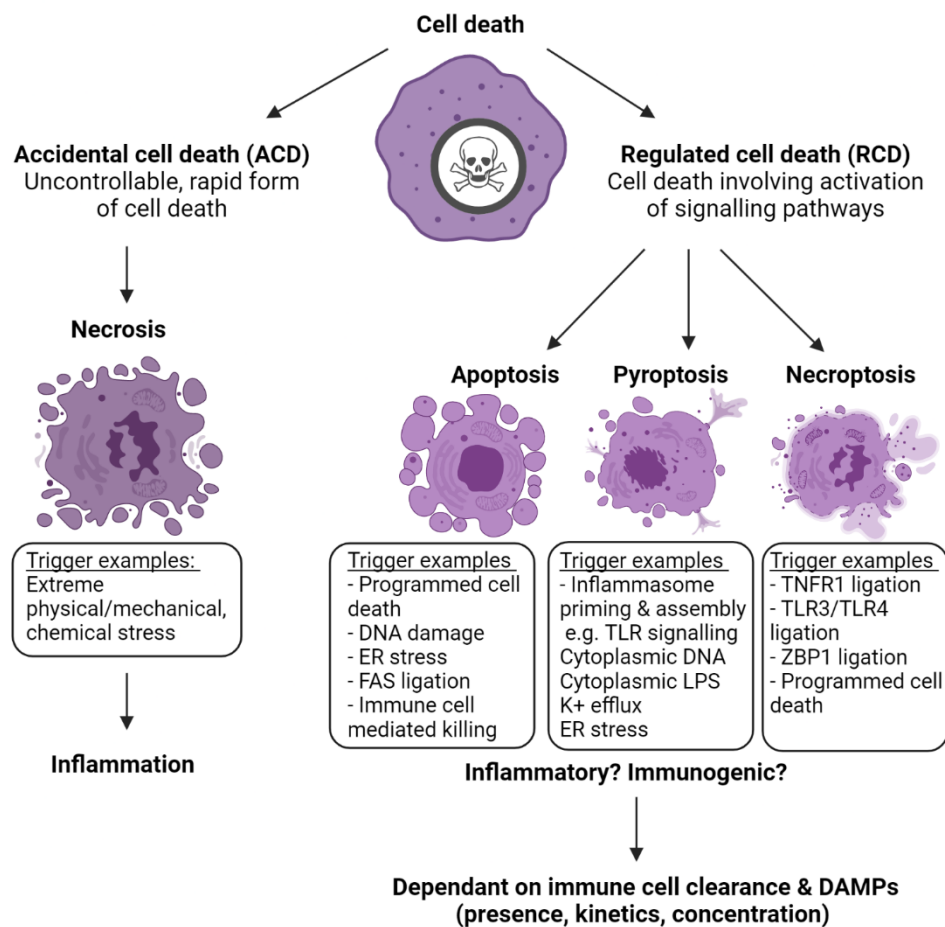


Figure 1.6 Main cell death modalities

Cell death can be principally categorised into accidental cell death (ACD) and regulated cell death (RCD). The former is uncontrolled and often arises in instances of extreme physiochemical cellular stress, leading to inflammatory immune responses but crucially, predominantly no induction of antigen specific adaptive immune responses. The activation of cell death signalling pathways is indicative of RCD, and this category of cell death encompasses multiple cell death modalities, with apoptosis, necroptosis and pyroptosis constituting the main forms. The immunogenicity of a cell dying through an RCD pathway is critically determined by the presence of DAMPs alongside their relative concentrations and kinetics of their release.

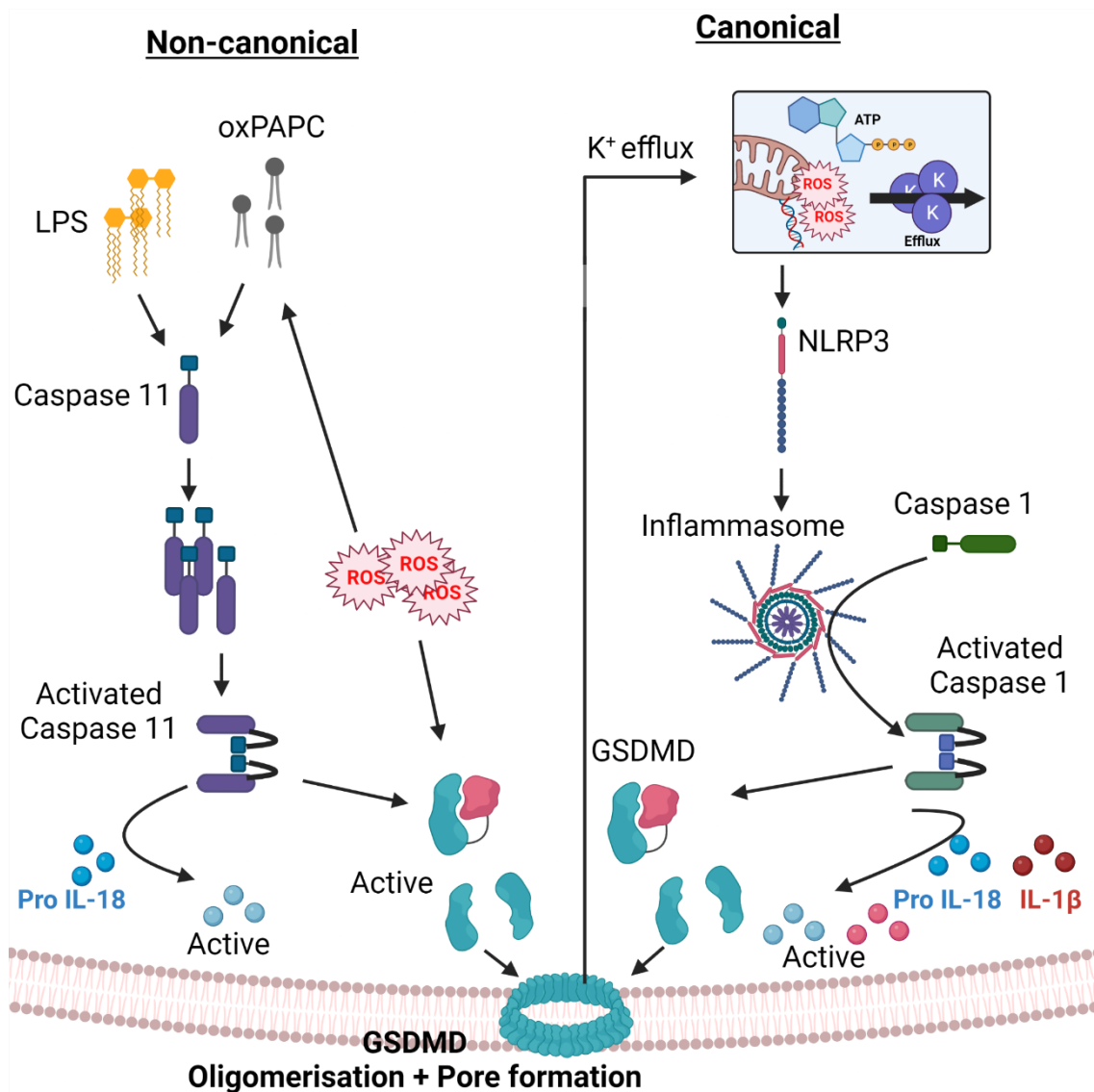


Figure 1.7 The canonical and non-canonical pyroptotic pathway

The non-canonical pyroptotic pathway predominantly involves the intracellular sensing of LPS by caspase 11 (caspase 4,5 in humans). Caspase 11 is then activated and can cleave IL-18 and GSDMD, leading to pore formation. The non-canonical pathway of inflammasome activation often leads to the activation of the canonical pathway of activation, with potassium ion efflux and subsequent NLRP3 activation implicated. Canonical pyroptosis occurs downstream of inflammasome priming and assembly, with the NLRP3 inflammasome used here as an example. Perturbations in cellular homeostasis leading to potassium efflux, extracellular ATP sensing and presence of mitochondrial ROS/DNA have all been implicated in the activation and assembly of the NLRP3 inflammasome. Caspase 1 is recruited to the inflammasome and is subsequently cleaved into its active form which cleaves IL-1, IL-18 and GSDMD into their active forms. GSDMD can form pores in cellular membranes through oligomerisation and can lead to membrane lysis and pyroptotic cell death.

1.3.1 Danger associated molecular patterns in ICD

DAMPs are essential molecules in promoting the recruitment of immune cells to sites of tissue damage and cellular stress. In the context of sterile inflammation, this plays a crucial role for recruitment of immune cells which will mediate a wound healing response. However on the other hand, in the context of infection and cancer, the release of DAMPs has been implicated in mounting adaptive immune responses against pathogens/ tumours²²⁹.

The immunogenicity of dying cells is critically determined by the adjuvanticity provided by the processing and release of DAMPs, including calreticulin (CALR) exposure, and release of ATP, high-mobility group box 1 (HMGB1), type I IFN, annexin A1 (ANXA1), and IL-1 family cytokines^{225,249} (Fig.1.6). The concentration of these DAMPs and kinetics involved in their release play a crucial role in determining whether adaptive immune responses are elicited.

DAMPs function predominantly in 1) promoting the recruitment of APCs to sites of ICD, alongside their maturation, for example ATP mediated recruitment and maturation of APCs via P2 receptors²⁵⁰, ANXA1 via formyl peptide receptor 1²⁵¹ and type I IFNs via IFNAR²⁵². 2) Encouraging the uptake of dying/dead cells via phagocytic/scavenger receptors e.g. CALR via LRP1^{253,254} and F-actin via Clec9a²⁵⁵ and subsequent presentation of antigens, with a particular emphasis on cross presentation (Fig.1.8). If tumour cells release these signals in sufficient concentrations, this can result in the induction of anti-tumour immunity, indeed this is what many traditional chemotherapies rely upon for efficacy^{228,229}. Similarly, some forms of ICD can result in the release of chemokines such as CXCL10, which can result in the recruitment of T cells directly to the site of cell death²²⁹, and DAMPs like type I IFN can promote the reactivation of T cells and DCs in the TME. While the importance of these DAMPs has been studied extensively in the context of cancer chemo- and radiotherapies, deciphering their role in the MOA of vaccine adjuvants is crucial¹⁶⁸. To this effect, the release of extracellular DNA and ATP have been implicated in the adjuvanticity of alum and MF59 respectively^{256,257}.

The IL-1 family of cytokines have been proposed as 'canonical DAMPs'²⁴⁹, and it has been proposed that the efficacy of many other DAMPs relies on their presence/ release²⁴⁹. IL-1 family cytokines are not actively secreted and are only released in the context of pore formation^{258,259} or membrane lysis. Furthermore, except for IL-1 α and IL-33, IL-1 family cytokines require enzymatic cleavage for activation, supporting their identification as inducible DAMPs. This provides a safeguard against autoinflammation and the potential for autoimmunity. The

differential expression and release of IL-1 family cytokines and the array of IL-1 family cytokine receptors shapes how these cytokines can modulate adaptive immune responses²⁴⁹. For example, IL-18 and IL-33 favour Th1 and Th2 polarisation respectively, owing to the differential expression of their cognate receptors on responding cell types, and IL-1 β has also been shown to promote the expansion of Th1 and CD8⁺ T cells^{260–262}. In the context of vaccine adjuvants, both IL-1 and IL-18 have been implicated in the mechanism of action of the saponin based ISCOMATRIX and the TLR4 agonist containing emulsion, GLA-SE¹⁸⁶. Indeed, previous research in the Lavelle Lab conducted by Dr Katie O’Grady identified IL-33 as a negative regulator of 50nm nanoparticle adjuvanticity²⁶³.

The combination of cell death pathway elicited, and DAMPs released, act in concert to provide immunogenicity to a cell’s death. It is therefore not only essential to determine the cell death pathway elicited by vaccine adjuvants, but also to investigate the precise role of associated DAMPs to gain insights into adjuvanticity.

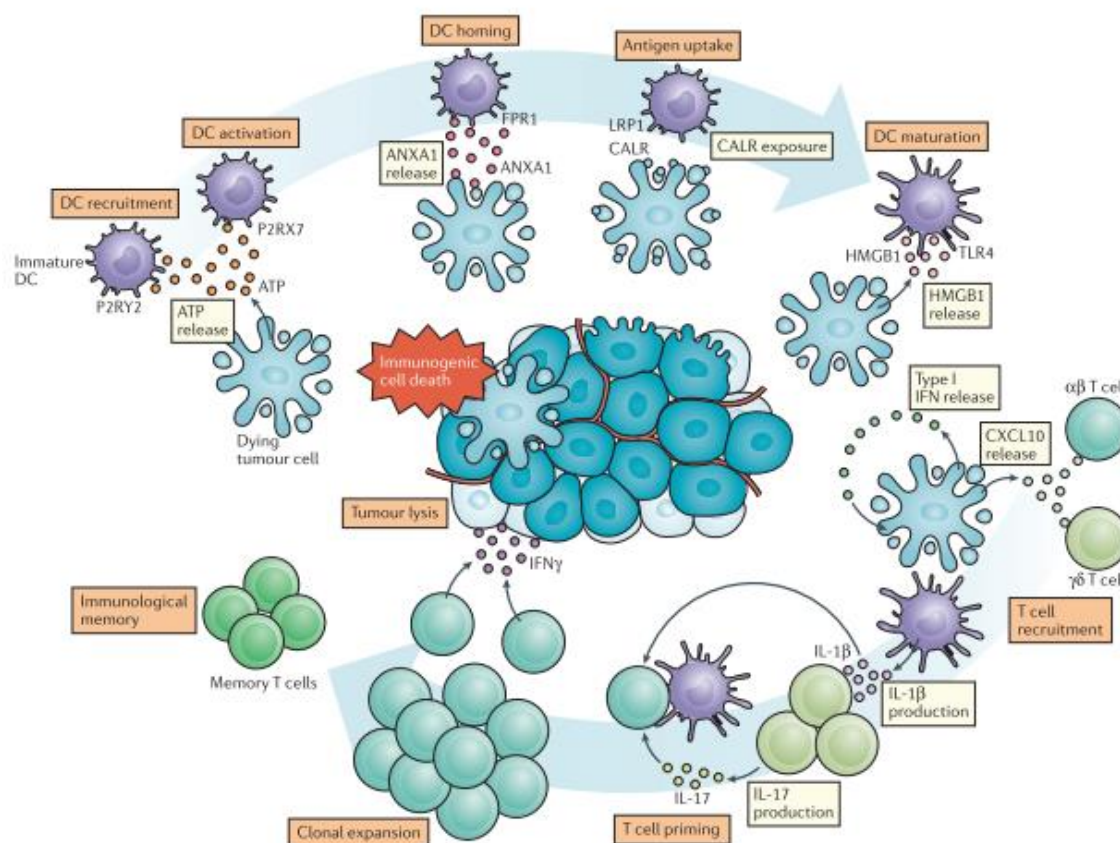


Figure 1.8 DAMP mechanisms contributing to ICD

Upon induction of ICD, cells release ‘find me’ DAMPs such as ATP and ANXA1 which contribute to the recruitment of immune cells to the site of insult. Exposure of CALR provides an ‘eat-me’ signal contributing

to enhanced uptake of dead/dying cells by APCs and Type I IFNs, IL-1 cytokines and HMGB1 contribute to APC maturation. DAMPs promote the upregulation of antigen presentation and the production of chemokines (e.g., CXCL10) which contribute to the recruitment of T cells. Finally, IL-1 family cytokines support the activation and expansion of T cell subsets. Figure taken from Galluzzi et al²²⁹.

1.4 The cGAS-STING pathway and anti-tumour immunity

cGAS is a widely conserved PRR that binds to cytoplasmic endogenous or exogenous double stranded DNA (dsDNA), frequently arising over the course of intracellular infection, cellular damage or frequently in tumour cells. cGAS upon dsDNA recognition and binding undergoes dimerisation and a conformational change in its activation loop switching it from its steady state autoinhibited form. This activation is threshold dependant, with clustering of complexes of cGAS and DNA into a liquid-like phase promoting activation of cGAS²⁶⁴. This dimerised active form of cGAS catalyses the formation of cyclic GMP-AMP (cGAMP), a cyclic dinucleotide (CDN) secondary messenger, from adenosine triphosphate (ATP) and guanosine triphosphate (GTP)²⁶⁵. cGAMP can then activate the adaptor protein STING, located on the ER membrane. STING activation results in oligomerisation and a conformational change, allowing the recruitment of TBK1, which phosphorylates STING in the C terminal domain²⁶⁶. IRF3 can then be recruited to the signalling complex and undergoes phosphorylation via TBK1, phosphorylated IRF3 undergoes dimerization. STING activation can also result in the activation of NFκB via IKK, and both activated IRF3 and NFκB translocate and act as transcription factors in the nucleus where they can promote the transcription of genes important in anti-viral and anti-tumour innate and adaptive immunity, for example type I IFN, downstream CXCL10 and multiple other interferon stimulated genes (ISGs). It is worth highlighting that for optimal induction of type I IFN signalling mediated by STING, STING translocation from the ER to the perinuclear region is required following activation²⁶⁷.

The cGAS-STING pathway is an attractive target for vaccine adjuvants aiming to elicit type I IFN responses, with chitosan a prominent example^{218,219}. While the role of cGAS-STING in promoting anti-tumour immunity will be focused on, it is worth noting that the cGAS-STING pathway has been implicated in the pathogenesis of some autoimmune diseases such as lupus, and indeed the inflammation associated with cGAS-STING activation has been tied to tumour progression and metastasis in some contexts²⁶⁸.

Owing to its anti-viral & anti-tumour functions, components of the cGAS-STING pathway are frequently downregulated in tumour cells²⁶⁹ or targeted by viral proteins²⁷⁰ as a mechanism of immune escape/evasion. cGAS activation and subsequent cGAMP production frequently arise in tumour cells in response to DNA leakage into the cytosol propagated by DNA and organelle damage. Recent studies have highlighted that the expression of cGAS alone is sufficient in tumour cells where STING signalling is impaired to initiate immune responses, as tumour derived

cGAMP can be taken up by phagocytes. This tumour derived cGAMP can be encountered upon lytic cell death, the uptake of dead/dying tumour cells, via its release in exosomes or via viral packaging²⁷¹, or via transfer to other cell types via gap junctions²⁷² (Fig.1.9).

Activation of the cGAS-STING pathway and subsequent type I IFN signalling plays a crucial role in promoting the induction and activity of anti-tumour immune responses. This is evident as STING signalling has been shown to be crucial for optimal NK cell mediated rejection of tumours in mice²⁵ as activation of cGAS-STING signalling in the TME was shown to promote NK cell activity via type I IFN signalling²⁵ (Fig.1.9) and similarly STING has been shown to be required for the effective clearance and early control of tumour cells by tumour infiltrating macrophages via phagocytosis²⁷³. The expression and activation of STING was also found to be required for the initiation of anti-tumour immune responses by tumour endothelial cells, mediated by downstream type I IFN production²⁷⁴ and similarly type I IFN production has also been shown be crucial for the spontaneous priming of cellular anti-tumour immunity mediated by the innate immune system^{275,276} principally mediated by CD8 α ⁺ DCs. In particular, the induction of spontaneous anti-tumour CD8⁺ T cell responses has been shown to be defective in STING^{-/-}, IRF3^{-/-}, and IFNAR^{-/-} mice^{275,277–280}.

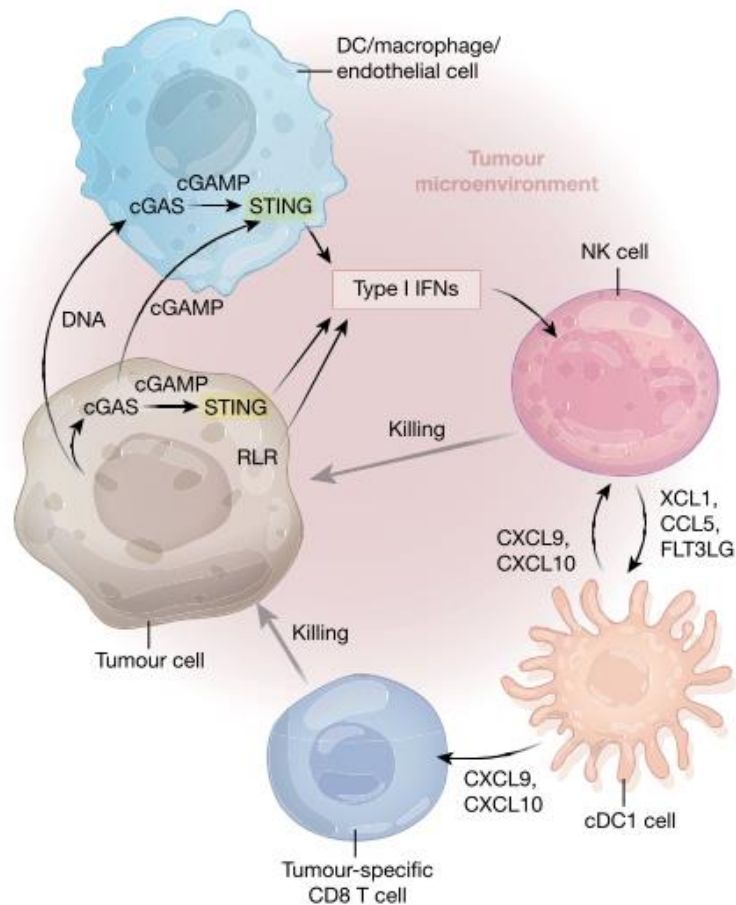


Figure 1.9 The cGAS-STING pathway plays a crucial role in the crosstalk between tumour cells and immune cells in the TME.

cGAMP is frequently produced in tumour cells and can activate STING intrinsically or in recruited immune cells via tumour cell uptake or cGAMP release via a variety of mechanisms. The cGAMP mediated activation of STING results in the production of Type I IFNs in the TME which potentiates NK cell anti-tumour activity and promotes the expression of cytokines (FLT3L) and chemokines (XCL1 and CCL5) which promote the infiltration of cDC1s and CD8⁺ T cells. In turn, the cDC1s produce chemokines (CXCL9 and CXCL10) which promote the infiltration of NKs and CD8⁺ T cells into the TME. This highlights the importance of the cGAS-STING pathway in the NK-cDC1-CD8⁺ T cell axis of tumour infiltration. Figure taken from Demaria et al²⁶.

1.4.1 Targeting the cGAS-STING pathway in cancer therapy

Traditional chemo and radiotherapies that elicit DNA damage likely activate cGAS STING via the presence of cytosolic DNA, and much of the efficacy of these therapies may be reliant on the downstream induction of immune responses²⁶⁸. Similarly the cGAS-STING pathway may play a role in the efficacy of checkpoint blockade therapies as cGAS KO mice have been shown to exhibit poor response to PD-L1 checkpoint blockade in a s.c B16 melanoma model, with responses restored and potentiated following co-administration of cGAMP²⁷⁹.

Directly targeting the cGAS-STING pathway for cancer therapy, utilising natural or synthetic CDNs or small molecule STING agonists has garnered much interest in academic and pharmaceutical led research. Proof of principle for these approaches has been provided in multiple preclinical studies involving intratumoral delivery of cGAMP and other STING agonists promoting effective anti-tumour immune responses, excellently reviewed by Le Naour et al¹⁵⁵. These results highlight the suitability of STING agonists for use as adjuvants in ISV approaches. However, despite preclinical success, so far clinical performances have been suboptimal with first generation STING agonists, with candidate agonists exhibiting poor clinical translatability. This is reflected in the fact that no cGAS-STING targeting candidate has successfully completed a phase III clinical trial. There are multiple next generation STING agonists in ongoing phase I/II clinical trials for use in ISV approaches (Table 1.6).

The most commonly known and trialled STING agonist is DMXAA, a modified derivative of flavone acetic acid (FAA)²⁸¹. Ultimately ISV with DMXAA alone or in combinatorial approaches has yielded clinically disappointing results¹⁵⁵. Subsequently DMXAA has been since shown to lack the capacity to activate the human STING protein²⁸², hence displaying poor mouse-human translatability. This translatability is an important consideration for the future development of STING agonists. In line with this, it should be noted that in contrast to the inbred mouse strains used for preclinical research, human STING expression is polymorphic²⁸³ and this has been shown to alter responsiveness to STING agonists. This necessitates agonists that can bind to the different STING haplotypes in the human population. To address this, next generation agonists have been developed. For example, E7766 is an intratumorally administered, macrocyclic bridge stabilised CDN agonist (stabilises the agonist in a conformation favourable to STING activation) that can activate the breadth of human STING haplotypes. E7766 recently completed a phase I clinical trial (NCT04144140) with results anticipated shortly.

Synthetic and natural CDNs have also been trialled clinically but have had underwhelming success. In addition to potential issues surrounding poor mouse-human translatability, poor bioavailability and stability²⁸⁴ have been proposed to underpin suboptimal clinical efficacy. ADUS100 is a prime example of a first generation CDN agonist which despite exhibiting promising preclinical anti-tumour activity when delivered intratumorally, ultimately had low efficacy in a clinical setting. CDNs are large, electronegative molecules that are as a result not conducive to cellular uptake, this is not only problematic due to a poor capacity to gain entrance and activate STING, but also that extracellular CDNs are rapidly degraded by phosphodiesterases like ENPP1. Pharmacokinetic data indicated that ADU-S100 was not retained at the intratumoral injection site, and was rapidly absorbed into the bloodstream²⁸⁵ which may be a valuable insight into its lack of efficacy. Next generation agonists have been developed to combat these issues surrounding cellular uptake, with a prominent example being exoSTING (Table 1.6) which aims to deliver CDNs into cells via packaging in exosomes.

Highly deacetylated chitin derived polymers may be attractive alternative cGAS-STING agonists for use as adjuvants in cancer vaccination approaches, possessing distinct advantages over the agonists detailed above. These polymers rely on endogenous DNA to activate the cGAS-STING pathway circumventing issues surrounding polymorphic STING expression in human populations. Furthermore, these polymers are readily taken up by cells and can activate STING in human cells²⁸⁶ which may indicate favourable mouse-human translatability and bioavailability (Data not shown).

1.5 Hypothesis and aims

The main hypothesis underlying this project is that 50nm polymeric nanoparticles and highly deacetylated chitin-derived polymers, are suitable vaccine adjuvants for use in cancer vaccination approaches.

The specific aims of this project are:

- To investigate mechanisms underlying 50nm NP adjuvanticity, with a particular emphasis on the potential role of pyroptotic machinery.
- To determine if 50nm NP induced adaptive immune responses have anti-tumour functionality, and if so to further characterise the contribution of specific cell types to this potential anti-tumour immunity.
- To establish whether highly deacetylated chitin-derived polymer-induced adaptive immune responses have anti-tumour functionality in ISV approaches.
- To identify the cellular mediators of this anti-tumour efficacy and the signalling pathways underlying their activation.

Chapter Two – Materials & Methods

2.1 Materials

2.1.1 General cell culture materials

Complete RPMI 1640 Medium (cRPMI)

Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo) was supplemented with 10% volume/volume (v/v) ultra-low endotoxin heat-inactivated filter sterilised foetal bovine serum (FBS) (MyBio), 2 mM L-Glutamine (Gibco), 50U/ml penicillin (Gibco) and 50µg/ml streptomycin (Gibco).

Complete DMEM culture medium (cDMEM)

Dulbecco's Modified Eagle Medium (DMEM) (Thermo) was supplemented with 2mM L-Glutamine, 50 U/mL penicillin and 50µg/ml streptomycin (Gibco) and 10% volume/volume (v/v) ultra-low endotoxin heat-inactivated filter sterilised FBS (MyBio).

Complete RPMI 1640 T cell Medium

RPMI 1640 medium (Thermo) was supplemented with 0.04mM β-mercaptoethanol (Gibco), 0.88mM sodium pyruvate (Gibco), 0.88mM L-Glutamine (Gibco), 4.4 U/mL penicillin (Gibco), 4.4µg/mL streptomycin (Gibco), 0.88% (v/v) 100X Minimum Essential Medium (MEM) Non-Essential Amino Acids (Gibco), 0.35% (v/v) 100X MEM Vitamins (Gibco) and 10% (v/v) heat-inactivated (56°C for 30 minutes) and ultra-low endotoxin heat-inactivated filter sterilised FCS (MyBio).

Granulocyte macrophage-colony stimulating factor (GM-CSF) was purchased from Peprotech.

Trypsin-EDTA was purchased from Sigma.

Hanks Balanced Salt Solution (HBSS) with sodium bicarbonate, without phenol red, calcium chloride and magnesium sulphate was purchased from Sigma

0.88% ammonium chloride (NH₄Cl)

8.8g of ammonium chloride was dissolved in 1L of endotoxin-free water (Baxter) and filter sterilised using a 0.22µm syringe-driven filter (Millipore).

Phosphate Buffered Saline (PBS) was purchased from Biosciences.

Cell counting

Cell viability was assessed via the Trypan Blue dye (Sigma) exclusion method, using a KOVA Glasstic cell counter slide with grids (VWR).

Table 2.1 Cell lines

Cell line	Source
B16F10	Prof Lydia Lynch, TBSI, TCD/ Harvard Medical School
B16F10-OVA	Prof Kinston Mills, TBSI, TCD
B16F10 (<i>Tmem173</i> ^{-/-})	Prof Roger Greenberg, Perelman School of Medicine, University of Pennsylvania
MC38	Kerafast
C2C12	ATCC

2.1.2 *In vitro* and *in vivo* treatments

Table 2.2: Adjuvants/Vaccine platforms used for *in vivo* studies

Adjuvant	Supplier
50nm Polystyrene (PS) nanoparticles	Phosphorex Inc, MA, USA
50nm poly-lactic co-glycolic acid (PLGA) nanoparticles	Prof Yvonne Perrie, University of Strathclyde
Alhydrogel (Alum)	Brenntag Biosector/ Croda
CpG ODN 1826	Invivogen
2'3'cGAMP	Invivogen

ProtasanCL213	Novamatrix, Norway
C90	Viscogel AB
C100	Viscogel AB
ChAdOX1-OVA	Dr Anita Milicic, Nuffield Department of Medicine, University of Oxford

Table 2.3: Antigens used for *in vivo* studies

Antigen	Supplier
Endotoxin-free ovalbumin (OVA)	Hyglos GmbH
MC38 peptide antigen: Aatf1 SFMAPIDHTTMSDDARTE	Thermo Fisher Scientific
MC38 peptide antigen: Adpgk HLELASMTNMELMSSIVHQ	Thermo Fisher Scientific
MC38 peptide antigen: Cpne1 GSNGDPSSPYSLHYLSPTGVNE	Thermo Fisher Scientific
MC38 peptide antigen: Rpl18 KAGGKILTFDRLALES PK	Thermo Fisher Scientific
MC38 peptide antigen: Rpl18 scr KGFGRLRSAPAITKLKEDL	Thermo Fisher Scientific

Table 2.4: Reagents used for *ex vivo* stimulations

Reagent	Supplier
Heat killed (HK) <i>E.coli</i> , strain BL21	Detailed below table
NA/LE Hamster anti-mouse CD3e	BD Pharmingen
Concanavalin A Type IV-S	Sigma

Preparation of HK *E. coli*

HK *E.coli* was previously prepared in the Lavelle Lab by previous lab member Dr Eimear Lambe. The BL21 strain of *E. coli* was spread onto an agar plate containing lysogeny broth (LB) and it was incubated overnight at 37°C. The culture was up-scaled to 100ml of LB broth which was

incubated for 2 hours while shaking at 37°C. Serial dilutions on LB agar plates were used to determine bacteria number and left to incubate overnight at 37°C, with subsequent enumeration of the number of colonies present. This was then multiplied by the dilution factor before being confirmed spectrophotometrically, measuring the optical density at 600nm. The bacteria were heat killed by incubation at 70°C for 15 minutes. The cells were pelleted by centrifugation then re-suspended in sterile PBS to make a final concentration of 1×10^9 cells/ml.

Table 2.5: Additional reagents used in *in vitro* studies

Reagent	Supplier
LPS (from <i>Escherichia coli</i> (<i>E.coli</i>) Serotype R515	Enzo life sciences
Nigericin sodium salt	Invivogen
Cycloheximide	Sigma
BV6 (SMAC mimetic)	Selleckchem
Z-VAD-fmk	Bachem
Necrosulfonamide (NSA)	Millipore
Dimethyl fumarate (DMF)	Sigma
Hydrogen Peroxide	Sigma

Table 2.6: Additional reagents used in *in vivo* studies

Reagent	Supplier
Cisplatin	Sigma
Ac-YVAD-cmk	Invivogen
Monoclonal anti-mouse CD8α antibody (Clone 2.43)	Bio X Cell/ Assay Genie
Monoclonal isotype control antibody (Clone LTF-2)	Bio X Cell/ Assay Genie
Monoclonal anti-mouse PD-1 antibody (Clone RMP1-14)	Bio X Cell/ Assay Genie
Monoclonal anti-mouse NK1.1 antibody	Assay Genie

(Clone PK136)	
Rat IgG2b isotype control	Assay Genie
Rat IgG2a isotype control	Assay Genie
TEMPOL	Tocris

2.1.3 Muscle/Tumour digestion reagents

Digestion media

Per sample: 1mL RPMI (with no FBS or HBSS with $\text{Ca}^{2+}/\text{Mg}^{2+}$), 20mg Type 1 collagenase (Gibco) and 100 μg DNase-1 .

5mM PBS-EDTA

500ml 1X PBS supplemented with 5ml of 0.5M EDTA (Gibco).

2.1.4 Flow Cytometry

FACS (Fluorescence activated cell sorting) buffer

FACS buffer consisted of 2% (v/v) FBS in 1X PBS.

OneComp eBeads used for single stains were purchased from eBioscience

Brilliant violet stain buffer was purchased from BD for use with brilliant violet (BV) antibodies.

4% Paraformaldehyde (PFA) was purchased from Santa Cruz Biotechnology

Table 2.7: Panel used for identification of antigen specific CD8^+ T cells following vaccination

Target	Clone	Fluorochrome	Volume per sample	Supplier
CD44	IM7	FITC	1 μl	BD Bioscience
F4/80 + B220	MCA497 + RA3-6B2	Al700	1 $\mu\text{l}/1\mu\text{L}$	BioRad + BD Bioscience

CD4	GK1.5	A780	1µl	eBioscience
Live/Dead	N/A	Aqua510	Final 1/500 dilution	BD Bioscience
CD11c	N418	BV605	1µl	eBioscience
CD3	17a2	BV650	1µl	Biolegend
CD8	H-2kb	PE	2.5µl	NIH Tetramer
SIINFEKL	tetramer		or	Core Facility
TCR	or		5µl	or
	H-2kb			ProlImmune
	pentamer			
CD8	MCA609P770	PE-A750	5µl	BioRad
*CD45	30-F11	APC	100µg per mouse for i.v or 1µl for standard staining	Biolegend
**NK1.1	PK136	BV421	1µl	Biolegend

* = used intravenously for residency experiment

** = used when including NK cell analysis

Table 2.8: Panel used to measure induction of cell ROS

Dye	Target	Concentration per sample	Supplier
CellROX™ Green	Cellular ROS	5µM	Thermo Fisher Scientific
MitoSOX™ Red	Mitochondrial ROS	1µM	Thermo Fisher Scientific
Aqua510	Viability	1/500 dilution	BD Bioscience

2.1.5 Enzyme-linked immunosorbent assay (ELISA) materials

IFN γ DuoSet® ELISA kit purchased from Biotechne/R&D

IL-10 ELISA MAX™ kit purchased from Biolegend

Sodium Carbonate buffer

4.2g NaHCO₃ and 1.78g Na₂CO₃ was dissolved in 1L distilled H₂O, pH 9.5.

Phosphate citrate buffer

10.19g anhydrous citric acid and 14.6g Na₂HPO₄ was dissolved in 1L distilled H₂O and brought to pH 5.5.

10X PBS

800g NaCl, 116g Na₂HPO₄, 20g KH₂PO₄ and 20g KCl was dissolved in 10L distilled H₂O and brought to pH 7.2.

Washing buffer

0.05% (v/v) Tween 20 in 1X PBS.

Blocking buffer

1% (w/v) of Bovine Serum Albumin (Fisher Scientific) in 1X PBS or 1% (w/v) of skimmed milk powder in 1X PBS.

ELISA substrate

o-Phenylenediamine dihydrochloride (OPD) prepared in phosphate citrate buffer at 0.4mg/ml. 7μl of H₂O₂ was added per 25ml of substrate solution. OPD substrate solution was used for both cytokine and antibody ELISAs.

Stop solution - 1M H₂SO₄

Reagent diluent for IFN γ ELISA 0.1 % (w/v) BSA and 0.01 % (v/v) Tween in PBS

Table 2.9: Detection antibodies used for measuring serum antibody titres

Detection antibody	Supplier	Working Concentration
Goat anti-mouse total IgG (HRP conjugated)	Southern Biotech	1/5000

Rat anti-mouse IgG1 (Biotinylated)	BD Pharmingen	1/4000
Rat anti-mouse IgG2b (Biotinylated)	BD Pharmingen	1/4000
Goat anti-mouse IgG2c (HRP conjugated)	AbD Serotec	1/2000

Horseradish peroxidase (HRP)-conjugated streptavidin

Purchased from BD Pharmingen and used at a dilution of 1/2000 where a detection antibody was not HRP-pre-conjugated.

2.1.6 Cell Death assays

CyQUANT™ LDH cytotoxicity assay (previously used Pierce™ Cytotoxicity assay - discontinued) was purchased from ThermoFisher Scientific

Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Merck

DMSO was purchased from Sigma

2.1.7 Immunoblotting

Bis-acrylamide and **N,N,N',N'-Tetramethylethylenediamine (TEMED)** were purchased from Merck

Ammonium persulfate (APS), **Tris base**, **Sodium Dodecyl sulfate (SDS)**, & **Methanol** were purchased from Sigma

Laemmli buffer, **Protease & Phosphatase inhibitors**, **Protein Ladder** and **filter paper** were purchased from Thermo Fisher Scientific

PVDF transfer paper, (0.2µM pore size) were purchased from Immobilon

Isopropanol was purchased from Acros Organic

Table 2.10: Buffer solutions for western blotting

Buffer	Composition
10% APS	10g APS in 100ml dH ₂ O
10% SDS	10g of SDS in 100ml dH ₂ O
1.5M Tris (pH 8.8)	18.15g Tris base in 100ml dH ₂ O
1M Tris (pH 6.8)	12.1g Tris base in 100ml dH ₂ O
10X Running buffer	144g Glycine, 30g Tris base, 10g SDS in 1000ml dH ₂ O
10X TBS-T	80g NaCl, 2g KCL, 30g Tris base, 10ml of Tween-20, in 990ml dH ₂ O
Transfer buffer	10.5g Glycine, 2.25g Tris base, 1g SDS, 200ml Methanol in 800ml dH ₂ O
3% Milk/BSA in TBS-T	3g of dried milk or 3g of BSA in 100ml 1X TBS-T

Table 2.11: Resolving gel composition (for 2 gels)

Composition	12% SDS acrylamide gel
H ₂ O	6.6ml
Acrylamide	8ml
1.5M Tris (pH 8.8)	5ml
10% SDS	200µl
10% APS	200µl
TEMED	8µl

Table 2.12: Stacking gel (for 2 gels)

Composition	12% SDS acrylamide gel
H ₂ O	5.5ml
Acrylamide	1.3ml
1M Tris (pH 6.8)	1ml
10% SDS	80µl
10% APS	80µl
TEMED	8µl

Table 2.13: Western blotting antibodies

Antibody	Dilution	Diluent	Incubation
Rabbit anti-mouse GSDMD (126-138) (Merck)	1/1000	3% Milk	Overnight at 4°C
Mouse anti-mouse β - actin (AC-74) (Merck)	1/5000	3% Milk	Overnight at 4°C
IRDye 800CW goat anti-rabbit IgG (Li-Cor)	1/20000	3% BSA	1 hour at room temperature
IRDye 680CW goat anti-mouse IgG (Li-Cor)	1/10000	3% BSA	1 hour at room temperature

2.1.8 Cell sorting with subsequent RNAseq profiling

Mouse CD8 α ⁺ T cell negative isolation kit and a **QuadroMACS™ separator** were sourced from Miltenyi Biotec

RNeasy Plus RNA isolation kits sourced from Qiagen

A **BDFACs Aria™** was used to sort fluorescently labelled antigen CD8⁺ T cells

Novosmart™, a R package developed by Novogene was used for data analysis

2.2 Methods

2.2.1 Mice

Mice were housed in the comparative medicine unit (CMU) of Trinity Biomedical Science Institute (TBSI), TCD. Mice were used for experimentation between the ages of 8-12 weeks in all experiments detailed in this project. All animals were maintained according to the European

Union and Irish Department of Health regulations. All experiments were conducted under the project authorisation number AE19136/P079, the individual licence number AE19136/I476 and were approved by the TCD Animal Research Ethics Committee (Ethical approval number 091210).

Table 2.14: Mice strains used

Strain	Background	Original Source
WT	C57BL/6J	CMU, TBSI, TCD
<i>Caspase11</i> ^{-/-}	C57BL/6J	Dr Emma Creagh, TBSI, TCD
<i>IL-1α</i> ^{-/-}	C57BL/6J	Prof. Kingston Mills, TBSI, TCD
<i>Nlrp3</i> ^{-/-}	C57BL/6J	Prof. Jürg Tschopp, University of Lausanne
<i>Ifng</i> ^{-/-}	C57BL/6J	Prof. Rachel McLoughlin, TBSI, TCD
<i>Gsdmd</i> ^{-/-}	C57BL/6NJ	Jackson Laboratory
<i>Ifnar</i> ^{-/-}		Prof Paul Hertzog, Monash University, Victoria, Australia
<i>Tmem173</i> ^{-/-}	C57BL/6J	Dr Lei Jin, Centre for Immunology and Microbial Disease, Albany Medical Centre, NY

2.2.2 *In vivo* studies

Analysis of adaptive immune responses following intramuscular vaccination

Mice were injected using BD tuberculin syringes permanently attached 27G needles via the intramuscular route with:

- PBS/Vehicle Control
- OVA antigen alone (10µg)
- 50nm PS NPs (1mg) and OVA (10µg)
- 50nm PLGA NPs (1mg) and OVA (10µg)

- C100 (200µg) and OVA (10µg)
- CpG 1826 (10µg or 50µg) and OVA (10µg)
- 2'3'cGAMP (2.5µg or 10µg) and OVA (10µg)
- Alum (100µg or 1mg) and OVA (10µg)
- ChAdOx1-OVA (10⁸ infectious units)
- 50nm PS NPs (1mg), OVA (10µg) and TEMPOL (25mg/kg)
- 50nm PS NPs (1mg), OVA (10µg) and 0.25mg Y-VAD-cmk
- or 50nm PS NPs (1mg) and OVA (10µg) and 0.25mg necrosulfonamide (NSA)

depending on the individual experiment. Doses were split between each leg, e.g., 0.5mg of 50nm NPs and 5µg OVA per leg. All mice received injections totalling 100µl, 50µl in each caudal thigh muscle. Mice received a priming vaccination on day 0 followed by a boosting vaccination two weeks later, on day 14. On day 21 mice were euthanised via CO₂ exposure and cervical dislocation. Spleens, caudal thigh muscle, inguinal lymph nodes (ILN) and blood samples were harvested. Serum was isolated from blood samples and was analysed for antigen-specific antibody titres via ELISA. Cells were isolated from tissues as described in the cell culture section 2.2.3. Cells were stained for flow cytometry or used for *ex vivo* antigen restimulation assays. Cytokine concentrations in cell culture supernatants were analysed via ELISA. Antigen was added to adjuvant formulations, vortexed briefly and left at RT for 40 minutes prior to immunisation.

Muscle residency analysis

Mice were injected using 27G tuberculin syringes via the intramuscular route with PBS or 50nm PS NPs (1mg) and OVA (10µg). Doses were split between each leg, e.g., 0.5mg of 50nm NPs and 5µg OVA per leg. All mice received injections totalling 100µl, 50µl in each caudal thigh muscle. Mice received a priming vaccination on day 0 followed by a boosting vaccination two weeks later on day 14. On day 21 mice were heated at 37°C for 5 minutes to encourage vasodilation, received an intravenous injection of 100µg of α-CD45-APC antibody and were culled one minute later via CO₂ exposure and cervical dislocation. Caudal thigh muscles were isolated and processed as outlined in section 2.2.3 and were stained for flow cytometry.

Prophylactic vaccination with tumour challenge model

Mice were injected using 27G tuberculin syringes via the intramuscular route with vaccine formulations indicated in the individual figures/figure legends depending on the individual experiment. Doses were split between each leg, e.g., 0.5mg of 50nm NPs and 5µg OVA per leg.

All mice received injections totalling 100µl, 50µl in each caudal thigh muscle. Mice received a priming vaccination on day 0 followed by a boosting vaccination 2 weeks later, on day 14. B16-OVA tumour cells were cultured and harvested as detailed in section 2.2.3. Two weeks later, on day 28 mice were injected subcutaneously with 3.5×10^5 B16-OVA or 5×10^5 MC38 tumour cells on the right flank which has been previously shaved, using 27G needles. Tumour growth was measured daily utilising a digital callipers and tumour volume was calculated as $(\text{Length} \times (\text{Width})^2)/2$. Mice were culled upon reaching the predetermined humane endpoint of tumour diameter $\geq 15\text{mm}$ or in the case of ulceration. Mouse survival was analysed using Kaplan-Meier analysis.

Prophylactic vaccination with tumour challenge model with CD8⁺ T cell depletion

Mice were injected using 27G tuberculin syringes via the intramuscular route with PBS, OVA antigen alone (10µg), or 50nm PS NPs (1mg) and OVA (10µg). Doses were split between each leg, e.g., 0.5mg of 50nm NPs and 5µg OVA per leg. All mice received injections totalling 100µl, 50µl in each caudal thigh muscle. Mice received a priming vaccination on day 0 followed by a boosting vaccination 2 weeks later, on day 14. On day 26 mice were injected i.p with 250µg of either a monoclonal $\alpha\text{-CD8}\alpha$ antibody (Clone 2.43) or a monoclonal isotype control antibody (Clone LTF-2). Mice received subsequent injections of 100µg of respective monoclonal antibody on days 29, 33, 37, 41 and 48. Blood was sampled from mice via the tail vein on days 23, 31 and 35 to detect presence of peripheral CD8⁺ T cells, with details outlined below in 2.2.4. B16-OVA tumour cells were cultured and harvested as detailed in section 2.2.3 and on day 28 mice were injected subcutaneously with 3.5×10^5 B16-OVA tumour cells on the right flank which has been previously shaved. Tumour growth was measured daily utilising a digital callipers and tumour volume was calculated as $(\text{Length} \times (\text{Width})^2)/2$. Mice were culled upon reaching the predetermined humane endpoint of tumour diameter $\geq 15\text{mm}$ or in the case of ulceration. Mouse survival was analysed using Kaplan-Meier analysis. (See illustrative experimental schematic Figure 4.5.1)

Therapeutic tumour model

Mice were injected subcutaneously with 3.5×10^5 B16-F10 or 5×10^5 MC38 tumour cells on the right flank which has been previously shaved. Tumour growth was measured daily utilising a digital callipers and tumour volume was calculated as $(\text{Length} \times (\text{Width})^2)/2$. Upon reaching 30-60mm³, mice were randomly assigned to experimental treatment groups, and received intratumoral injections of PBS, C100, C90, Protasan™, or CpG 1826, with doses depending on the individual experiment, with treatments repeated 4 days later on day 4, and on day 8 for a

total of 3 intratumoral treatments (See illustrative experimental schematic Figure 5.5.2A). In one experiment a group of mice received 3mg/kg of cisplatin via the intraperitoneal route one day prior to intratumoral treatment (See illustrative experimental schematic Figure 5.6.1). In experiments utilising α -PD1 therapy, 200 μ g of α -PD1 monoclonal antibody was given on the day of intratumoral treatment (See illustrative experimental schematic 5.8.1A). Tumour growth was monitored daily. Mice were culled upon reaching the predetermined humane endpoint of tumour diameter ≥ 15 mm or in the case of ulceration with survival analysed using Kaplan-Meier analysis.

Therapeutic tumour model with depletion of CD8⁺ T cells or NK cells

Mice were injected subcutaneously with 3.5×10^5 B16-F10 or 5×10^5 MC38 tumour cells on the right flank which has been previously shaved. Tumour growth was measured daily utilising a digital callipers and tumour volume was calculated as $(\text{Length} \times (\text{Width})^2)/2$. Upon reaching 30-50mm³, mice were randomly assigned to experimental treatment groups, with treatments repeated 4 days later on day 4, and on day 8 for a total of 3 intratumoral treatments. In experiments utilising α -PD1 therapy, 200 μ g of α -PD1 monoclonal antibody was given on the day of intratumoral treatment. To deplete CD8 α^+ T cells mice were injected with i.p with 250 μ g of either a monoclonal α -CD8 α antibody (Clone 2.43) or a monoclonal isotype control antibody (Clone LTF-2) 2 days before initial treatment (Day -2). Mice received subsequent injections of 100 μ g of respective monoclonal antibody every 4 days to maintain cell depletion (See illustrative experimental schematic Figure 5.9.1A). Blood was sampled from mice via the tail vein on day 0 to detect presence of peripheral CD8⁺ T cells, with details outlined below in 2.2.4. To deplete NK cells mice were injected with i.p with 400 μ g of either a monoclonal α -CD8 α antibody (Clone PK136) or a monoclonal isotype control antibody 2 days before initial treatment (Day -2). Mice received subsequent injections of 200 μ g of respective monoclonal antibody every 4 days to maintain cell depletion (See illustrative experimental schematic Figure 5.9.4A). Blood was sampled from mice via the tail vein on day 0 to detect presence of peripheral NK T cells, with details outlined below in 2.2.4. Tumour growth was monitored daily. Mice were culled upon reaching the predetermined humane endpoint of tumour diameter ≥ 15 mm or in the case of ulceration with survival analysed using Kaplan-Meier analysis.

Isolation via MACS & FACs of antigen specific CD8⁺ T cells, with subsequent RNAseq profiling

Mice were injected using 27G tuberculin syringes via the intramuscular route with PBS, OVA antigen alone (10 μ g), 50nm PS NPs (1mg) and OVA (10 μ g), or ChAdOx1-POVA. Doses were split between each leg, e.g., 0.5mg of 50nm NPs and 5 μ g OVA per leg. All mice received injections

totalling 100µl, 50µl in each caudal thigh muscle. Mice received a priming vaccination on day 0 followed by a boosting vaccination 2 weeks later, on day 14. On day 21 mice were euthanised via CO₂ exposure and spleens were harvested. Cells were isolated from tissues as described in the cell culture section 2.2.3. CD8α⁺ T cells were isolated using magnetic-activated cell sorting (MACs) and negative selection according to manufacturer's instructions (Miltenyi). These isolated cells were then stained for flow cytometry including class I SIINFEKL tetramer staining. CD44^{hi} Class I SIINFEKL Tetramer⁺ CD8α⁺ T cells were then isolated through fluorescence-activated cell sorting (FACs) via acquisition and gating on the BD FACS Aria™. RNA was isolated from these sorted cells using the RNeasy Plus RNA isolation kit from Qiagen according to manufacturer's instructions. RNA quality and quantity was determined using a NanaDrop™. RNA was subsequently frozen and sent to Novogene for bulk coding RNA sequencing using a HiSeq 4000 platform (Illumina). Data was further analysed & visualised using the Novosmart™ R package provided by Novogene.

2.2.3 Cell culture

Cell culture

All cells were cultured at 37°C with 95% humidity and 5% CO₂, using a cell culture incubator

Cell counting

For all *in vitro* and *in vivo* experiments cells were counted using trypan blue, disposable KOVA Glasstic haemocytometer slides and a light microscope. Cell viability was ascertained using trypan blue dye exclusion. Cell suspensions were diluted in trypan blue, ranging from 1/10 – 1/50 dilution depending on setting, and 10µl of trypan blue cell dilution was added to chamber. Cells were counted in three large diagonal squares to obtain average square count, with care to exclude cells on the borders of outside squares. Cell counts were determined according to the following formula:

Number of cells/ml = average cell number X 10⁴ X trypan blue dilution factor.

Isolation of cells from spleens and ILNs

Spleens and ILNs were isolated from euthanised mice, and single cell suspensions were obtained through passing the spleens and ILNs through 40µM cell strainers, with washing with cRPMI. Cells were centrifuged at 1200rpm for 5 minutes at 4°C, and additionally splenocytes were resuspended in 1ml of 0.88% ammonium chloride (NH₄Cl) for 2 minutes to lyse red blood cells

(RBCs), with subsequent quenching of reaction with cRPMI and repeat of centrifugation. Cells were resuspended in cRPMI and counted as detailed above. For *ex vivo* antigen restimulation assays, cells were plated in sterile U-bottom 96 well plates in 200µl of T cell media at 2×10^6 cells/ml. Cells were then restimulated with appropriate antigen concentrations and controls as detailed in the figure legends for each experiment. For tetramer analysis via flow cytometry, 2×10^7 cells were stained. Care was taken to ensure that samples used for tetramer staining were not placed on ice in the isolation process.

Digestion & Isolation of single cell suspension from caudal thigh muscle

Following intramuscular injection, the caudal thigh muscle where the injection site is located (vastus lateralis thigh muscle) was isolated, with particular attention to remove the popliteal lymph nodes located at the back of the knee. These samples were dried, weighed, added to 12 well plates and minced into small pieces using sterile scissors to aid the digestion process. 1ml of digestion buffer, detailed above, was added to each sample well and plates were put onto an orbital shaker in an incubator set to 37°C, 95% humidity and 5%CO₂ for 30-40 minutes. PBS-EDTA (5mM) was used to quench the digestion reaction and samples were passed through a 100µM cell strainer to obtain a single cell suspension. Samples were centrifuged at 400g for 10 minutes at 15°C. Cells were resuspended in 1ml of cRPMI and all of sample was taken for tetramer staining protocol. Care was taken to ensure that samples used for tetramer staining were not placed on ice in the isolation process.

Digestion & Isolation of single cell suspension from tumour

Tumours were dissected from mice, with care taken to remove as much other tissue from tumour as possible. Tumours were then dried, weighed and added to petri dishes. Remaining skin, hair and connective tissue was removed using a forceps and scalpel. The tumours were then minced using a scalpel/dissection scissors until obtaining a homogenous mixture of small pieces of tissue. Minced tumours were then added to 2ml microcentrifuge tubes with 1 ml of digestion buffer. Tubes were added to a tube adapted wheel rotator placed in an incubator set to 37°C, 95% humidity and 5%CO₂ for 30-40 minutes. PBS-EDTA (5mM) was used to quench the digestion reaction and samples were passed through a 100µM cell strainer to obtain a single cell suspension. Samples were centrifuged at 400g for 10 minutes at 15°C. In the case of B16 but not MC38 tumours, an RBC lysis step was additionally utilised. Cells were resuspended in 1ml of cRPMI and all of sample was taken for staining protocol. Care was taken to ensure that samples used for tetramer staining were not placed on ice in the isolation process.

Isolation of serum from blood samples

Whole blood was collected from mice, and was left to coagulate overnight at 4°C. The next day the blood was centrifuged at 11000g for 11 minutes at 4°C and serum was harvested for analysis.

Preparation of B16F10-OVA/B16F10/MC38 cells and preparation for tumour challenge

B16F10 and B16F10-OVA cell lines were cultured in cDMEM and upon reaching logarithmic growth between 50-70% confluency were used for experimentation. Cells were taken off flasks using a wash with pre-warmed PBS, followed with trypsin-EDTA treatment for 5 minutes at 37°C. Cells were then harvested and centrifuged at 1200rpm for 5 minutes at room temperature. In the case of *in vitro* experiments cells were resuspended in cDMEM and counted and were plated in 24W plates with 2×10^5 cells per well. Cells were then allowed to adhere for at least 2 hours before stimulation. In the case of use for *in vivo* tumour challenge, cells were resuspended and washed twice in ice cold HBSS. Cells were then counted and then adjusted to ideal concentration in ice cold HBSS and added to pre-cooled tubes, ready for tumour implantation. Additionally, care was taken to ensure cells had been split at least twice before use *in vivo* and stocks with low passage numbers were preferentially used.

Culture of bone marrow derived dendritic cells (BMDCs)

On day 1, the femurs and tibiae were removed from culled mice, and were cleaned of muscle tissue with a sterile scissors, tissue and 70% ethanol (EtOH). The separated bones were then briefly washed in petri dishes containing 70 % EtOH and rinsed in cRPMI. A 20 mL syringe was filled with cRPMI and attached to a 27G needle. The tips of the femur and tibia bone were minimally cut using a sterile scissors and the bone marrow was flushed out into a petri dish utilising a 20ml syringe filled with cRPMI and a 27G needle. Cell clumps were disrupted by repeat gentle pipetting with a 19G needle and the same syringe. The cells were transferred to 50 mL falcon tubes and centrifuged at 1200 rpm for minutes at room temperature. The supernatant was removed, and the cell pellet was resuspended in 1 mL of 0.88 % NH_4Cl de (NH_4Cl) for RBC for 2 minutes, with subsequent quenching of reaction with cRPMI and repeat of centrifugation. Supernatants were discarded and the cell pellet was resuspended in 10 mL cRPMI. Cells were counted as outline above and cell suspension volume was adjusted with cRPMI containing 20 ng/mL GM-CSF to give a final concentration of 4.25×10^5 cells/ml. Cells were transferred to T175 flasks (12.75×10^6 cells/flask) gently handling to avoid DC activation. On day 3 of culture protocol, 30 mL of cRPMI containing 20 ng/mL GM-CSF was added to each T175 flask. On day 6 flask supernatants were removed and replaced with 30 mL fresh cRPMI containing 20 ng/mL GM-CSF.

On Day 7, 30 mL of cRPMI containing 20 ng/mL GM-CSF was added to each T175 flask. On day 10, loosely adherent cells were removed by gentle repeat pipetting. Cells were transferred to 50 mL falcon tube and centrifuged at 1200rpm for 5 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 10 mL cRPMI. Cell suspensions were adjusted to a final concentration of 6.25×10^5 cells/mL in cRPMI with 10 ng/mL GM-CSF. Cells were plated in round bottom 96 well plates at a volume of 200 μ L/well. The plates were incubated for at least 2 hours at 37°C, 95% humidity and 5% CO₂ before stimulation.

Culture and differentiation of C2C12 cells

C2C12 culturing protocol was based on the cell line product data sheet²⁸⁷. C2C12 cells were cultured at 37°C, 95% humidity and 5% CO₂ in T75/T175 flasks in cDMEM, and it is crucial to avoid cells becoming confluent over course of their subculture. To remove cells from flasks they were first washed with pre-warmed PBS, followed with trypsin-EDTA treatment for 5 minutes at 37°C. Cells were then harvested and centrifuged at 1200rpm for 5 minutes at 37°C, and the cell pellet was resuspended in cDMEM for cell counting as described above. Cells were then plated in 24W plates for cytotoxicity studies. C2C12 cells were plated at 5×10^3 cells per cm² of cell culture surface area. These cells were allowed to become confluent, usually after 3-4 days of culture and following confluency media was changed from standard cDMEM to cDMEM supplemented with 2% horse serum instead of 10% FBS, to encourage differentiation, usually occurring within another 3-4 days of culture. C2C12 cells undergo differentiation from myoblasts into myotubules, with indicative changes in morphology towards tightly packed, striated cells. Fully differentiated cells were then used for *in vitro* experimentation.

2.2.4 Flow Cytometry

Compensation was achieved utilising single stains on cells or control compensation beads. Auto-fluorescence was accounted and corrected for using unstained samples as a control and in fluorescence-minus-one controls (FMOs), where samples are stained for all fluorochromes in a panel except for one. For all experiments where flow cytometry was carried out samples were acquired using FACs DIVA software on a FACSCANTO II cytometer or an LSRFortessa cytometer, specified in each individual experiment. Subsequent analysis was carried out using FlowJo software. The gating strategies utilised for each experiment are illustrated as figures below in results section.

Measuring cell death in BMDCs

Cell culture plates were centrifuged at 1200rpm for 5 minutes at room temperature and supernatants were discarded or kept for subsequent analysis. Cells were re-suspended using ICE cold PBS-EDTA, following incubation on ice for 5 minutes. The cells were transferred to labelled FACs tubes and washed with PBS. FACs tubes were centrifuged at 1200rpm for 5 minutes at room temperature, supernatants were removed, and cells were stained in BV510 fixable viability stain (1/500 dilution in PBS) on ice for 30 minutes in the dark. For a cell death control for staining, cells were killed with 70% EtOH prior to staining. Cells were washed as before with PBS and were resuspended in 200µL FACs buffer and were acquired immediately on the BDFACSCANTO II. Debris was excluded from analysis using FSC-A vs SSC-A plots, with doublets subsequently excluded via FSC-H vs FSC-A plots.

Measuring cellular and mitochondrial reactive oxygen species (ROS) in BMDCs

Cells were incubated with CellROX™ Green and MitoSOX™ Red for 30 minutes at 37°C 5% CO₂. Cell culture plates were then centrifuged at 1200rpm for 5 minutes at room temperature, and supernatants were discarded. Cells were removed from wells using ice cold PBS, transferred to labelled FACs tubes, and washed with 2 mL PBS. Once washed, cells were stained with BV510 viability stain for 15 minutes at RT. Cells centrifuged as before and were washed twice in PBS and were re-suspended in 200µL FACS buffer and acquired immediately on the BDFACSCANTO II.

Measuring CD8⁺T or NK cell depletion efficacy via peripheral blood sampling and staining

Peripheral blood was sampled from depleted and control group mice on days 23, 31 and 35 from the tail vein, using a lance to sample 2-3 drops of blood. Blood was collected into tubes containing 100µL of 3mg/ml EDTA on ice to prevent coagulation. Samples were then transferred to FACs tubes and 0.88% NH₄Cl was used to lyse RBC for 2 minutes, with reaction quenched with cRPMI. FACs tubes were centrifuged at 1200rpm for 5 minutes at room temperature, supernatants were removed, and cells were stained in BV510 fixable viability stain (1/500 dilution in PBS) on ice for 30 minutes in the dark. Cells were then washed as before and were resuspended in PBS supplemented with Fc block and were left for 10 minutes in dark on ice. Fluorochrome labelled surface stain antibodies were then added in a master mix to samples and individually to FMOs and single stains (SS). Samples were incubated for 30 minutes in the dark and were then washed as before and resuspended in 200µL of FACs buffer and acquired immediately via BDFACSCANTO II. It is worth noting that the CD8 clones used for depletion and

for staining were different, as this is crucial in obtaining an accurate measure of depletion efficacy.

Analysis of the induction of antigen-specific CD8⁺ T cell responses following intramuscular vaccination via Class-I tetramer staining

Splenocytes and cell suspensions obtained from muscle digestion were obtained as outlined above in 2.2.3. Cells were stained with PE-labelled H-2Kb SIINFEKL tetramers (15µg/ml) in 100µl of T cell medium for 90 minutes on an orbital shaker at 37°C, 95% humidity and 5% CO₂. After 50 minutes of this incubation, anti-CD16/CD32 monoclonal antibodies (Fc block) were added to samples to block FcγRII/III which can lead to background staining (0.5µl per sample in 50µl of T cell medium). For the final 30 minutes of tetramer staining, fluorochrome labelled cell surface staining antibodies were added to samples. Samples were then washed with PBS and centrifuged at 400g for 8 minutes. Supernatants were removed, especially careful in muscle samples due to the presence of fat. Samples were resuspended in live dead Aqua stain 1/500, and were incubated for 30 minutes in the dark at 4°C. Cells were then washed as before, resuspended in 2% paraformaldehyde in PBS and were incubated for 15 minutes at room temperature in the dark to fix samples. Cells were then washed as before and resuspended in FACs buffer ready for acquisition via LSRFortessa or the BD FACSAria in the case of cell isolation.

2.2.5 ELISA

Measuring cytokine secretion in cell culture supernatants

R&D and Biolegend ELISA's were performed according to the kit manufacturer's instructions. In summary high binding plates were coated overnight at 4°C with capture antibody diluted in PBS or sodium carbonate buffer, depending on the cytokine of interest (50µl/well). After washing with wash buffer 3 times (1X PBS supplemented with 0.05% tween), the plates were incubated for 1 or 2 hours (kit dependent) at room temperature with 150µl of the blocking solution (usually 1% BSA) to block non-specific binding sites. The plates were washed with washing buffer and then 50µl of undiluted/diluted supernatant or serially diluted recombinant standard was added to the plates and incubated for 2 hours at room temperature. The plates were then washed with washing buffer before detection antibody (50µl/well) was added. The plates were incubated for either 1 or 2 hours (kit dependent) at room temperature. After another wash step, HRP-conjugated streptavidin or avidin (50µl/well) was added to the plates and left to incubate for 20 or 30 minutes (kit dependent) at room temperature, in darkness. Finally, the

plates were washed and OPD substrate, supplemented with 0.7µl of hydrogen peroxide (H₂O₂) per 1mg of OPD was added (50µl/well). The enzyme reaction was stopped by the addition of 1M H₂SO₄ (25µl/well). The optical density (OD) values were determined by measurement of absorbance at 492nm using a plate reading spectrophotometer. Cytokine concentrations were determined using a standard curve prepared from the OD values of the recombinant standard.

Measuring antigen-specific serum antibodies (Total IgG, IgG1, IgG2b and IgG2c)

Medium binding ELISA plates were coated overnight at 4°C with 10µg/ml OVA protein diluted in 0.1M sodium carbonate buffer (50µl/well). Plates were washed with 3 times with wash buffer (1X PBS supplemented with 0.05% Tween). Plates were blocked with blocking buffer (1% milk in PBS), 150µl per well for 2 hours at room temperature. Plates were washed as before, and serial dilutions of serum samples (Table 2.10) were made across plate in blocking buffer. Plates were incubated for 2 hours at room temperature. Detection antibodies (outlined below) were diluted in blocking buffer and added 50µl per well, plates were incubated for 1 hour at room temperature in the dark. Plates were washed as before and in the case of IgG1 and IgG2B, streptavidin-HRP was added, at 1/2000 dilution to plates (50µl/well), this step is not required for IgG2C and total IgG as detection antibodies are conjugated to detection antibodies. Finally, the plates were washed as before and OPD substrate, supplemented with 0.7µL of hydrogen peroxide (H₂O₂) per 1mg of OPD was added (50µl/well). The enzyme reaction was stopped by the addition of 1M H₂SO₄ (25µl/well). The optical density (OD) values were determined by measurement of absorbance at 492nm using a plate reading spectrophotometer.

Table 2.15: Serum dilutions used for serum antibody detection

Antibody	Starting serum dilution	Subsequent serial dilutions
IgG	1:500	1:2
IgG1	1:500	1:2
IgG2c	1:250	1:2
IgG2b	1:250	1:2

2.2.6 Cell death assays

LDH assay in cell culture supernatants

LDH assay was carried out according to the manufacturers instructions²⁸⁸. Firstly, a set of triplicates wells were lysed using addition of 10X lysis buffer and incubation for 45 minutes at

37°C, 95% humidity and 5% CO₂. 600µl of supplied assay buffer was added to 11.4ml of supplied substrate stock solution and was mixed to make up the reaction mixture and kept in the dark. Alternatively pre-made-up reaction was thawed. Assay was conducted to fresh cell supernatants to ensure greater accuracy. 50µl of supernatants were added to a 96 well flat-bottom plate in triplicate samples. 50µl of reaction mixture was added to each well and the plate was incubated for 30 minutes in the dark at room temperature. Following incubation, 50µl of supplied stop solution was added to each well, and the optical density (OD) values were determined by measurement of absorbance at 492nm using a plate reading spectrophotometer. Additionally, the absorbance was read at 680nm to account for any background absorbance values. This is especially apparent with larger PS nanoparticles (>100nm). Similarly, the vehicle control of added stimulants was added to a control set of triplicates to account for spontaneous LDH activity. % Cytotoxicity was calculated as follows for each absorbance:

$$\% \text{Cytotoxicity} = \left(\frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{100\% \text{ lyse control LDH activity} - \text{Spontaneous LDH activity}} \right) \times 100$$

Then the values obtained for cytotoxicity determined from the 680nm wavelength were subtracted from this obtained from those measured at the 492nm wavelength to account for background.

MTT assay

Firstly, a set of triplicates wells were lysed using addition of 10X lysis buffer and incubation for 45 minutes at 37°C, 95% humidity and 5% CO₂. The MTT reagent was made up to 1mg/ml with sterile PBS and was weighed in a sterile manner. Cell culture 96W plates were centrifuged at 1200rpm for 5 minutes at room temperature, supernatants were removed, and cells were subsequently washed with PBS and centrifuged as before. The PBS was removed and replaced with 200µl of the 1mg/ml MTT reagent. The plate was wrapped in tinfoil and incubated at 37°C, 95% humidity and 5% CO₂ for 3 hours (at least 2 hours minimum is necessary). Following incubation, the MTT reagent is removed with a multichannel pipette. 200µl of DMSO is added to each well and the plate is wrapped in tinfoil and incubated for 20 minutes on an orbital shaker for 20 minutes, the DMSO functions to lyse cells and solubilise the formazan crystals formed. The optical density (OD) values were determined by measurement of absorbance at 595nm using a plate reading spectrophotometer. %Viability is calculated as follows:

$$\% \text{Viability} = 100 - \left(\frac{100\% \text{ lyse control MTT assay value}}{\text{Compound-treated MTT assay value}} \right) \times 100$$

2.2.7 Washing of 50nm PS nanoparticles and antigen addition

Particles were washed twice using centrifugation utilising an ultracentrifuge and resuspension in sterile apyrogenic PBS to remove surfactant. Centrifugation velocity and time was calculated according to Stoke's Law (45000rpm for 45 minutes at 15°C). Particles were then resuspended to desired concentration in PBS/vehicle control and were mixed with antigen 40 minutes prior to immunisation.

2.2.8 50nm PLGA nanoparticle synthesis and QC (conducted by members of Prof Yvonne Perrie Lab, University of Strathclyde)

50nm sized PLGA nanoparticles were produced using a continuous flow microfluidic platform (NanoAssemblr® produced by Precision Nanosystems Inc). PLGA 50:50 and DOPS or DOTAP were mixed at a final concentration of 0.5mg/ml in DMSO. A flow rate ratio of 1:1 (DMSO/Tris buffer) and a total flow rate of 10ml/min was used to produce nanoparticles. In addition, excel solvent was removed and concentration of nanoparticles was adjusted using a Krosflo Research Ili tangential flow filtration system (Spectrum Labs) with a 500kD mPES column. Particle size (including polydispersity index) and zeta potential (indicating charge) were measured using a Zetasizer Nano ZS (Malvern Instruments).

2.2.9 Endotoxin quantification

Endotoxin levels in the nanoparticles used for experimentation were quantified using a Pierce Chromogenic endotoxin kit as per manufacturer's instructions.

2.2.9 Statistical analysis and *in vivo* experimental design

Sample size was determined through existing power calculations based off of pilot studies, these calculations are detailed in approved HPRC project licence AE19136/P079. Experimental blocking was utilised in some instances to achieve the appropriate sample size.

All statistical analysis was carried out using GraphPad Prism software (version 9) the statistical tests used are detailed in each relevant figure's figure legend.

Chapter Three - Investigating the underlying mechanisms of 50nm nanoparticle adjuvanticity

3.1 Introduction

Nanoparticulate adjuvants have been extensively studied in the field of vaccinology, and the size of nanoparticulates has emerged as a key parameter in dictating the immune responses triggered^{204,205}. Previous research conducted in the Lavelle Lab has identified nanoparticulate size as crucial for the successful induction of cellular immunity. Research conducted by Dr Claire Hearnden identified 50nm sized polystyrene nanoparticles (PS NP) as inducers of antigen-specific CD8⁺ T cells when injected intraperitoneally (i.p)²⁸⁹. Subsequent research conducted by Dr Katie O'Grady, using the more clinically relevant intramuscular (i.m) injection route, confirmed the induction of antigen-specific CD8⁺ T cells alongside robust IFN γ ⁺ Th1 cells^{263,290}. This research identified 50nm PS NPs as suitable adjuvants for the induction of cellular immunity, however the translatability of these findings to other materials, the functionality of these cellular immune responses, the benchmarking of their adjuvanticity against alternative adjuvants/vaccine platforms and the mechanism for their induction remained to be elucidated.

While most licensed adjuvants have been discovered and incorporated into vaccines according to empirical approaches, this can yield little mechanistic insight and is not conducive to rational adjuvant design. To have the capacity to improve vaccine adjuvants and understand their efficacy, mechanisms of action must be determined. This crucially allows us to define how certain immune responses are shaped, why certain injection routes may be favoured, and what vaccine applications an adjuvant is suitable for.

Research conducted by Dr Fiona Sharp characterised cell death surrounding the injection site when 50nm PS NPs were injected subcutaneously (s.c)²⁹¹, and Dr Katie O'Grady identified IL-33, a DAMP, as a negative regulator of the cellular immune response observed^{263,290}, these data suggests that cell death may be playing a role in the adjuvanticity of 50nm PS NPs. Immune responses can be differentially regulated by diverse forms of cell death, more immunologically quiet forms of death like early apoptosis can favour anti-inflammatory, tolerogenic immune responses,^{292,293,230} and more lytic forms of cell death promoting inflammation²⁹⁴.

It is worth noting however that not all inflammatory forms of cell death can elicit immune responses favourable to vaccination. ICD is required to prime adaptive immune responses in certain contexts and thus, the potential role of 50nm PS NP induced ICD in their adjuvanticity was investigated.

Furthermore, since vaccine safety is such a priority, vaccine adjuvant research must prioritise the use of biocompatible materials e.g., poly lactic-co-glycolic acid (PLGA). As such, 50nm PLGA NP were examined, to confirm that the adjuvanticity of PS NP is a result of particle size and not the composition of the polymer. In addition, 50nm NP adjuvanticity was compared to alternative adjuvants including CpG and 2'3'cGAMP and also the ChAdOx1 vector, representing a gold standard alternative vaccine platform.

3.2 Hypothesis

The hypothesis underlying the research in this chapter was that vaccination with antigen and 50nm NP results in the local induction of ICD leading to priming of antigen-specific cellular immunity.

3.3 Aims

- To establish if 50nm NP adjuvanticity is retained when using biocompatible, biodegradable PLGA instead of PS.
- To establish the cytotoxic capacity of 50nm NPs *in vitro* and characterise the cell death pathway elicited.
- To investigate the importance of this cell death pathway in vaccine induced cellular immunity.
- To benchmark 50nm NP adjuvanticity against other vaccine adjuvants and other vaccine platforms.

3.4 50nm polymeric NP adjuvants promote the induction of systemic antigen-specific cellular immunity, with some preferential T cell homing to the injection site

The Lavelle Lab has previously characterised size as a crucial parameter in NP adjuvanticity, and 50nm PS NPs as potent inducers of cellular immunity^{263,289}. This impact of size on adjuvanticity has been suggested to principally relate to induction of cellular immune responses with less influence on the induction of antigen specific antibody responses²⁸⁹. To address this issue here, C57BL6/J mice were vaccinated intramuscularly according to our standard prime boost regimen as detailed in Fig 3.4.1A, with analysis of antigen-specific CD8⁺ T cells in the spleens of vaccinated mice via FACS analysis 21 days following initial priming vaccination (Fig.3.4.2B). An MHC Class I SIINFEKL Tetramer was used to identify antigen-specific T cells and CD44 was included as a marker of T cell activation²⁹⁵.

Vaccination with 50nm PS NP + OVA (50nm PS OVA) led to significantly higher induction of splenic antigen-specific CD8⁺ T cells than vaccination with antigen alone in terms of frequency (% CD44⁺ SIINFEKL Tetramer⁺/splenic CD8⁺ T cells) (Fig.3.4.2A) and total cell number (Fig.3.4.2B). Furthermore, vaccination with OVA and 50nm NPs promoted significantly higher antigen specific IFN γ responses than vaccination with antigen alone upon splenocyte restimulations (Fig.3.4.3). These results confirm the utility of 50nm NPs as suitable adjuvants to drive cellular immune responses.

While vaccination with antigen and 50nm PS NPs can promote robust splenic cellular immune responses, the presence of antigen-specific CD8⁺ T cells locally at the caudal thigh injection site, following isolation 21 days after initial priming had previously been observed by Dr Natalia Muñoz-Wolf. In order to provide an initial insight into whether these cells are tissue resident, a pilot study was executed where C57BL6/J mice were injected according the standard i.m. prime boost regime, and on day 21, mice were injected intravenously with an α -CD45 APC-conjugated antibody, 1 minute prior to culling and processing (Fig.3.4.4A). Tissue resident/Non-resident antigen-specific CD8⁺ T cells were characterised utilising anti-CD45 antibody staining, and an MHC Class I SIINFEKL Pentamer. Cells staining positive for CD45 are deemed to be non-resident as only cells present in the vasculature will stain positive. Antigen-specific CD8⁺ T cells were detected in the muscle of 50nm PS OVA immunised mice as previously observed (Fig.3.4.5A). Roughly 80-90% of these cells were identified as being CD45⁺ and as a result, tissue non-resident (Fig.3.4.5B+C). These data suggest that the antigen-specific CD8⁺ T cells found at the

intramuscular injection site following vaccination with 50nm PS OVA, may not be tissue resident but instead may have preferential homing to the site of intramuscular injection when in circulation. This warrants further investigation to better characterise these cell types with a focus on their potential functionality, and definitively characterise non-residency/residency.

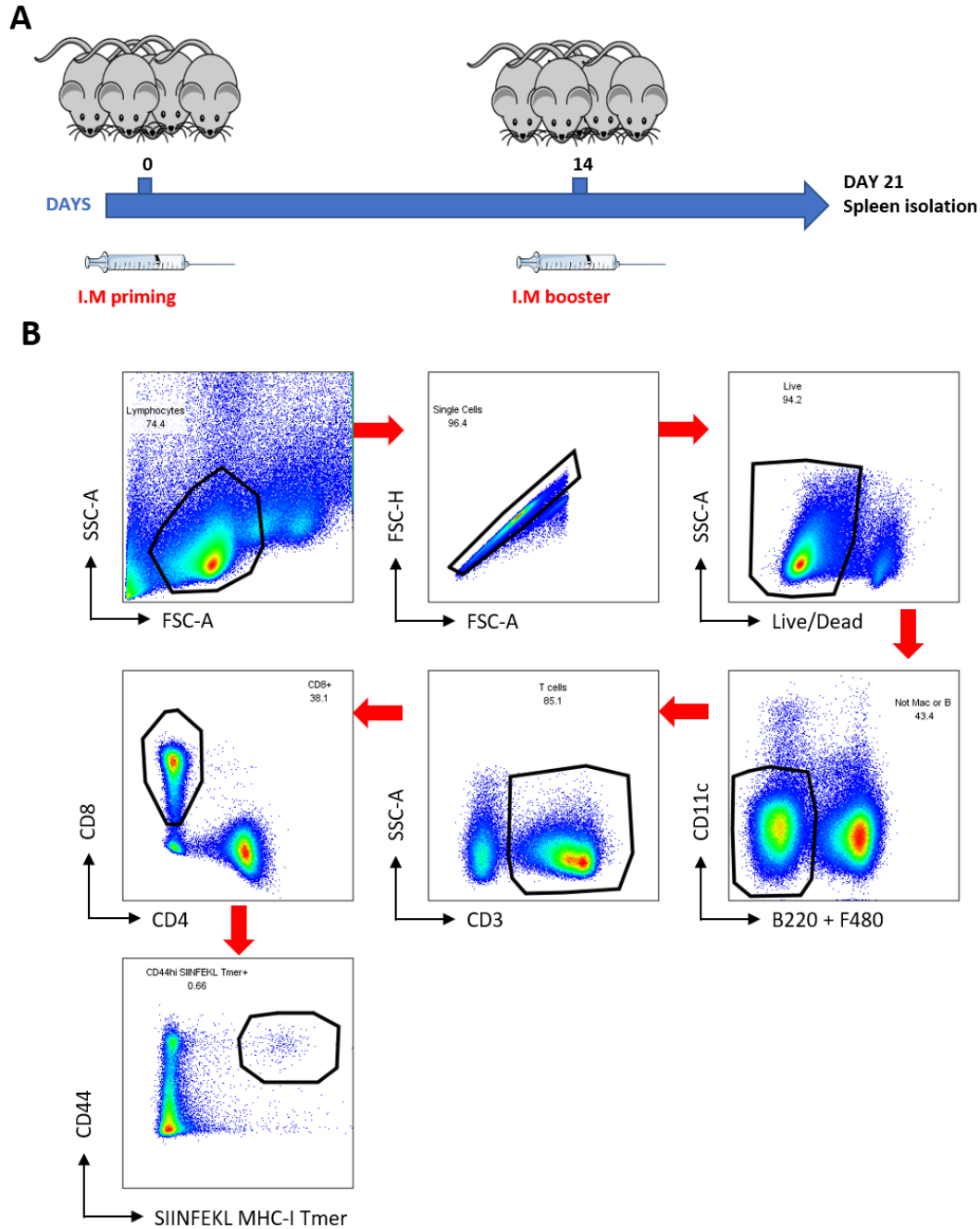


Figure 3.4.1 Prime boost vaccine schematic with FACS gating strategy

A) Vaccination scheme, C57BL/6 mice received a priming vaccination intramuscularly on day 0 followed by a booster on day 14. B) Spleens were isolated on day 21, processed and antigen-specific T cells characterised via flow cytometry. Cells were acquired using LSR Fortessa and analysed according to gating strategies presented. Splenocytes were also plated for 72-hour antigen restimulations.

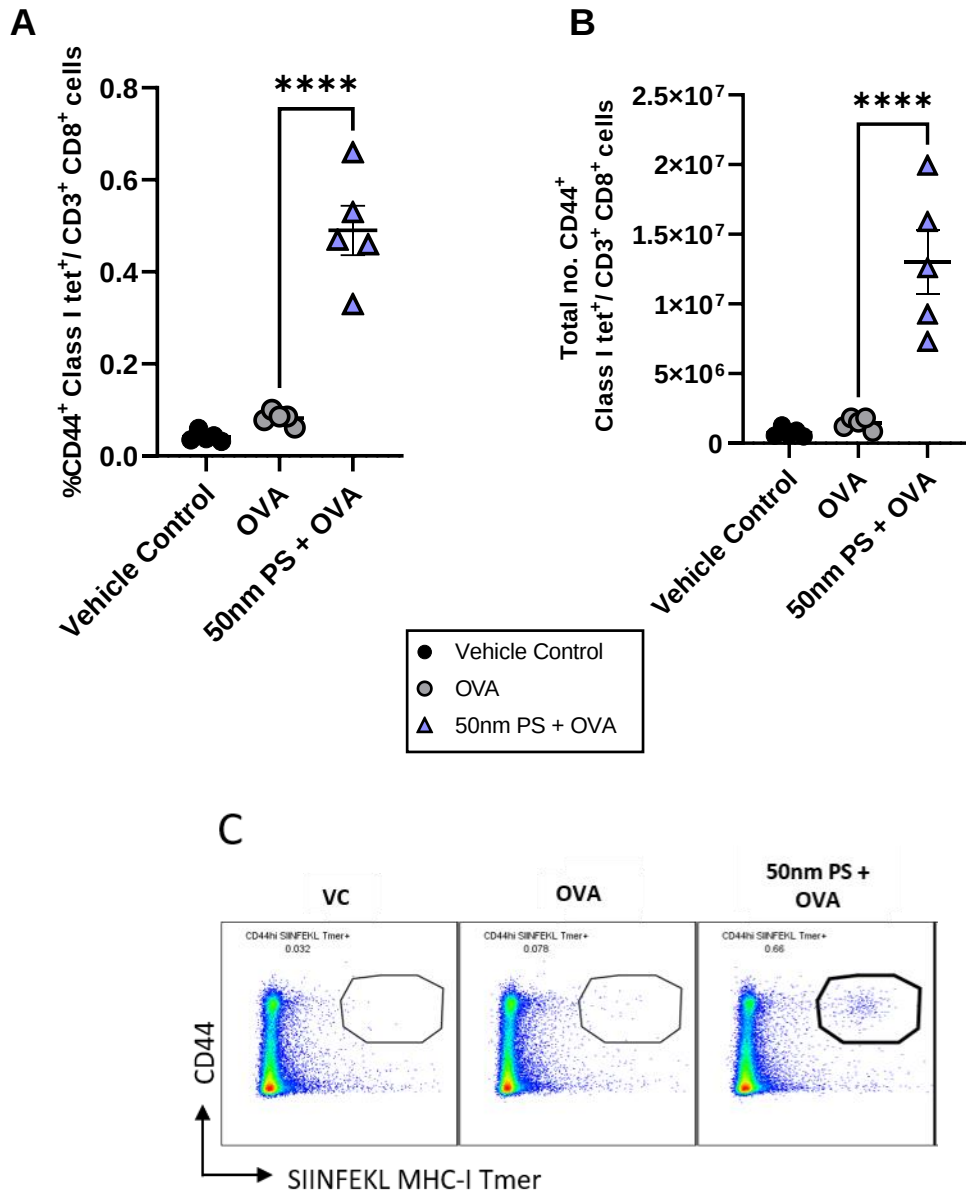


Figure 3.4.2. Vaccination with antigen and 50nm PS drives splenic antigen-specific CD8⁺ T cell responses

C57BL/6 mice were vaccinated intramuscularly with PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), via the intramuscular route according to Fig.3.4.1. On day 21, antigen-specific CD8⁺ T cell responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. 7.5% trehalose in PBS or OVA alone were used as vehicle or antigen alone controls respectively. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen (C) Representative FACS plots showing antigen-specific CD8⁺ T cells from each experimental group. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001. ** P<0.01, * P<0.05.

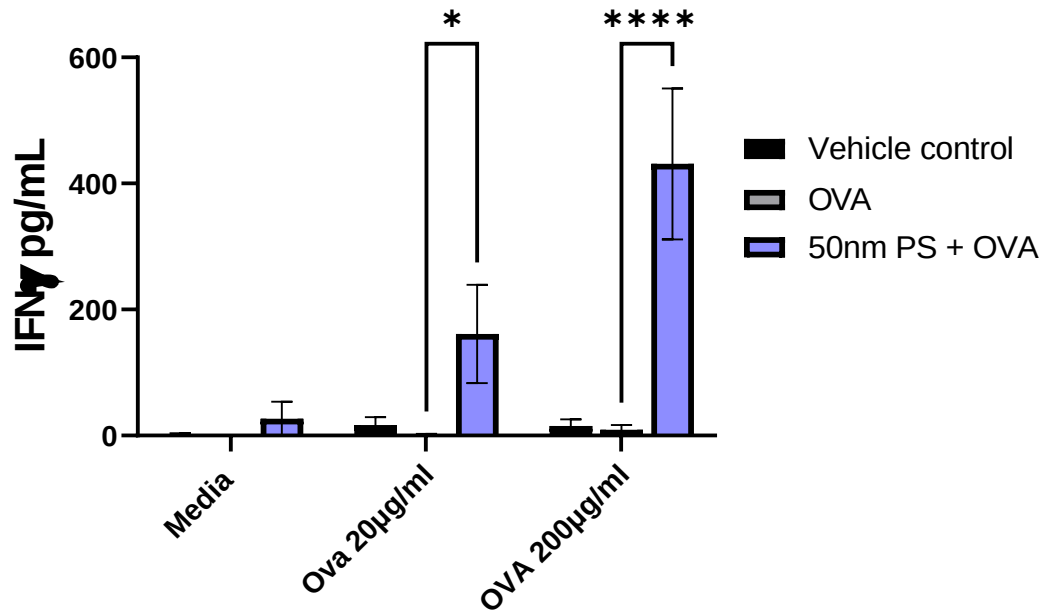


Figure 3.4.3. Vaccination with antigen and 50nm PS drives antigen specific IFN γ responses

C57BL/6 mice were vaccinated intramuscularly with PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), via the intramuscular route according to Fig.3.4.1. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media, ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001. ** P<0.01, * P<0.05.

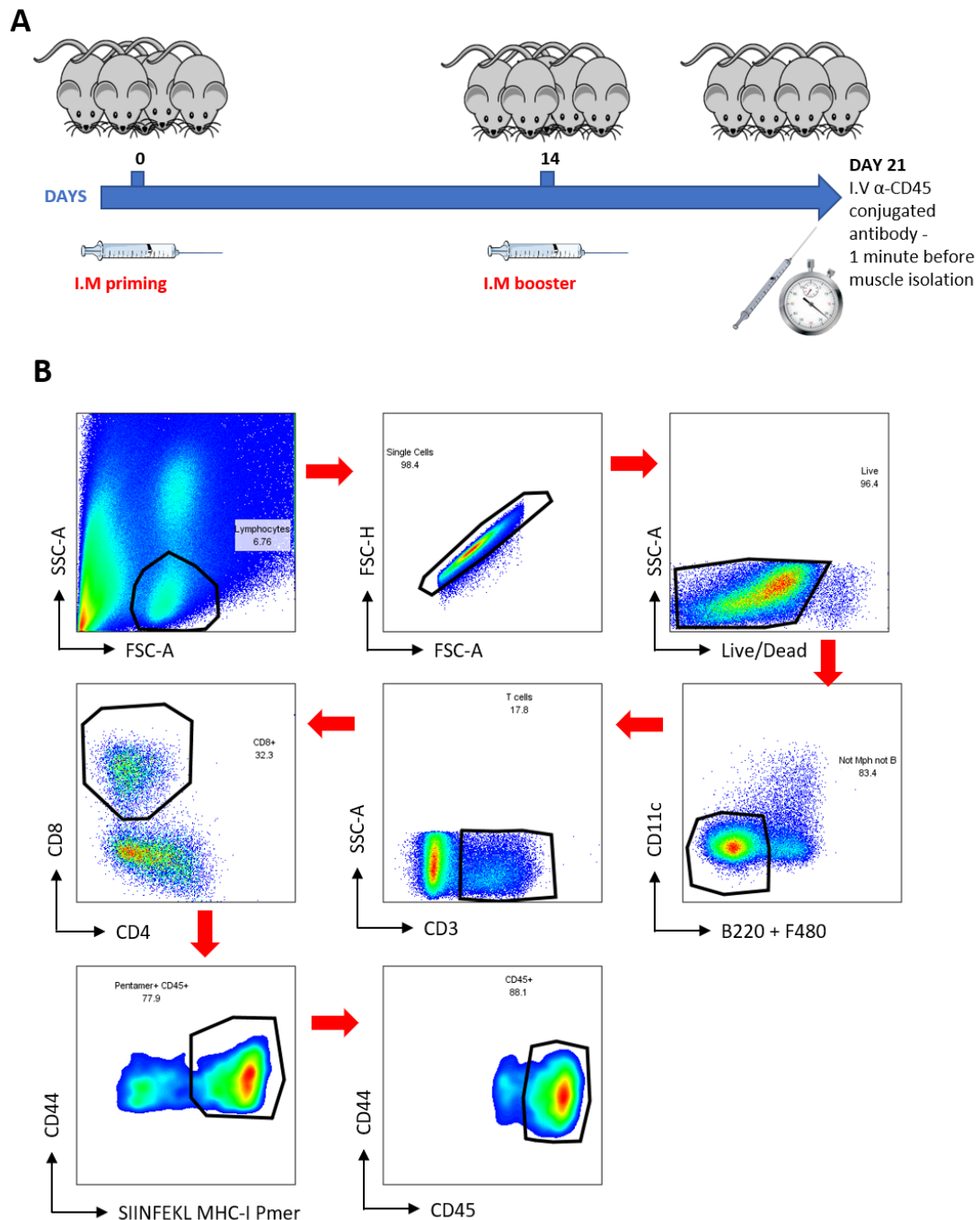


Figure 3.4.4 Assessment of tissue resident antigen-specific CD8⁺ T cells following i.m. vaccination with antigen and 50nm PS NP

A) Vaccination scheme. C57BL/6 mice received a priming vaccination intramuscularly on day 0 following by a booster on day 14. On day 21, mice were injected i.v with 100 μ g of α -CD45-APC antibody and culled 1 minute later. B) Caudal thigh muscles were subsequently isolated, processed, and digested utilising collagenase IV and DNase. Single cell suspensions were obtained for staining to characterise tissue resident and circulating antigen-specific T cells via flow cytometry. Cells were acquired using LSR Fortessa and analysed according to the gating strategies presented.

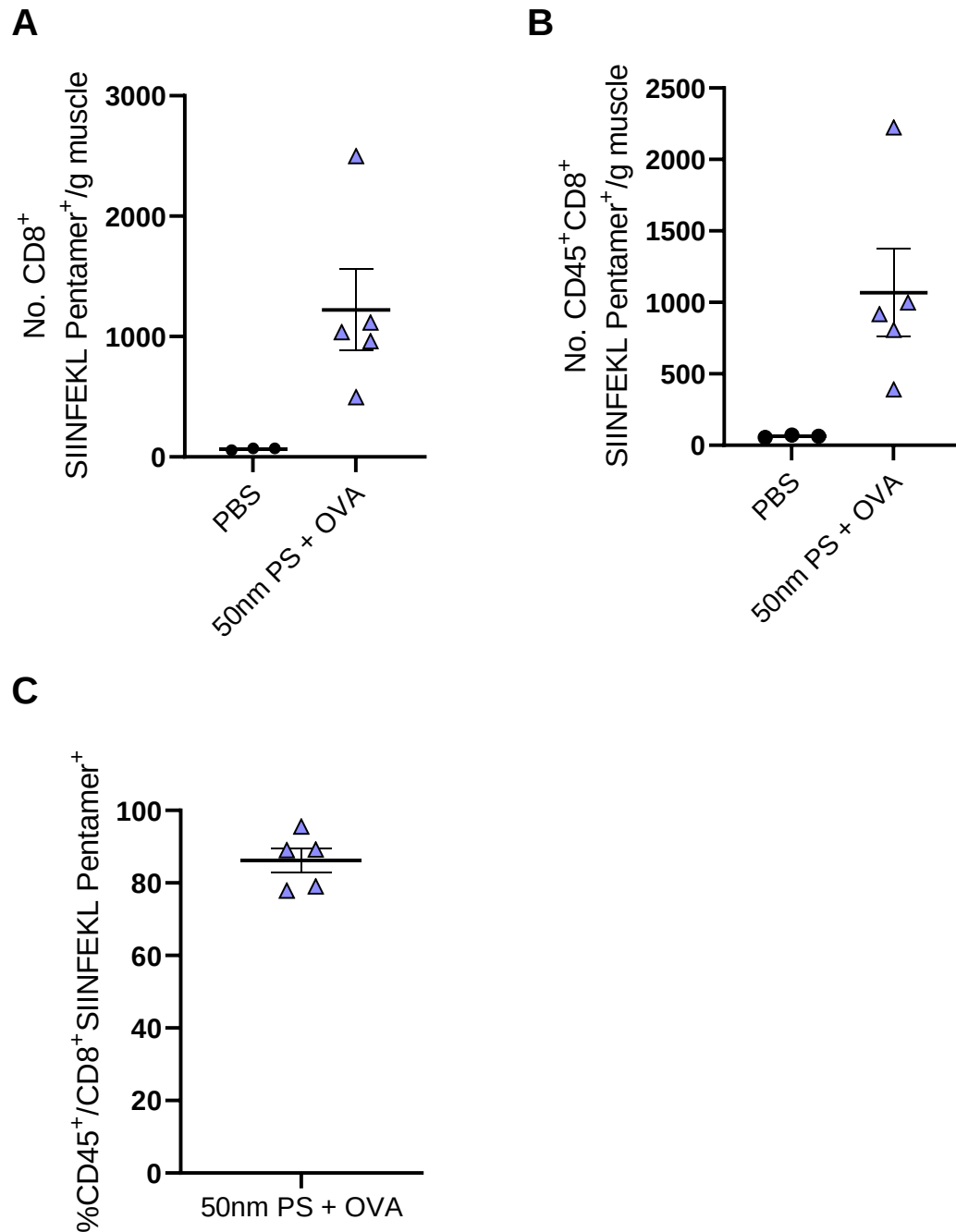


Figure 3.4.5 Intramuscular injection with 50nm PS NPs results in homing of antigen-specific CD8⁺ T cells to the muscle

C57BL6/J mice were vaccinated on days 0 and 21, muscle tissue was processed and analysed as outlined in Fig 3.4.2. An MHC-I SIINFEKL Pentamer used to identify antigen-specific CD8⁺ T cells, and intravenous CD45⁺ used to determine tissue residency. A) Number of antigen-specific CD8⁺ T cells per gram of muscle. B) Number of CD45⁺ non-resident antigen-specific CD8⁺ T cells per gram of muscle. C) % of CD45⁺ non-resident antigen-specific CD8⁺ T cells/ Total CD8⁺ T cells in muscle.

3.5 50nm NPs retain adjuvanticity when the more biodegradable and biocompatible negatively charged PLGA is used instead of PS

The Lavelle Lab has previously characterised size as a crucial parameter in NP adjuvanticity, and 50nm PS NPs as potent inducers of cellular immunity²⁶³. PS is an excellent polymer candidate for this work due to consistencies in size and excellent stability, and as a result is used for the bulk of experiments in this project. However, it is also vitally important to validate this adjuvanticity with a more biocompatible polymer like PLGA²¹⁴, validation of adjuvanticity here would improve translatability to clinical vaccine applications. Through close collaborations with Prof Yvonne Perrie, University of Strathclyde, freeze-dried 50nm PLGA NP with different charges made through microfluidics technology were obtained for use. Both positive and negatively charged 50nm PS NPs have been shown previously in our lab to possess similar adjuvanticity (data not shown), but the importance of charge of 50nm PLGA NPs remained to be determined.

C57BL6/J mice were vaccinated intramuscularly according to our standard prime boost regimen as detailed in Fig 3.4.1A, with analysis of antigen-specific CD8⁺ T cells in the spleens of vaccinated mice via FACS analysis 21 days following initial priming vaccination (Fig.3.5.1). An MHC Class I SIINFEKL Tetramer was used to identify antigen-specific T cells and CD44 was included as a marker of T cell activation²⁹⁵. The PLGA NPs were supplied in 7.5% trehalose, as this serves as a cryoprotectant during the freeze-drying process. Therefore 7.5% trehalose was used as the vehicle control and was included in all vaccine formulations tested, including antigen alone and the 50nm PS + OVA control.

Due to different supply times of the freeze dried PLGA NPs arising from production issues, two different experiments had to be used to examine their adjuvanticity. In the first experiment, positively charged dimethyldioctadecylammonium bromide stabilised PLGA NP (PLGA:DDA) were trialled, and in the second experiment, negatively charged 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine stabilised PLGA NP (PLGA:DOPS) and neutrally charged PLGA NPs were trialled. In both experiments 50nm PS NP were used as positive control to compare adjuvanticity. In both experiments, vaccination with 50nm PS NP + OVA (50nm PS OVA) led to significantly higher induction of splenic antigen-specific CD8⁺ T cells than vaccination with antigen alone in terms of frequency (%CD44⁺ SIINFEKL Tetramer⁺/splenic CD8⁺ T cells) (Fig.3.5.1A+B) and total cell number (Fig3.5.1A+B). In contrast, vaccination with antigen and the positively charged 50nm PLGA:DDA NP failed to elicit higher frequencies (Fig.3.5.1A) or total

numbers (Fig.3.5.1B) of antigen-specific CD8⁺ T cells when compared to antigen alone. Similarly, neutrally charged PLGA 50nm NP also failed to enhance induction of antigen-specific CD8⁺ T cells when compared to antigen (OVA) alone (Fig.3.5.1C+D). However, significantly enhanced induction of antigen-specific CD8⁺ T cells was observed in mice vaccinated with 50nm PLGA:DOPS NP + OVA in terms of frequency (Fig.3.5.1C) and total cell numbers present in the spleen (Fig.3.5.1C), albeit to a lesser extent than following vaccination with 50nm PS NP and OVA. This lack of adjuvanticity in terms of cellular immune responses elicited following vaccination with antigen and neutral PLGA NP or PLGA:DDA NP was also observed when antigen specific IFN γ was assayed in supernatants from splenocyte restimulations from vaccinated mice (Fig.3.5.2A+B). Enhanced antigen specific IFN γ was only detected in restimulated splenocytes from mice vaccinated with antigen and 50nm PS or negatively charged 50nm PLGA:DOPS NP. Vaccination with 50nm PLGA:DDA + OVA also elicited significantly lower titres of antigen-specific IgG when compared to vaccination with 50nm PS + OVA (Fig.3.5.3A) assayed from the sera of vaccinated mice, while no significant differences in IgG2c titres were observed (Fig.3.5.3B). Vaccination with antigen and 50nm PLGA:DOPS NP elicited somewhat comparable titres of antigen specific IgG (Fig.3.5.4A) and IgG2c (Fig.3.5.4B) to those in mice vaccinated with OVA and 50nm PS NP, while vaccination with antigen and neutrally charged PLGA NP + OVA elicited titres compared to vaccination with antigen alone.

These results validate the suitability of NPs in the 50nm size range as adjuvants capable of driving enhanced cellular immune responses, in a biocompatible polymer (PLGA) suitable for clinical translation. However, this adjuvanticity in PLGA NP, looks to be particle charge dependant, with negatively charged PLGA:DOPS identified as the most suitable candidate.

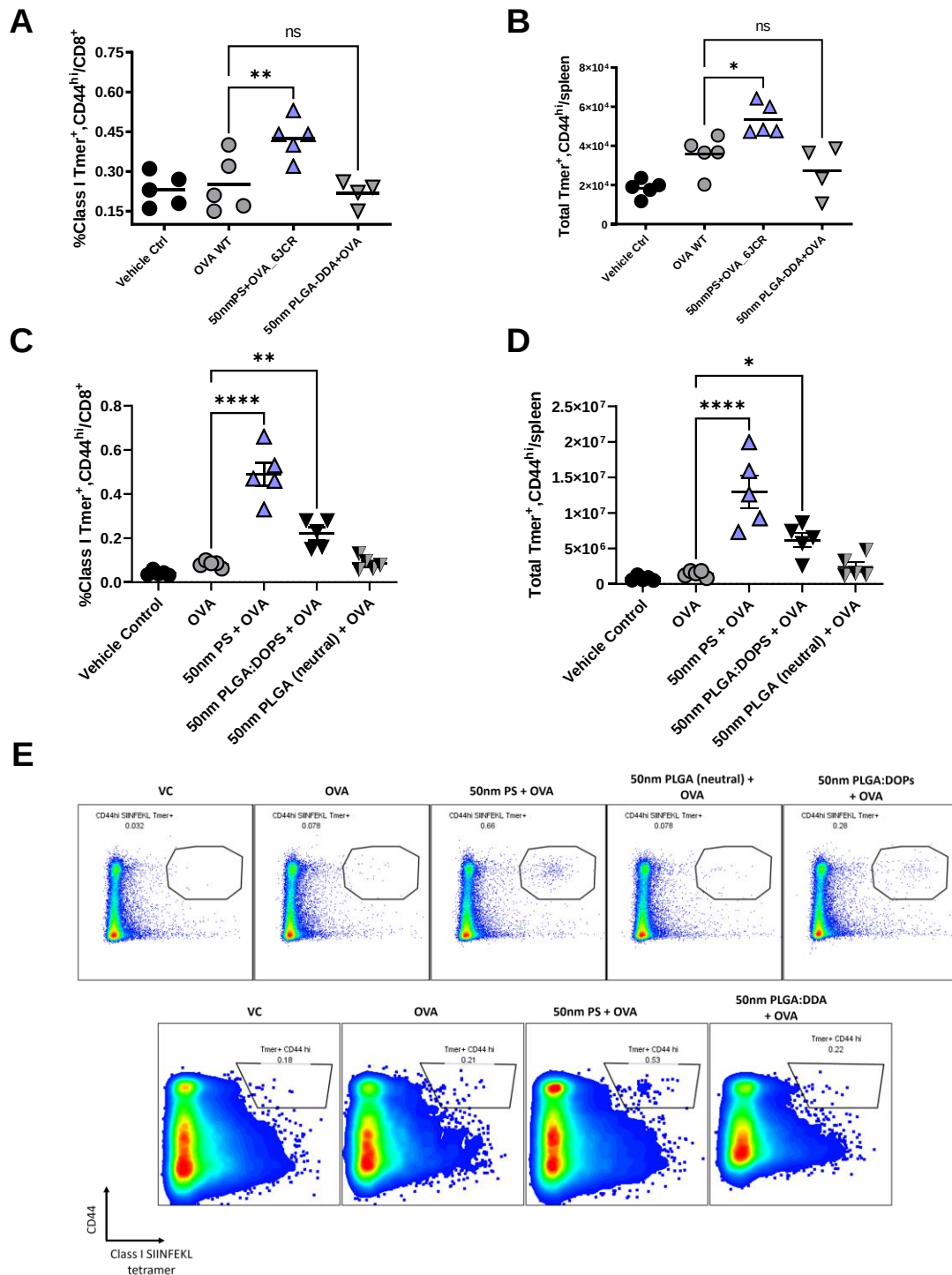


Figure 3.5.1. Vaccination with antigen and 50nm PS or 50nm negatively charged PLGA (PLGA:DOPS) drives splenic antigen-specific CD8⁺ T cell responses

C57BL/6 mice were vaccinated intramuscularly with 7.5% trehalose PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), 50nm neutral PLGA NP (1mg) + OVA (10 μ g), 50nm PLGA:DOPS NP + OVA (10 μ g), or PLGA:DDA NP +OVA via the intramuscular route according to Fig.3.4.1. On day 21, antigen-specific CD8⁺ T cell responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. 7.5% trehalose in PBS or OVA alone were used as vehicle or antigen alone controls respectively. (A+C) Percentage of OVA-

specific CD8⁺ over total CD8⁺ T cells; (B+D) Total number of OVA-specific CD8⁺ T cells in the spleen (C) Representative FACS plots showing antigen-specific CD8⁺ T cells from each experimental group in both experiments. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

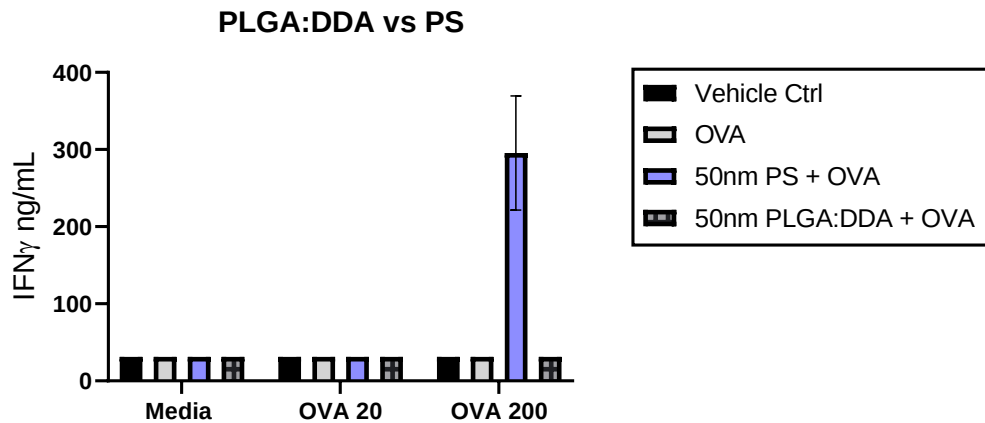
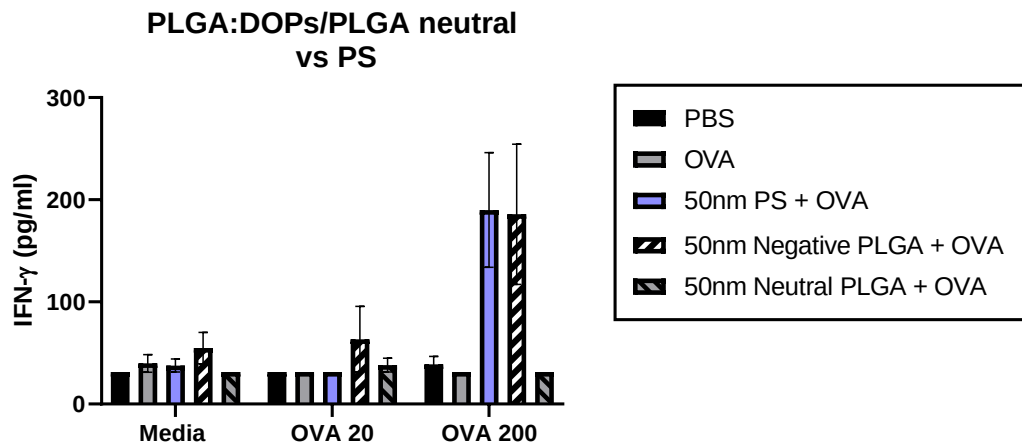
A**B**

Figure 3.5.2. Vaccination with antigen and 50nm PS NP or negatively charged PLGA (PLGA:DOPS) NP promote antigen specific IFN γ responses

C57BL/6 mice were vaccinated intramuscularly with 7.5% trehalose PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), 50nm neutral PLGA NP (1mg) + OVA (10 μ g), 50nm PLGA:DOPS NP + OVA (10 μ g), or PLGA:DDA NP + OVA (10 μ g) via the intramuscular route according to Fig.3.4.1. 7.5% trehalose in PBS or OVA alone were used as vehicle or antigen alone controls respectively. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media or ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. (A+B) Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

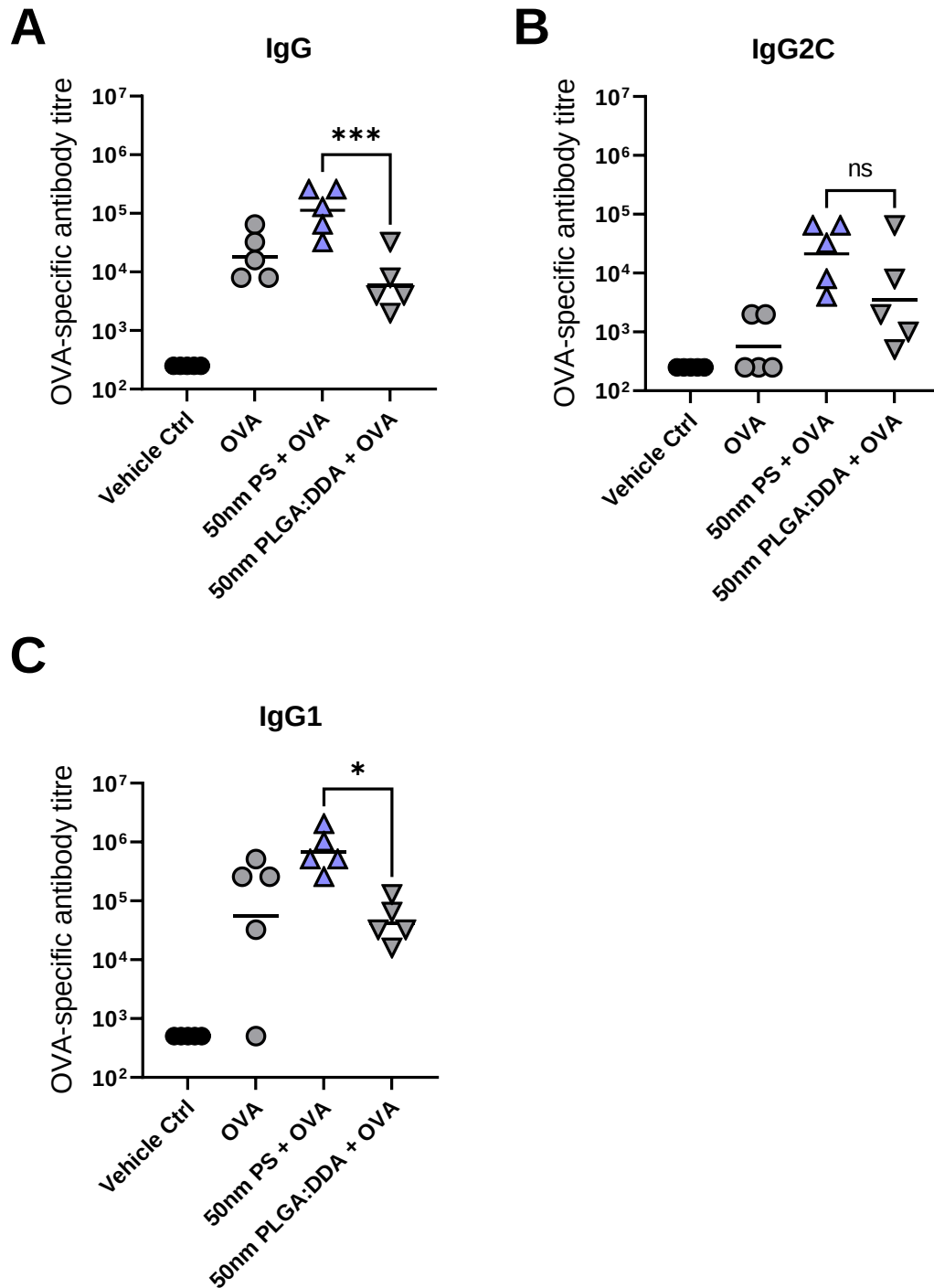


Figure 3.5.3. Vaccination with antigen and 50nm positively charged PLGA (PLGA:DDA) NP drives lower titres of antigen specific IgG than vaccination with antigen and 50nm PS NP

C57BL/6 mice were vaccinated intramuscularly with 7.5% trehalose PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), or 50nm PLGA:DDA NP + OVA (10 μ g) via the intramuscular route according to Fig.3.4.1. 7.5% trehalose in PBS or OVA alone were used as vehicle or antigen only controls respectively. On day 21, mice were sacrificed, blood was collected and serum antigen-specific A) Total IgG, B) IgG2c, C) IgG1 titres

were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

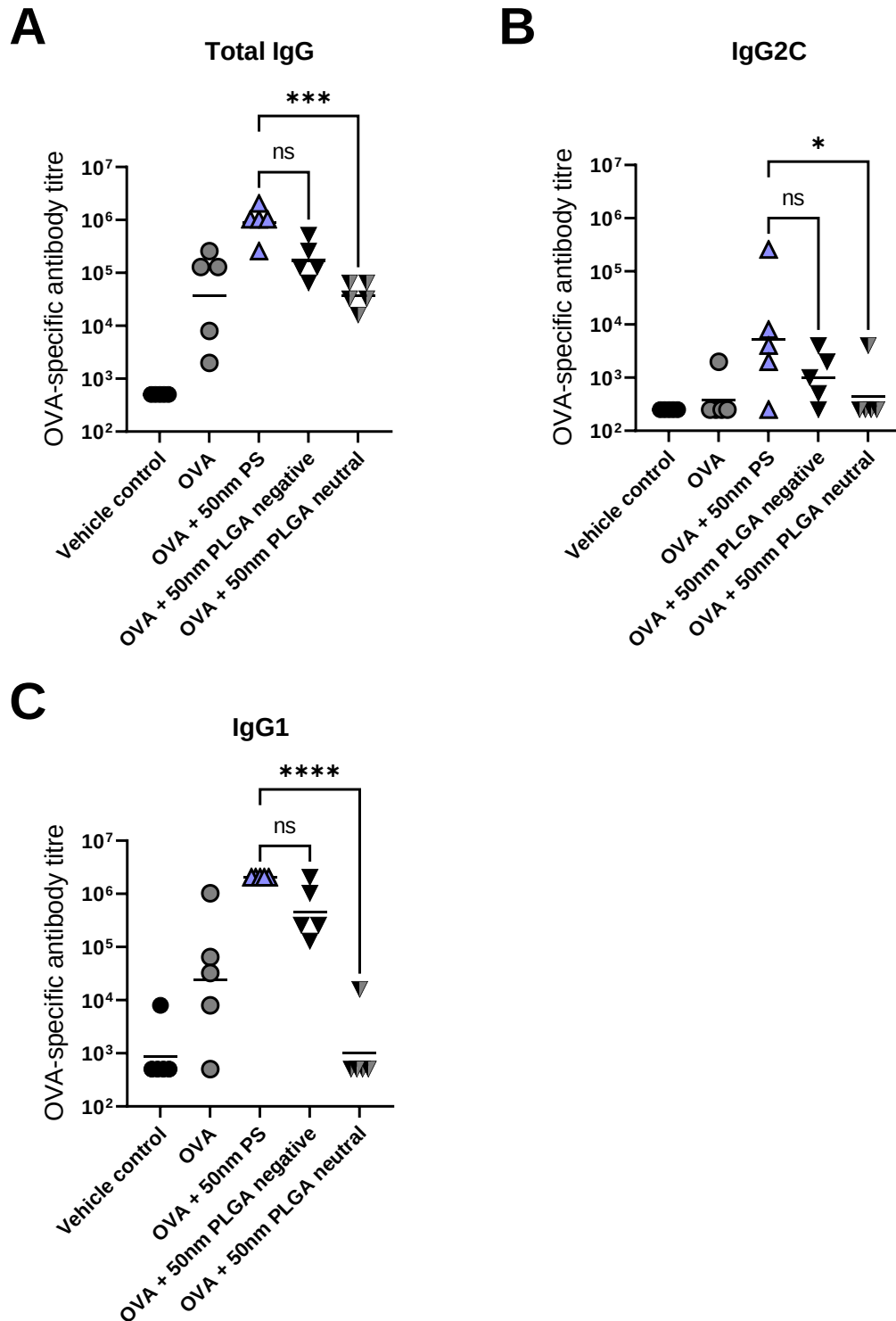


Figure 3.5.4. Vaccination with antigen and 50nm negatively charged PLGA NP (PLGA:DOPs) drives comparable antigen specific antibody titres to vaccination with antigen and 50nm PS NP.

C57BL/6 mice were vaccinated intramuscularly with 7.5% trehalose PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), 50nm neutral PLGA NP (1mg) + OVA (10 μ g), or 50nm PLGA:DOPs NP + OVA (10 μ g) via the intramuscular route according to Fig.3.4.1. 7.5% trehalose in PBS or OVA alone were used as vehicle or

antigen alone controls respectively. On day 21, mice were sacrificed, blood was collected and serum antigen-specific A) Total IgG, B) IgGc, C) IgG1 titres were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001. ~~** P<0.01, * P<0.05.~~

3.6 50nm nanoparticles drive pyroptotic cell death *in vitro*

As previously discussed, research in the Lavelle Lab conducted by Dr Fiona Sharp characterised cell death and immune cell infiltration at the injection site of mice vaccinated i.p. with 50nm PS NP²⁹¹. However, the i.m route is the dominant route of vaccination in humans. The muscle (injection site) is composed of skeletal striated muscle cells accompanied by transient immune cell populations, including APCs such as DCs. In theory, vaccine components may come into contact with any cell types present, therefore BMDCs and C2C12 cells (a differentiable murine myoblast cell line²⁸⁷) were used as representative cells for experimentation.

Cell death/viability was determined utilising three distinct assays 1) An LDH assay measuring the presence of extracellular LDH, released upon loss of cellular membrane integrity, 2) An MTT assay measuring cellular metabolic activity and by proxy, cell viability, and 3) Live dead staining and gating via flow cytometry.

Cycloheximide treatment was used as a control for apoptosis induction^{296,297}, here apoptotic body formation can be observed as early as 4 hours, as can be measured via flow cytometry and FSC-A vs SSC-A plots. This indicative apoptotic body formation was not observed in 50nm PS NP-stimulated cells. A combination of BV6 (a SMAC mimetic) and LPS in Z-VAD-fmk treated cells was used as a control for necroptotic cell death²⁹⁸, peaking in cytotoxicity at 18 hours (Fig. 3.6.1A+B). Nigericin (a potassium ionophore) and LPS stimulation was used as a control for pyroptosis induction^{299,300}, with death apparent at 1 hour, peaking at the 18 hour timepoint. While BMDCs were susceptible to all controlled death stimuli, C2C12 cells are particularly susceptible to pyroptotic cell death (Fig. 3.6.1C).

Incubation with 50nm PS NP induced dose dependant cell death in both BMDCs and C2C12 cells (Fig. 3.6.1). Cell death was detectable at 4 hours post treatment and continued to increase up to 18-hours (Fig. 3.6.1A + B). A timepoint of 18-24 hours was subsequently used in all following experiments as a result.

While it is important to determine the cytotoxicity of vaccine adjuvants, it is crucial to determine the modality of cell death induced to ascertain any mechanistic insights. To achieve this, inhibitors commonly used to dissect cell death modalities were used. Z-VAD-fmk, a pan-caspase inhibitor blocking apoptotic and pyroptotic pathways, necrosulfonamide (NSA), a potent inhibitor of murine gasdermin D (GSDMD)³⁰¹ and subsequently pyroptosis, and dimethyl fumarate (DMF) another GSDMD and pyroptosis inhibitor³⁰² were used.

Interestingly, pre- treatment with all the inhibitors tested resulted in a reduction in 50nm PS NP mediated cytotoxicity (Figure 3.6.2A + 3.6.2B), as measured via LDH assay and flow cytometry live dead staining. NSA and DMF pre-treatment had the most potent inhibitory effects, with Z-VAD-fmk exerting a modest reduction in cytotoxicity. Unfortunately for the experiments where Z-VAD-fmk and NSA were used as inhibitors there was no pyroptotic control included for study, as controls at that time in the project were focused on apoptosis and necroptosis.

Intriguingly, 50nm NP induced cytotoxicity was only modestly reduced at the highest concentration tested when comparing BMDCs from WT and GSDMD KO mice (Fig. 3.6.2C). Importantly, there was also residual cytotoxicity in the Nigericin + LPS positive control treated cells in GSDMD KO BMDCs (Fig.3.6.2C) and in DMF treated BMDCs (Fig.3.6.2B) , hinting at compensatory death pathways being elicited.

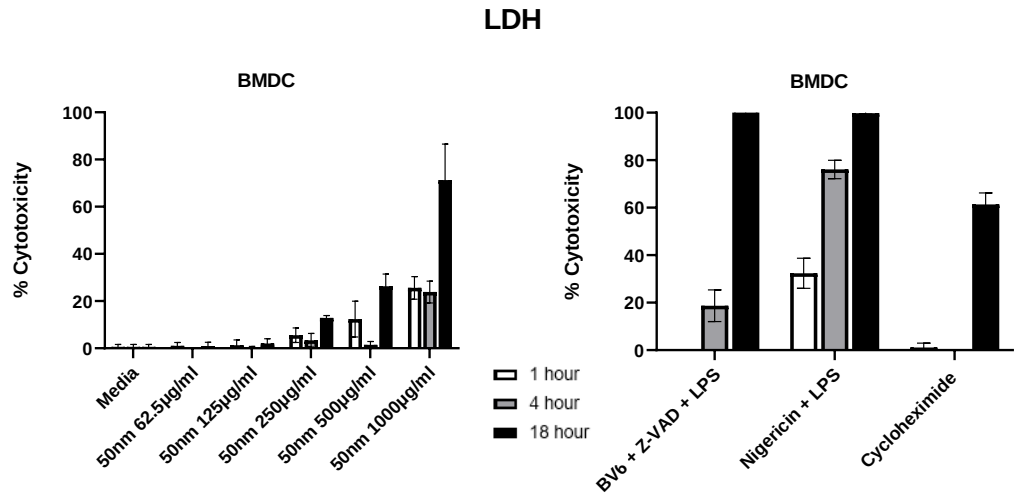
Importantly through western blotting, 50nm NPs were identified as being able to mediate GSDMD cleavage in BMDCs when combined with LPS (Fig.3.6.3) and this was shown to be a particle size dependant phenomenon as 1 μ m sized PS could not mediate this effect. Furthermore, LPS treatment alone could not induce GSDMD cleavage but importantly GSDMD cleavage was observed in the cells treated with nigericin and LPS (positive control). In both LPS primed and unprimed BMDCs, treatment with 50nm PS NP could mediate cleavage of GSDMD. 50nm NPs could not mediate the cleavage of GSDMD alone (Data not shown).

Pyroptosis can be elicited via a canonical or non-canonical pathway, with the former reliant on inflammasome assembly with downstream caspase 1 activation and the latter reliant on activation of caspase 11 (caspase 4/5 in humans)^{238,239}. Cellular ROS has been implicated in the elicitation of the pyroptotic pathway through generation of oxidised ligands for Caspase 11/4/5 activation and through direct oxidation of the Cys191/Cys192 residue of GSDMD^{236,237}. To investigate if 50nm NPs were enhancing cellular ROS in stimulated cells and if this was a particle size dependent phenomenon, CellROX reagent staining and FACS analysis was utilised (Fig.3.6.4A). BMDCs were stimulated with 50nm, 200nm or 1 μ m PS particles for 6 hours and 18 hours alongside hydrogen peroxide (H₂O₂) alone, as a positive control for ROS induction. 50nm PS NPs were found to induce cell ROS from 6 hours onwards, peaking at 18 hours post incubation (Fig.3.6.4B+E). In contrast incubation of cells with 200nm or 1 μ m sized PS NP did not induce cellular ROS above levels observed basally in unstimulated BMDCs (Fig.3.6.4C+D+E). There was some difficulty in measuring cell ROS levels in BMDCs stimulated with higher concentrations of 50nm NPs (>150 μ g/ml), potentially arising due to increased cytotoxicity in these samples (Data not shown). The ROS generated following 50nm NP stimulation appears not to be mitochondrial in origin, as determined via MitoSOX staining via flow cytometry (Fig.3.6.5). There was no

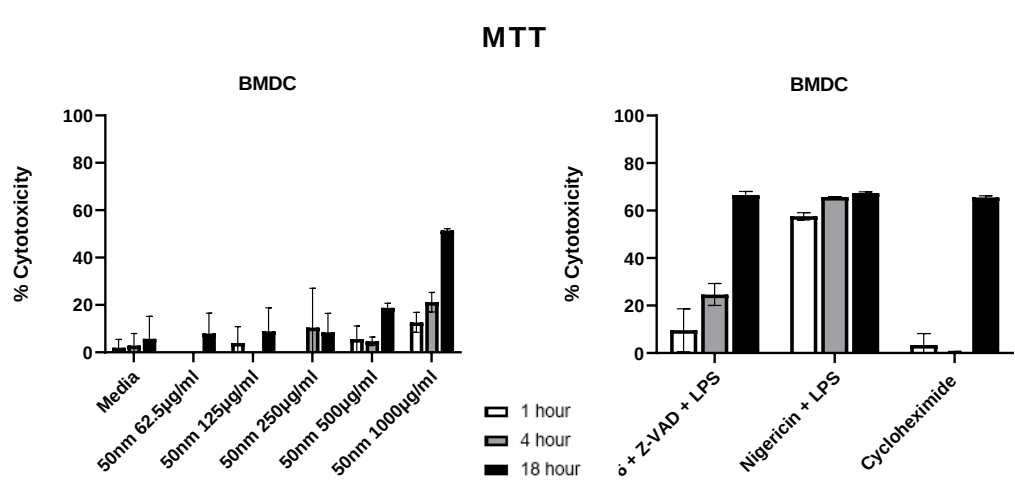
observed increase in MitoSOX intensity in 50nm NP treated BMDCs, while an increase was observed in C100 treated cells acting as a positive control.

Altogether, these results suggest that 50nm PS NP induce pyroptotic cell death and indicate a particle size dependent induction of cell ROS and GSDMD cleavage, supporting *in vivo* studies to address the role of these pathways in 50nm PS NP adjuvanticity.

A



B



C

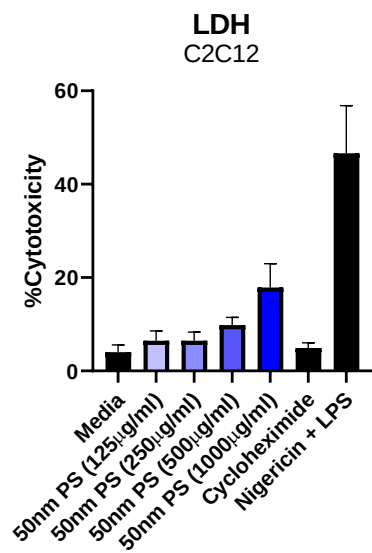


Figure 3.6.1 Incubation with 50nm PS NPs exerts dose dependant cytotoxic effects on BMDCs and C2C12 cells.

A+B) 6×10^5 BMDCs were stimulated with 50nm PS NP concentrations from $62.5 \mu\text{l/ml}$ - $1000 \mu\text{g/ml}$, or $25 \mu\text{g/ml}$ cycloheximide (Apoptosis control), or $2.5 \mu\text{M}$ BV6/ $10 \mu\text{M}$ Z-VAD-fmk/ 10ng/ml LPS (Necroptosis control), or $10 \mu\text{M}$ nigericin/ 10ng/ml LPS (Pyroptosis control) for 1 hour, 4 hours or 18 hours as indicated. Cell death was analysed using A) LDH assay on supernatants and B) MTT assay on cells. C) Confluent, differentiated C2C12 cells in 24W plates were stimulated with a range of particle concentrations from $125 \mu\text{g/ml}$ – $1000 \mu\text{g/ml}$, $25 \mu\text{g/ml}$ of cycloheximide, or 10ng/ml LPS/ $10 \mu\text{M}$ nigericin for 18 hours, cell death was analysed using an LDH assay on supernatants. A+B+C) Data representative of 3 independent experimental repeats.

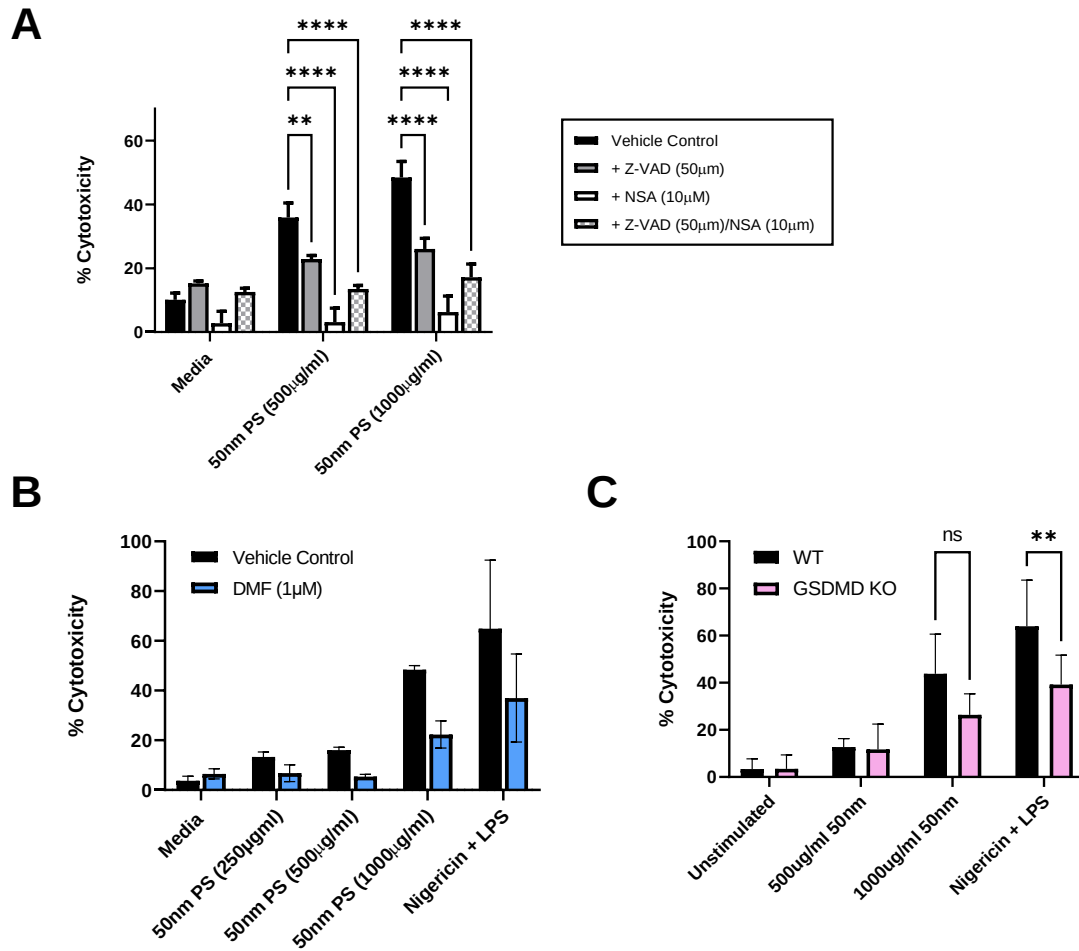


Figure 3.6.2. 50nm PS NP mediated cytotoxicity is partially inhibited through pan-caspase and GSDMD inhibition

A+B+C) 6×10^5 BMDCs were plated and pre-treated for 1 hour with: A) Media, 10μM NSA, 50μM Z-VAD-fmk or both inhibitors in combination B) Media, NSA 10μM or 1μM DMF. Cells were then stimulated with 250μg/ml, 500μg/ml and 1000μg/ml concentrations of 50nm PS NPs for 18 hours. Cell death was analysed using A) Live/dead staining and gating via flow cytometry, B+C) LDH assay on culture supernatants. Data are displayed as mean cytotoxicity $\pm$ SD. A+C) Data is representative of two independent experimental repeats. B) Data is representative of one experiment.

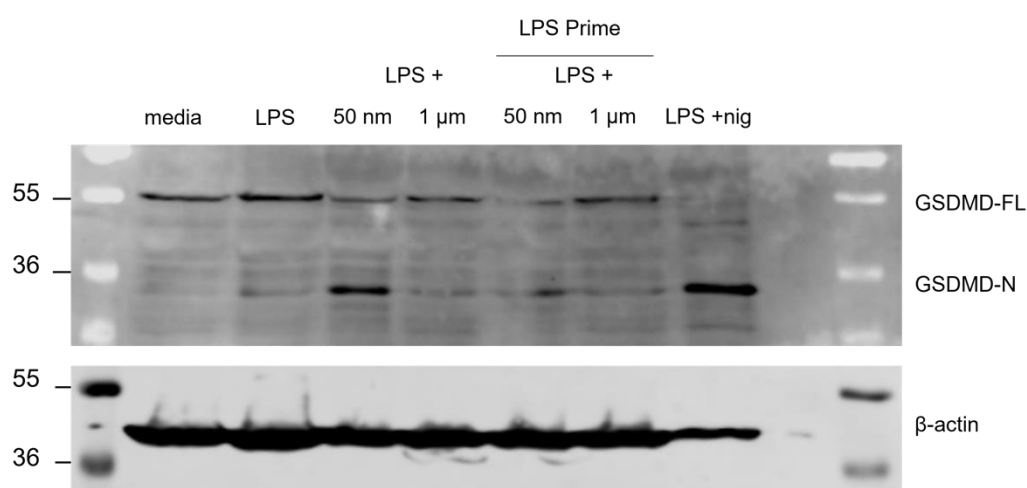


Figure. 3.6.3 Incubation of BMDCs with 50nm PS NPs but not larger particles can enhance the cleavage of GSDMD

BMDCs were primed with LPS (10ng/ml) or left unprimed and were then stimulated with 50nm or 1μm particles (500μg/ml) in combination with LPS (10ng/ml), or LPS alone (10ng/ml) for 18 hours. Nigericin (10μM) and LPS (10ng/ml) treatment was used as a positive control. Cell lysates were probed for full length and cleaved GSDMD protein, β-actin was used as a loading control. LPS + Nigericin treated BMDCs were used as positive control for GSDMD cleavage. Data representative of 3 independent experimental repeats *Blots performed by Dr Katharine Shanahan/Coralie Guy

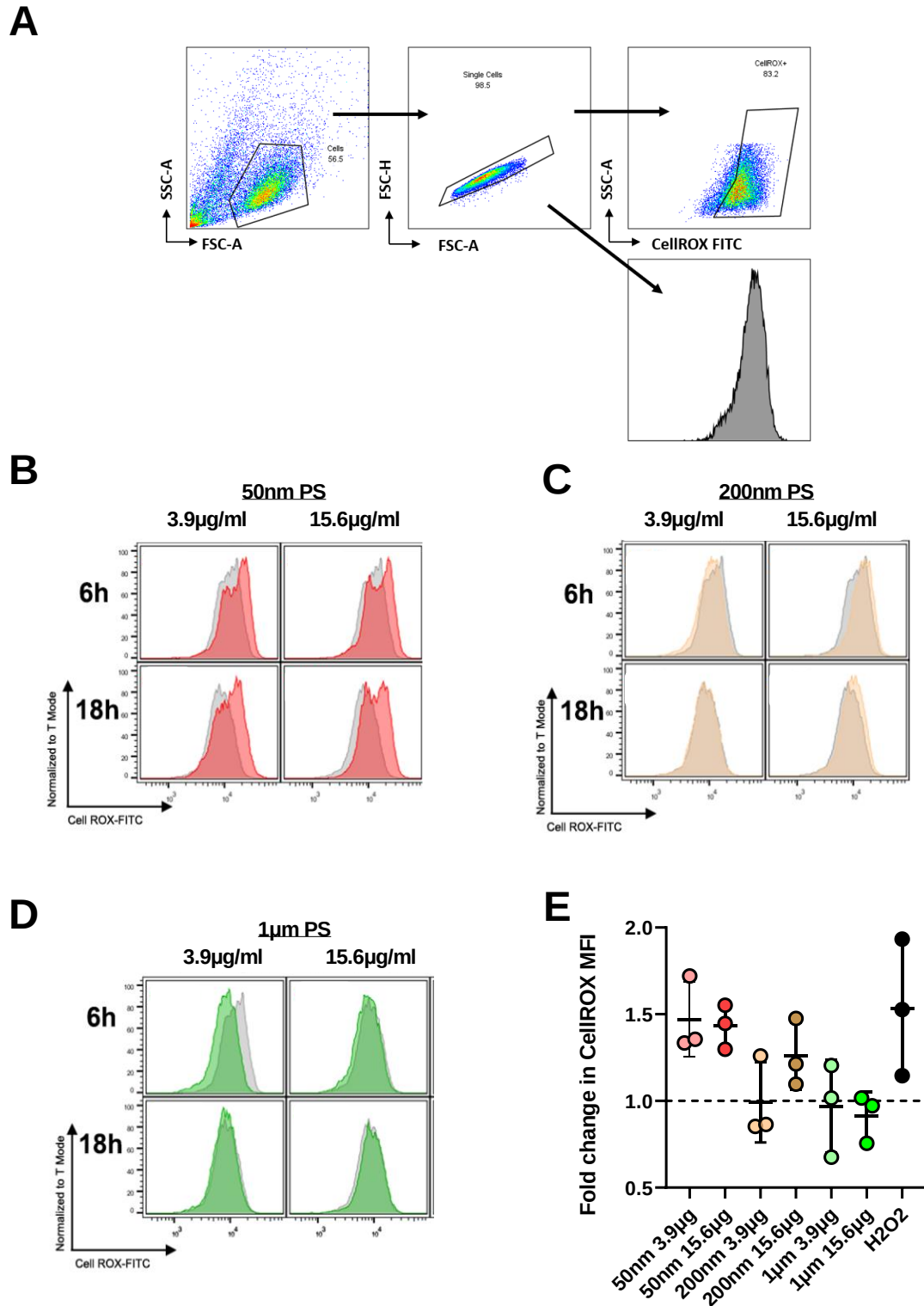


Figure 3.6.4 Incubation of BMDCs with 50nm PS NP but not 200nm or 1µm PS NP results in enhanced cellular ROS.

6x10⁵ BMDCs were plated and stimulated with media, 3.9µg/ml or 15.6µg/ml of 50nm PS NPs or 100nM H₂O₂ for 6 hours or 18 hours. A) Cells were then removed from the plates and the presence of cell ROS was analysed using CellROX Green and the BD FACSCANTO II according to the gating strategy shown. B)

Representative histogram plots of 50nm PS NP treated cells, with the grey histogram representing media treated cells and red coloured histograms representing 50nm PS NP treated cells. C) Representative histogram plots of 200nm PS NP treated cells, with the grey histogram representing media treated cells and brown coloured histograms representing 50nm PS NP treated cells. D) Representative histogram plots of 1 μ m PS NP treated cells, with the grey histogram representing media treated cells and green coloured histograms representing 50nm PS NP treated cells. A+B+C) Representative data of 3 independent experimental repeats. D) Mean fold change in CellROX MFI compared to unstimulated BMDC controls at the 18hour timepoint is displayed +/- SD of the 3 independent experimental repeats.

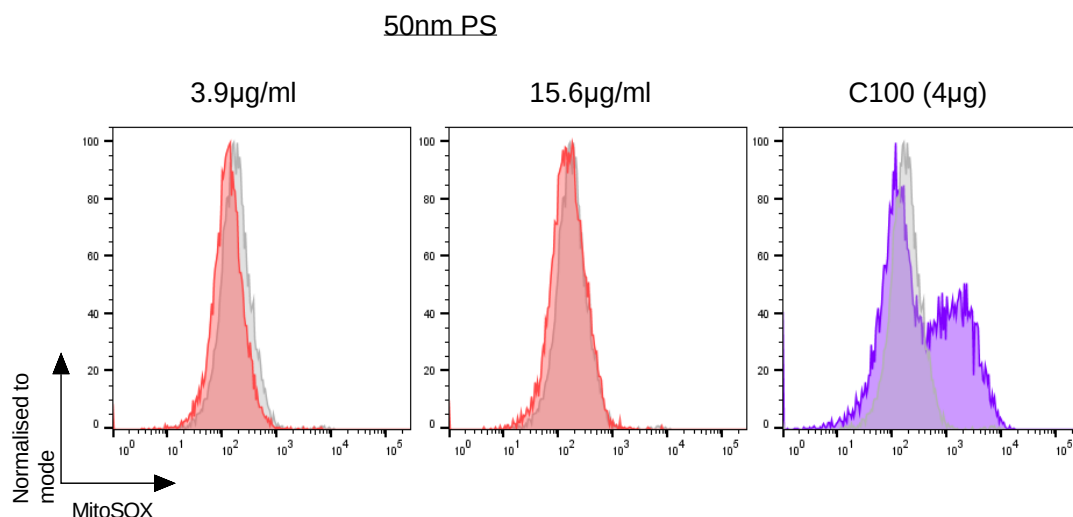


Figure 3.6.5 ROS induced following incubation with 50nm NP is not mitochondrial.

6×10^5 BMDCs were plated and stimulated with media, 3.9µg/ml or 15.6µg/ml of 50nm PS NP, or 4µg of C100 as a positive control 18 hours. Cells were then removed from the plates and the presence of mitochondrial ROS was analysed using MitoSOX Red on the BD FACSCANTO II. Representative histogram plots of 50nm PS NP or C100 treated cells, with the grey histogram representing media treated cells and red coloured histograms representing 50nm PS NP treated cells, and purple representing C100 treated cells. Data is representative of one experiment.

3.7 Caspase 11 is required for the ability of 50nm PS NPs to promote antigen specific cellular immune responses

With *in vitro* data suggesting that 50nm NPs elicit pyroptotic cell death and recent literature identifying ICD as an effective driver of adaptive immunity, the role of pyroptosis in nanoparticle induced adaptive immunity was assessed.

As previously described, pyroptosis can be initiated in a canonical and non-canonical setting, canonical pyroptosis can therefore be blocked through targeting inflammasome activation and assembly, caspase 1 activation and GSDMD oligomerisation. The non-canonical route can be targeted by inhibiting caspase 11 and GSDMD activation.

To ascertain the impact of blocking pyroptosis, caspase 11 deficient mice and C57BL/6 mice injected with Ac-YVAD-cmk (Caspase 1 inhibitor) were vaccinated i.m. as in Figure 3.7.1A and responses were compared to those in untreated WT C57BL6/J mice. Muscle tissue and spleens from these mice were isolated 21 days post initial priming and were digested and stained for OVA-specific CD8⁺ T cells using Class I MHC-SIINFEKL tetramer staining. Populations were gated as displayed in Figure.3.7.1B + C in spleen and muscle samples respectively.

Caspase 11 KO mice vaccinated with 50nm PS nanoparticles and OVA had a significantly lower proportion of splenic CD44⁺ antigen-specific CD8⁺ T cells, represented in terms of frequency (Fig.3.7.2A + Fig.3.7.3) and in total cell number (Fig.3.7.2B) when compared to WT mice vaccinated with 50nm PS and OVA. There was also a lower frequency (Fig.3.7.2C + Fig.3.7.3) and lower total number of CD44⁺ antigen-specific CD8⁺ T cells per g in the muscle of the caspase 11 KO mice, albeit not significant different compared to the immunised WT mice.

Antigen-specific IFN γ production by splenocytes from caspase 11 deficient mice vaccinated with 50nm PS and OVA was significantly lower than in WT mice following 72-hour antigen restimulation (Fig.3.7.4). In contrast the capacity of vaccination with 50nm PS NP and OVA to enhance antigen-specific IgG titres remained intact in caspase 11 KO mice (Fig.3.7.5).

Caspase 1 inhibition (Ac-YVAD-cmk) led to a reduction in the frequency of splenic CD44⁺ antigen-specific CD8⁺ T cells (Fig.3.7.2A) but not in total numbers present (Fig.3.7.2B) in mice immunised with 50 PS NP and OVA when compared to immunised untreated WT counterparts. No difference in induction of CD44⁺ Class I Tmer⁺ CD8⁺ T cells was observed in the muscle of caspase 1 inhibited mice when compared to immunised WT.

However, antigen-specific IFN γ production by splenocytes isolated from mice immunised with 50nm PS NP and OVA and treated with a caspase 1 inhibitor was significantly lower than in vaccinated but untreated WT counterparts following 72-hour antigen restimulation (Fig.3.7.4). The ability of vaccination with 50nm PS NP and OVA to promote antigen-specific antibody responses was largely intact following caspase 1 inhibition, except for a significant decrease in antigen-specific IgG1 induction (Fig.3.7.5).

In contrast to the clear role for caspase 11, there was no reduction in 50nm PS NP + OVA-mediated splenic antigen-specific CD8 $^{+}$ T cells in immunised IL-1R1 KO or NLRP3 KO mice (Fig.3.7.6), in fact IL-1R1 KO mice had a significant increase in splenic CD44 $^{+}$ antigen-specific CD8 $^{+}$ T cells (Fig.3.7.6A). However, both IL-1R1 and NLRP3 KO mice had significantly less antigen-specific IFN γ production in splenocytes isolated following 72-hour antigen restimulation when compared to WT counterparts (Fig.3.7.6 C+D).

Overall, these data suggest a pivotal role for caspase 11 in the capacity of 50nm PS NPs to drive antigen-specific cellular immunity. Previous work in the Lavelle Lab by Dr Katie O'Grady²⁹⁰ has characterised antigen-specific CD4 $^{+}$ and not CD8 $^{+}$ T cells as the source of IFN γ following vaccination with antigen and 50nm PS NP. Therefore, this indicates that caspase 11 is essential for the induction of CD8 $^{+}$ and CD4 $^{+}$ T cells following vaccination with 50nm PS NP and OVA, suggesting a potential role for the non-canonical pyroptotic pathway. However, this inferred effect on the induction of antigen-specific CD4 $^{+}$ T cells in caspase 11 KO mice should be confirmed through Class II tetramer staining analysis.

Caspase 1, IL-1R1 and NLRP3 when absent or inhibited had a limited impact on induction of antigen-specific CD8 $^{+}$ T cells in the spleens of immunised mice. However, these same mice had a diminished capacity for production of IFN γ following antigen restimulation of splenocytes. This suggests that the NLRP3-caspase1-IL-1 pathway may be required for CD4 $^{+}$ T cell induction or the maintenance of IFN γ production in these cells. It is worth noting that these molecules can all be involved in the execution of and downstream signalling following canonical pyroptosis.

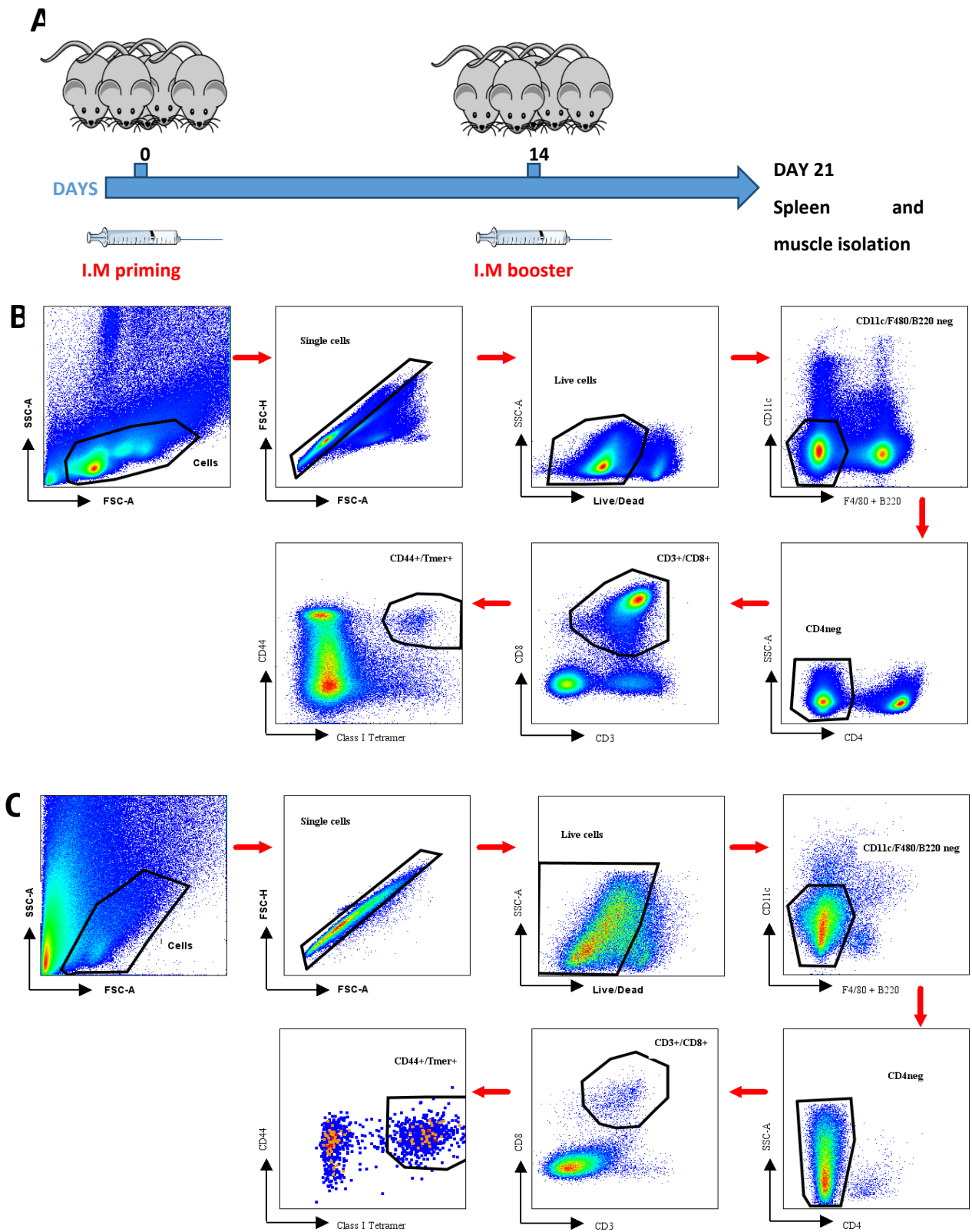


Figure 3.7.1 Vaccination scheme and gating strategy for the identification of antigen-specific CD8⁺ T cells

A) Vaccination scheme for study, C57BL/6 mice received a priming vaccination intramuscularly on day 0 followed by a booster on day 14. Spleens (B) and caudal thigh muscle (C) were isolated on day 21, processed, and additionally digested in the case of muscle samples. Single cell suspensions were obtained for staining to characterise antigen-specific T cells via flow cytometry. Cells were acquired using LSR Fortessa and analysed according to gating strategies presented. Splenocytes were also plated for 72-hour antigen restimulations.

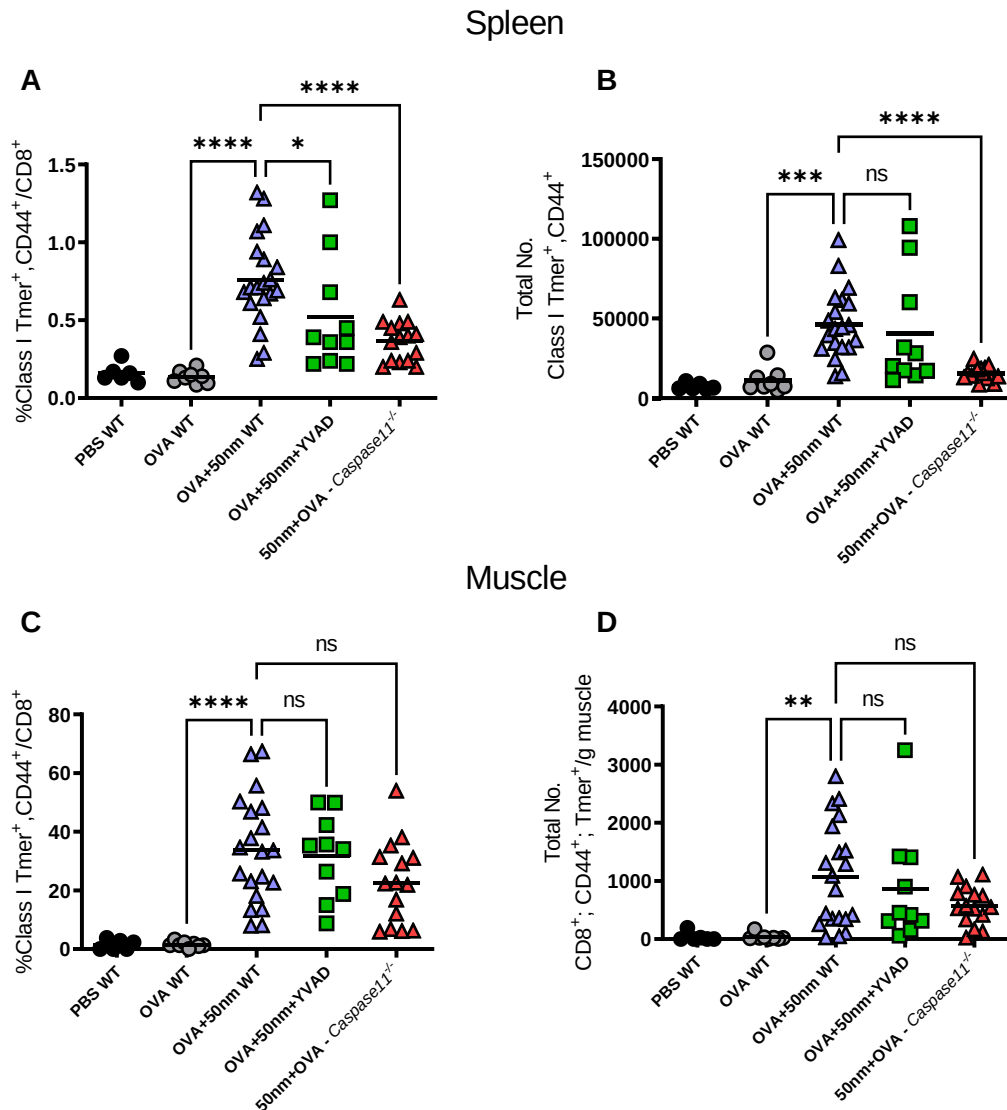


Figure 3.7.2. Caspase 11 is required for optimum induction of antigen-specific CD8 responses following vaccination with 50nm PS NP and OVA

Caspase 11 KO or WT mice were vaccinated intramuscularly with PBS, OVA (10 μ g), 50nm PS NP (1mg) + OVA (10 μ g), or 50nm PS NP (1mg) + OVA (10 μ g) + 0.25mg of caspase 1 inhibitor Y-VAD via the intramuscular route according to Fig.3.7.1. On day 21, antigen-specific responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen alone controls. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen. (C) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells in the muscle; (D) Total number of OVA-specific CD8⁺ T cells per gram of muscle. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

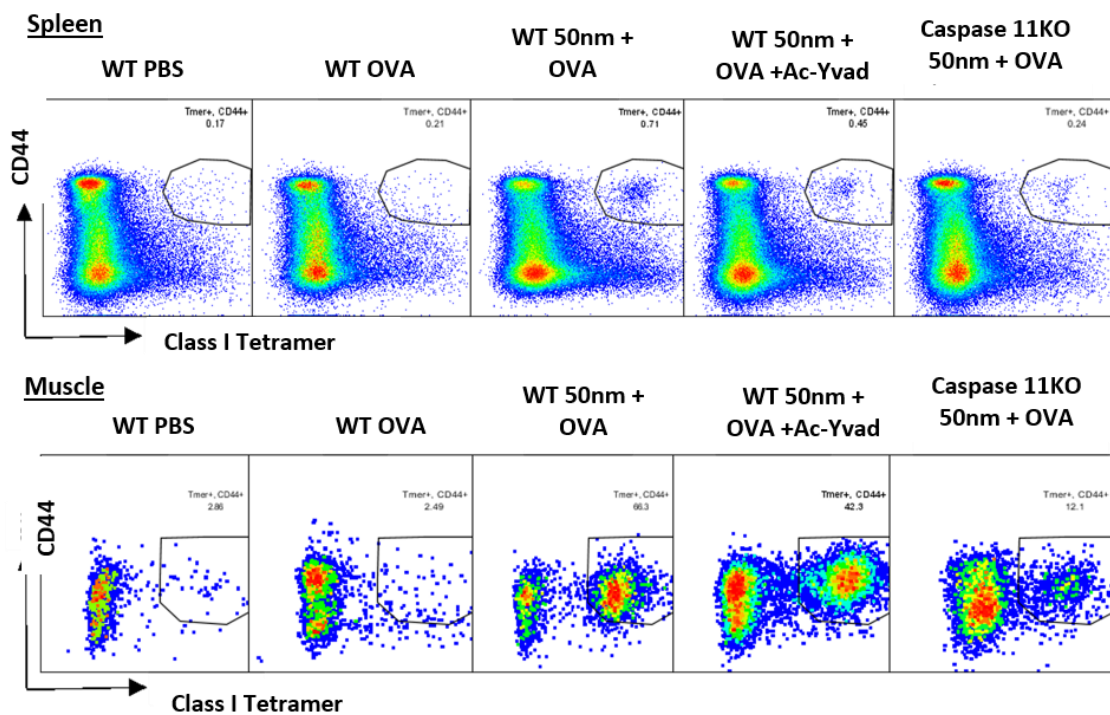


Figure 3.7.3 Representative FACS plots of the muscle and spleen from the 50nm PS NP vaccination study in Caspase 11KO and Ac-YVAD-cmk treated mice.

FACS plots are gated on Class I Tetramer⁺/CD44⁺ CD8⁺ T cells.

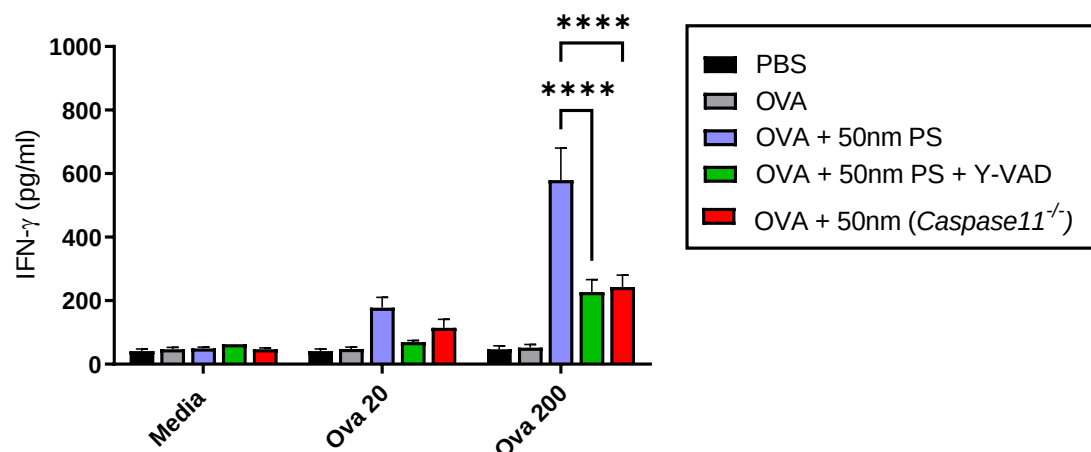


Figure 3.7.4 Caspase 11 and caspase 1 activity are required for optimum antigen-specific IFN γ production in splenocytes following vaccination with 50nm PS NP and OVA.

Caspase 11 KO or WT mice were vaccinated intramuscularly with 1mg of 50nm PS NP plus 10 μ g of OVA, or particles, OVA and 0.25mg of caspase 1 inhibitor Y-VAD via the intramuscular route according to Fig.3.7.1. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media or ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicates significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

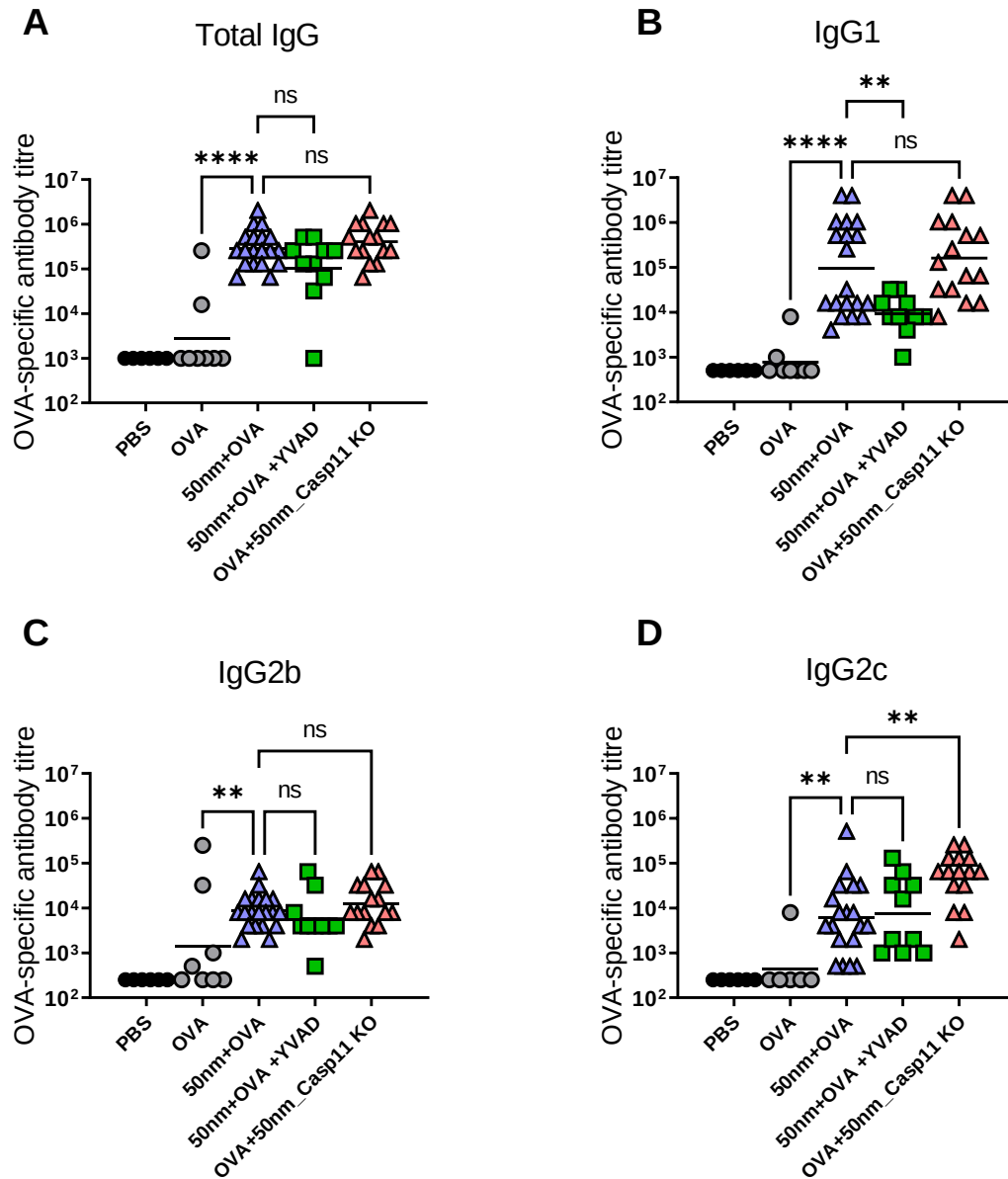


Figure 3.7.5 Caspase 11 is not required for induction of antigen-specific antibodies following vaccination with 50nm PS NP and OVA

Caspase 11 KO or WT mice were vaccinated intramuscularly with 1mg of 50nm particles plus 10 μ g of OVA, or particles, OVA and 0.25mg of caspase 1 inhibitor Y-VAD via the intramuscular route according to Fig.3.7.1. On day 21, mice were sacrificed, blood was collected and serum antigen-specific A) Total IgG, B) IgG1, C) IgG2b and D) IgG2c titres were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

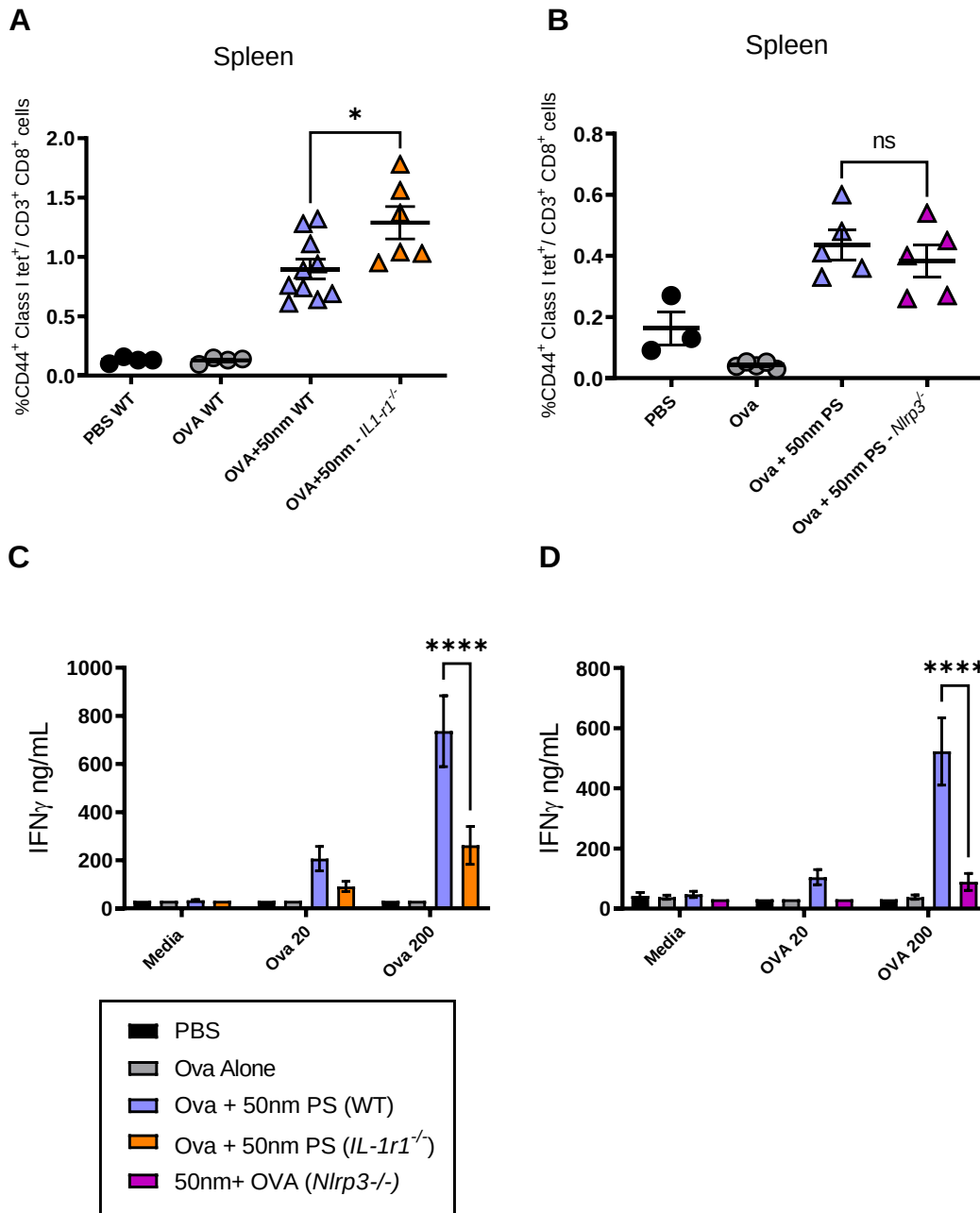


Figure 3.7.6 IL-1R1 and NLRP3 are dispensable for induction of antigen-specific CD8⁺ T cells but are required for optimum induction of antigen-specific IFN γ production following vaccination with 50nm PS NP and OVA.

IL-1R1 KO, NLRP3 KO and WT mice were vaccinated intramuscularly with 1mg of 50nm PS particles plus 10 μ g of OVA, or particles, OVA and 0.25mg of caspase 1 inhibitor Y-VAD via the intramuscular route according to Fig.3.7.1. On day 21, antigen-specific responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen alone controls. (A+B) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells. Splenocytes were also plated and re-

stimulated *ex vivo* with cRPMI media or ova (20µg/mL or 200µg/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. (C+D). Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

3.8 Inhibition of ROS locally at the intramuscular injection site using TEMPOL impairs the capacity of 50nm NPs to mount antigen specific CD8⁺ T cell responses

As the capacity of 50nm PS NP to drive antigen-specific cellular immunity was diminished in caspase 11 KO mice, it implicated the non-canonical inflammasome pathway in mediating 50nm NP adjuvanticity. Cellular ROS is intricately linked to the activation of this pathway through the generation of oxidised endogenous ligands for caspase 11²⁴² and downstream in the direct oxidation of GSDMD at the Cys191/192 residue²³⁷, sensitising GSDMD for subsequent activation and characterising GSDMD as a sensor for cellular redox. To examine the potential role for ROS in 50nm NP adjuvanticity, the ROS scavenger TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) was utilised. TEMPOL is a membrane permeable SOD mimetic that acts as free radical scavenger, possessing anti-oxidant properties³⁰³. It has been used extensively *in vivo* in preclinical rat and murine models of disease and is currently being trialled clinically treating COVID-19 (NCT04729595) and side effects associated with cancer chemo/radiotherapy (NCT03480971).

TEMPOL was either injected intraperitoneally into mice or intramuscularly 30 minutes prior to intramuscular immunisation to promote ROS scavenging upon vaccination, and again 24 hours following vaccination to maintain ROS scavenging early in the resulting immune response. Injection of TEMPOL intramuscularly prior to vaccination with 50nm PS NP and OVA significantly reduced splenic antigen-specific CD8⁺ T cell induction compared to the non-treated 50nm PS NP + OVA vaccinated group in terms of frequency of CD44^{hi} Tmem⁺ CD8⁺ T cells (Fig.3.8.1A) and in total number of these cells (Fig.3.8.1B). This indicates a mechanistic dependency on local ROS at the injection site following vaccination with 50nm NPs

In contrast there was no apparent effect of local ROS inhibition on the production of antigen specific IFN γ by splenocytes from mice vaccinated with 50nm NPs + OVA following 72-hour restimulation (Fig.3.8.2), however, a slight increase was observed in mice which received systemic TEMPOL. Similarly, there was no observed effect on the 50nm NP mediated induction of antigen specific IgG (Fig.3.8.3A) or IgG2c (Fig.3.8.3B) when mice received local or systemic TEMPOL.

Overall, these results indicate that local ROS production plays a role in mediating 50nm PS NP adjuvanticity in terms of antigen specific CD8⁺ T cell induction, but not in the induction of antigen specific IFN γ or antibody responses.

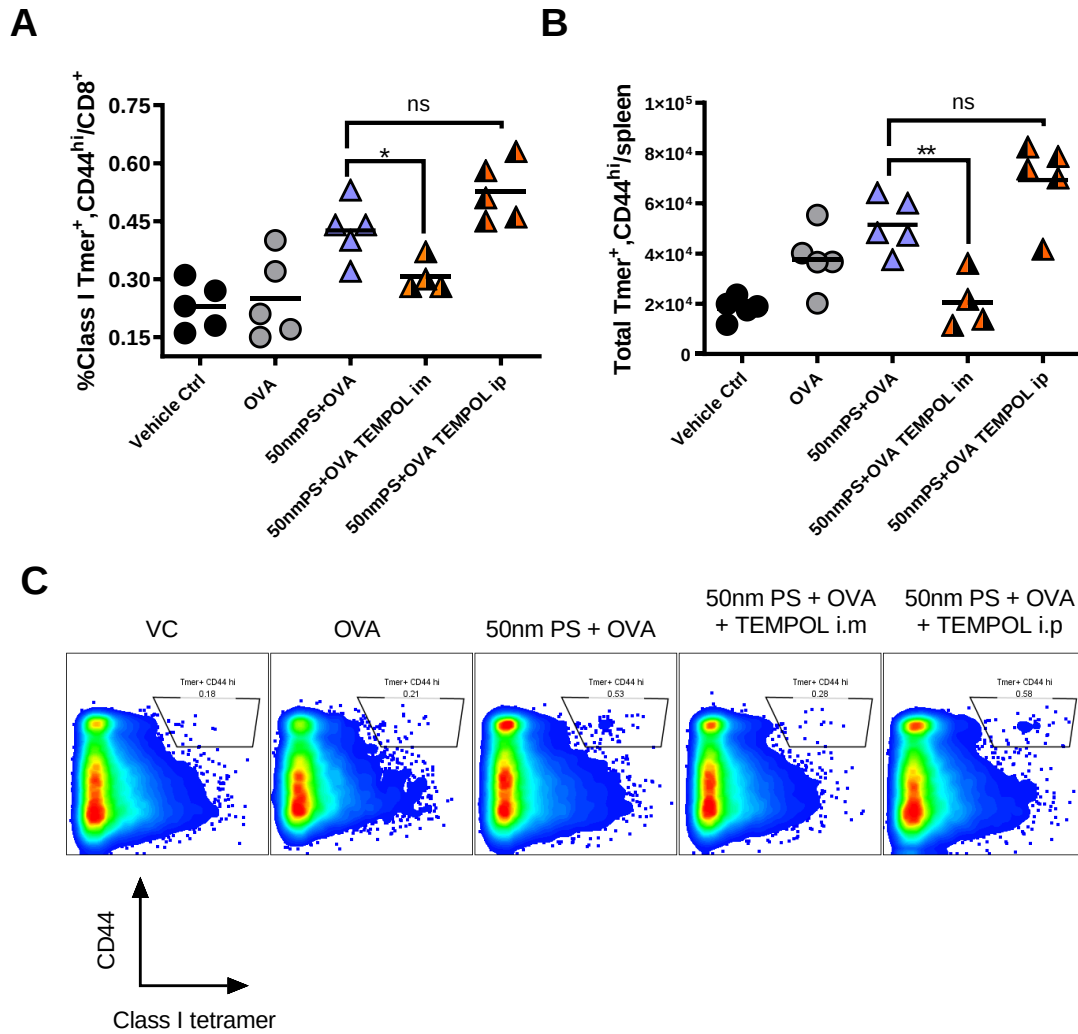


Figure 3.8.1. Inhibition of ROS locally at the intramuscular injection site impairs the ability of 50nm NPs to mount antigen specific-CD8⁺ T cell responses

C57BL/6JCR mice were injected with either PBS or TEMPOL (100mg/kg) intraperitoneally and PBS or TEMPOL (25mg/kg) intramuscularly 30 minutes prior to vaccination, and again 24 hours following vaccination. Mice were subsequently vaccinated with either PBS, OVA (10 μ g), or 50nm PS NP (1mg) + OVA (10 μ g). according to the schematic detailed in Fig.3.7.1. On day 21, antigen-specific responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen alone controls. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen. (C) Representative FACS plots, gated on CD44^{hi} Class I SIINFEKL tetramer⁺ CD8⁺ T cells. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

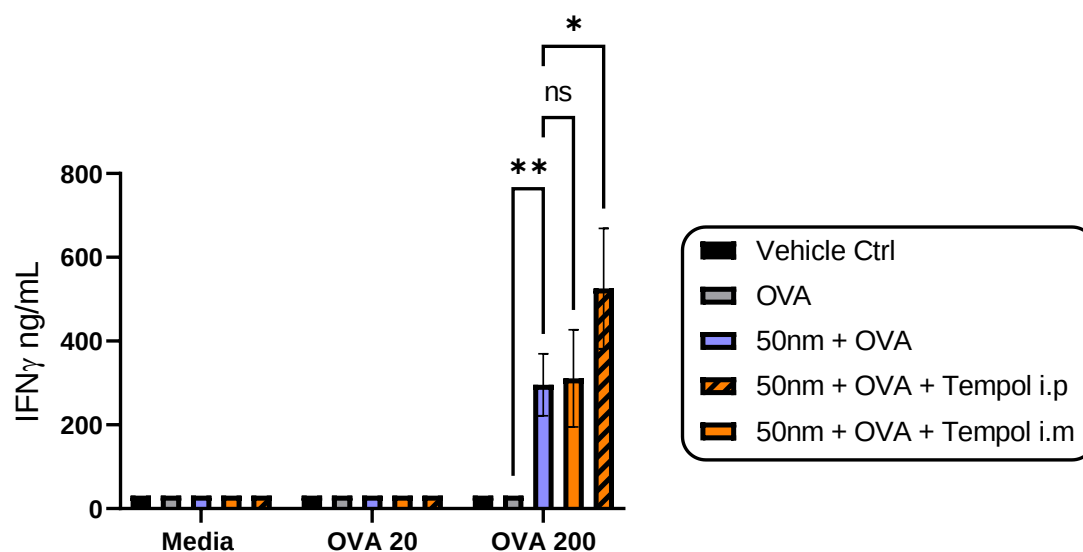


Figure 3.8.2. Inhibition of ROS locally or systemically does not impair the induction of antigen-specific IFN γ responses in splenocytes following vaccination with 50nm PS NP and OVA.

C57BL/6JCR mice injected with either PBS or TEMPOL (100mg/kg) intraperitoneally and PBS or TEMPOL (25mg/kg) intramuscularly 30 minutes prior to vaccination, and again 24 hours following vaccination. Mice were subsequently vaccinated with either PBS, OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g). according to the schematic detailed in Fig.3.7.1. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media or ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

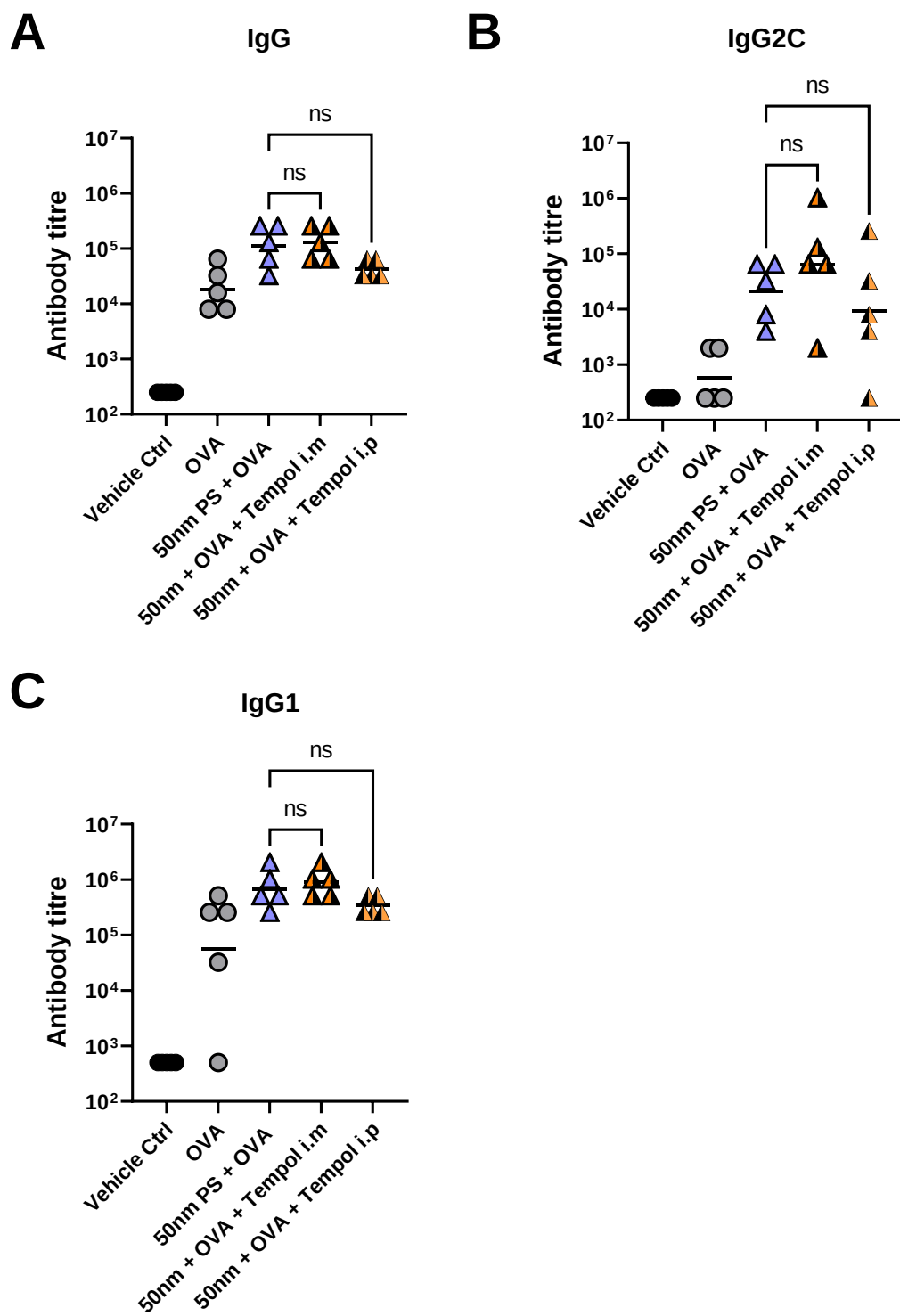


Figure 3.8.3. Inhibition of ROS locally or systemically does not impair the ability of 50nm NPs to induce antigen specific IgG or IgG2c responses.

C57BL/6JCR mice injected with either PBS or TEMPOL (100mg/kg) intraperitoneally and PBS or TEMPOL (25mg/kg) intramuscularly 30 minutes prior to vaccination, and again 24 hours following vaccination. Mice were subsequently vaccinated with either PBS, OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g). according to the schematic detailed in Fig.3.7.1. On day 21, mice were sacrificed, blood was collected and serum

antigen-specific A) Total IgG, B) IgG2c, C) IgG1 titres were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

3.9 Chemical inhibition of GSDMD locally at the injection site abrogates the adjuvanticity of 50nm NPs *in vivo*

While the capacity of 50nm PS NP to drive antigen-specific cellular immunity was diminished in caspase 11 KO mice and partly blocked in NLRP3 KO, IL1-R1 KO mice or in those treated with ROS scavengers or caspase 1 inhibitors. It remained to be determined whether blockade of the pyroptotic signalling cascade was responsible for reduced responses and not another function of these molecules. To address the specific role for pyroptosis, co-injection of NSA with 50nm PS NP was used to block local induction of GSDMD activation and subsequent pyroptosis. NSA was originally developed as an inhibitor of human MLKL and subsequent necroptosis, however Liu *et al*, discovered that NSA binds to GSDMD and prevents oligomerisation following dimerization³⁰¹. This in turn prevents the GSDMD pore formation in cells that ultimately would lead to pyroptotic cell death. As shown in section 3.5, NSA pre-treatment reduced the cytotoxic capacity of 50nm PS NP *in vitro*.

Co-injection of NSA intramuscularly with the 50nm PS NP and OVA blocked the induction of antigen-specific CD8⁺ T cells. This was apparent in the spleens and muscles of these mice with complete ablation of the response when compared to vehicle injected, 50nm NP + OVA immunised mice (Fig.3.9.1 + Fig.3.9.2). This is represented in terms of the frequency (Fig.3.9.1A) and total CD44⁺ antigen-specific CD8⁺ T cell number present in the spleen (Fig.3.9.1B), and the frequency (Fig.3.9.1C) and total CD44⁺ antigen-specific CD8⁺ T cell number per g in the muscle (Figure 3.9.1D).

Co-injection of NSA also significantly diminished the production of antigen-specific IFN γ in splenocytes following 72-hour antigen restimulation (Fig.3.9.3), but only slightly reduced the magnitude of total antigen-specific IgG (Fig.3.9.4A).

To further interrogate the role of GSDMD in 50nm PS NP mediated adjuvanticity, GSDMD KO mice were sourced from Jackson Laboratories, which carry a deletion in exon 5 of GSDMD. This strain was originally derived and maintained on a C57BL/6NJ background, and as a result these mice were sourced as the appropriate controls. Previous experiments involving the use of WT mice had been performed in C57BL/6JCR mice. It is important to use these relevant controls as C57BL/6NJs were first separated from C57BL/6Js around 75 years ago and there are now significant genetic differences between these substrains³⁰⁴, with some differences in phenotypes arising. An important difference is a decreased susceptibility of C57BL/6NJ mice to

LPS induced lethal shock when compared to C57BL6/J mice³⁰⁵ which could arise through mutations in LPS sensing, including innate sensing pathways of importance to this project.

However, C57BL/6JCR and C57BL/6NJ did not exhibit significant differences with regard to the induction of antigen specific CD8⁺ T cells (Fig.3.9.5), antigen specific IFN γ responses in restimulated splenocytes (Fig.3.5.6) or in antigen specific antibody production (Fig.3.5.7). Surprisingly, GSDMD KO mice vaccinated with 50nm NPs + OVA did not display lower induction of antigen specific CD8⁺ T cells in terms of frequency of splenic CD44^{hi} Class I Tmer⁺/CD8⁺ T cells (Fig.3.5.5A) or in total numbers of these cells (Fig.3.5.5B). A decrease, albeit not statistically significant, was noted in splenocyte antigen-specific IFN γ in GSDMD KO mice compared with WT mice (Fig.3.5.6). There was no reduction in the titres of antigen specific IgG detected in the serum of 50nm NP + OVA vaccinated GSDMD KO mice when compared to WT, in fact titres were higher in the GSDMD deficient mice.

Altogether, these data suggest that a local cellular pyroptotic response at the site of injection following immunisation with antigen and 50nm PS NP could be crucial for the downstream cross-priming of CD8⁺ T cells and optimum antigen-specific IFN γ responses. This potentially provides a key mechanistic insight into their adjuvanticity. However, results from the use of GSDMD KO mice confounds these observations, where no impact on CD8⁺ T cell induction was observed. This warrants further investigation, exploring possible compensatory cell death mechanisms, but also additional targets when using NSA as an inhibitor *in vivo*.

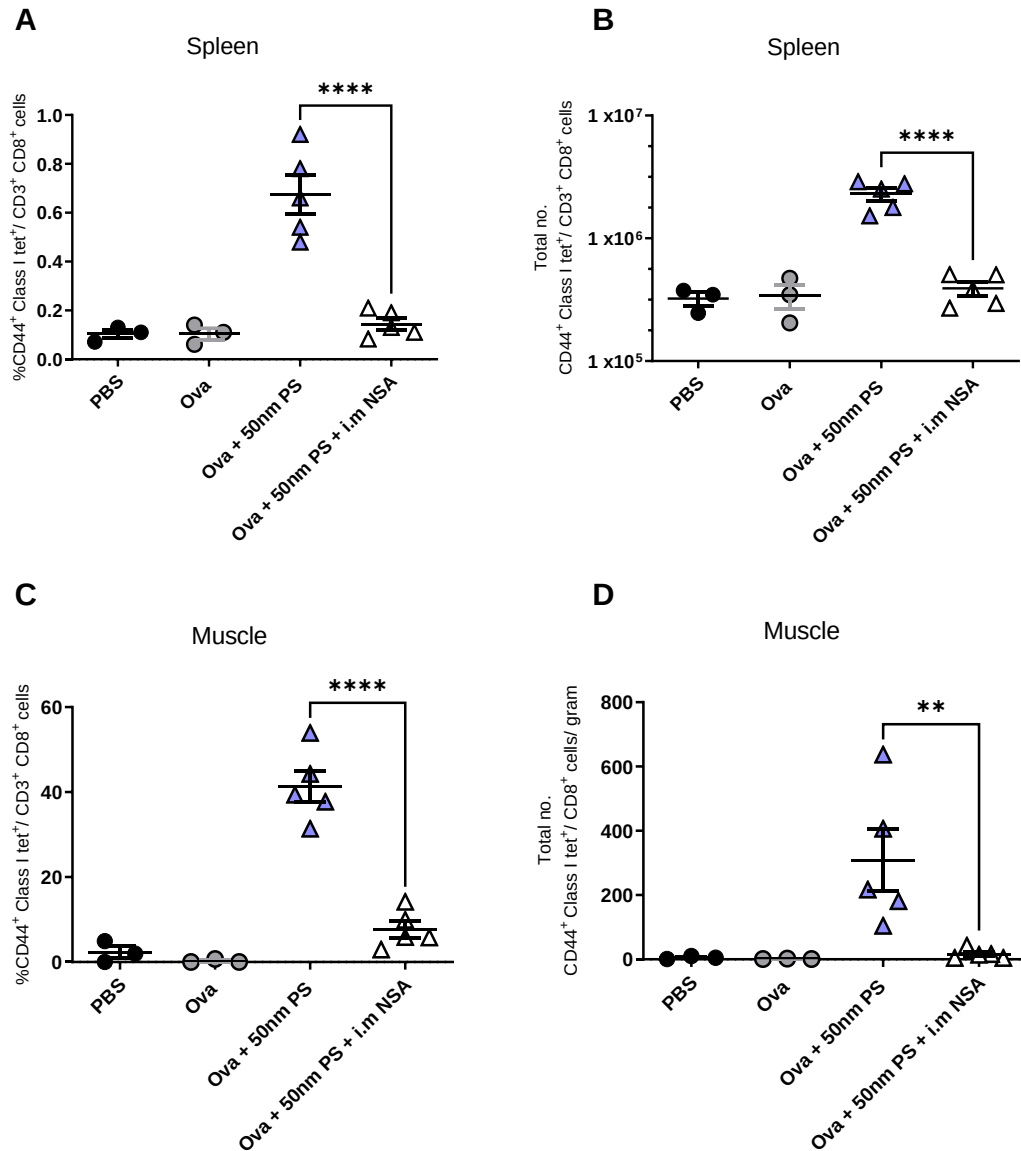


Figure 3.9.1 Inhibition of GSDMD locally at the site of vaccination leads to complete abrogation of the ability of 50nm PS NP to enhance antigen-specific CD8⁺ T cell responses.

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g) + 0.25mg of the GSDMD inhibitor, NSA via the intramuscular route according to Fig.3.7.1. On day 21, antigen-specific CD8⁺ T cell responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen only controls. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen. (C) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells in the muscle; (D) Total number of OVA-specific CD8⁺ T cells per gram of muscle. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences, not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

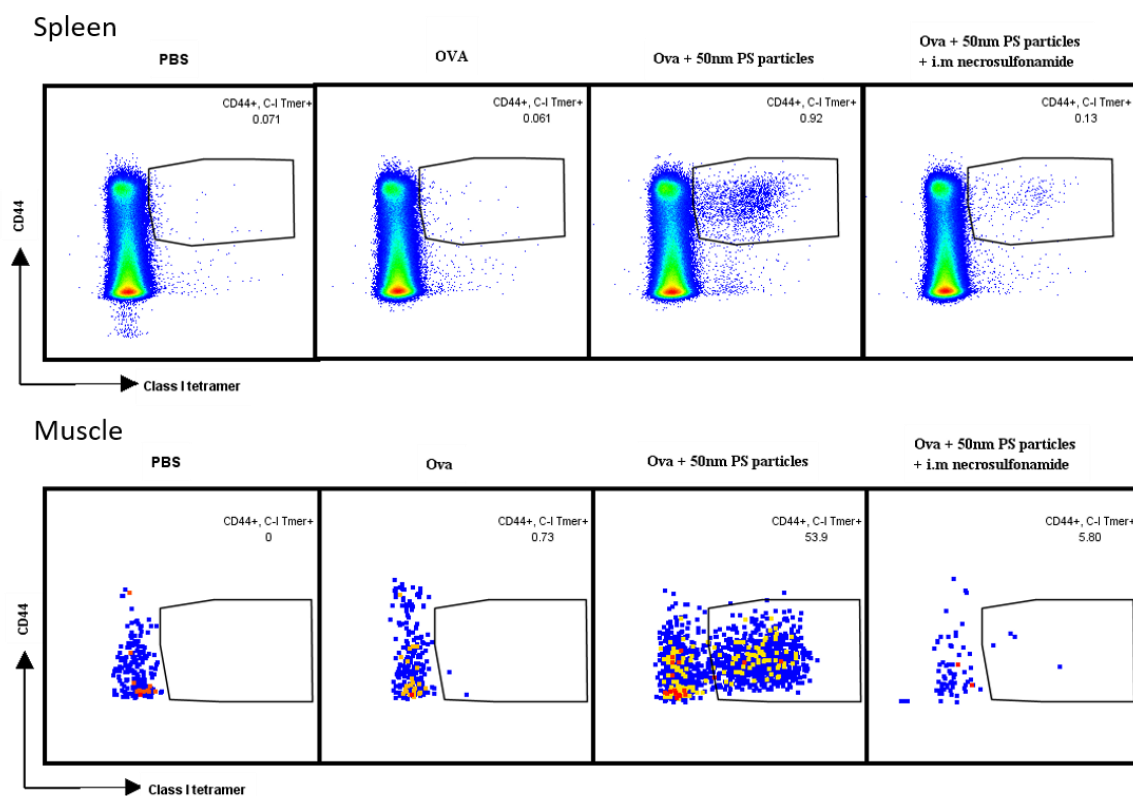


Figure 3.9.2 Representative FACS plots of the spleen and muscle from the 50nm PS NP vaccination study described in Fig.3.9.1

FACS plots are gated on Class I Tetramer⁺/CD44⁺ CD8⁺T cells.

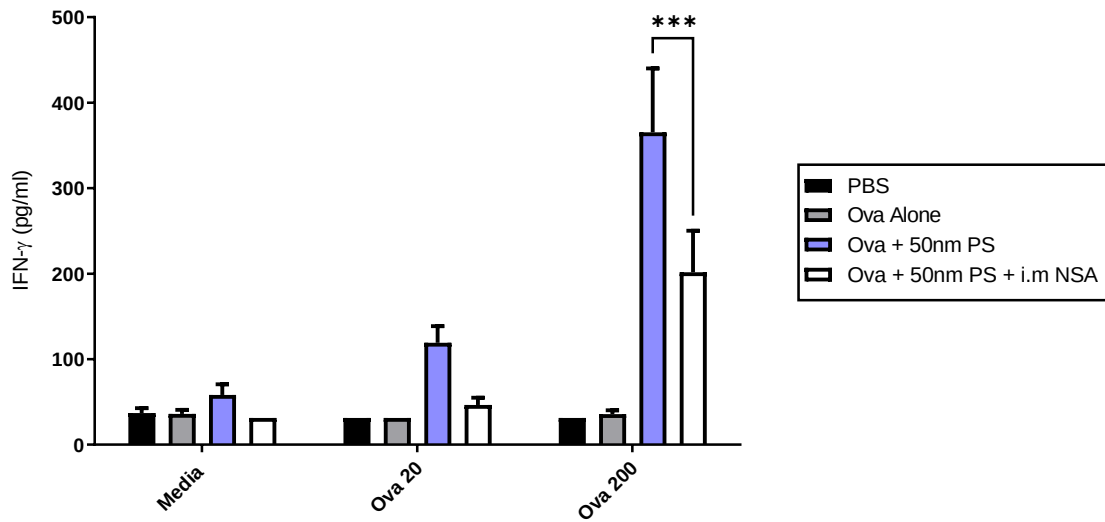


Figure 3.9.3 NSA treated mice display lower splenocyte antigen-specific IFN γ production following vaccination with 50nm PS NP + OVA.

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g) + 0.25mg of GSDMD inhibitor NSAD via the intramuscular route as outlined in Fig.3.7.1. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media or ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns. P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

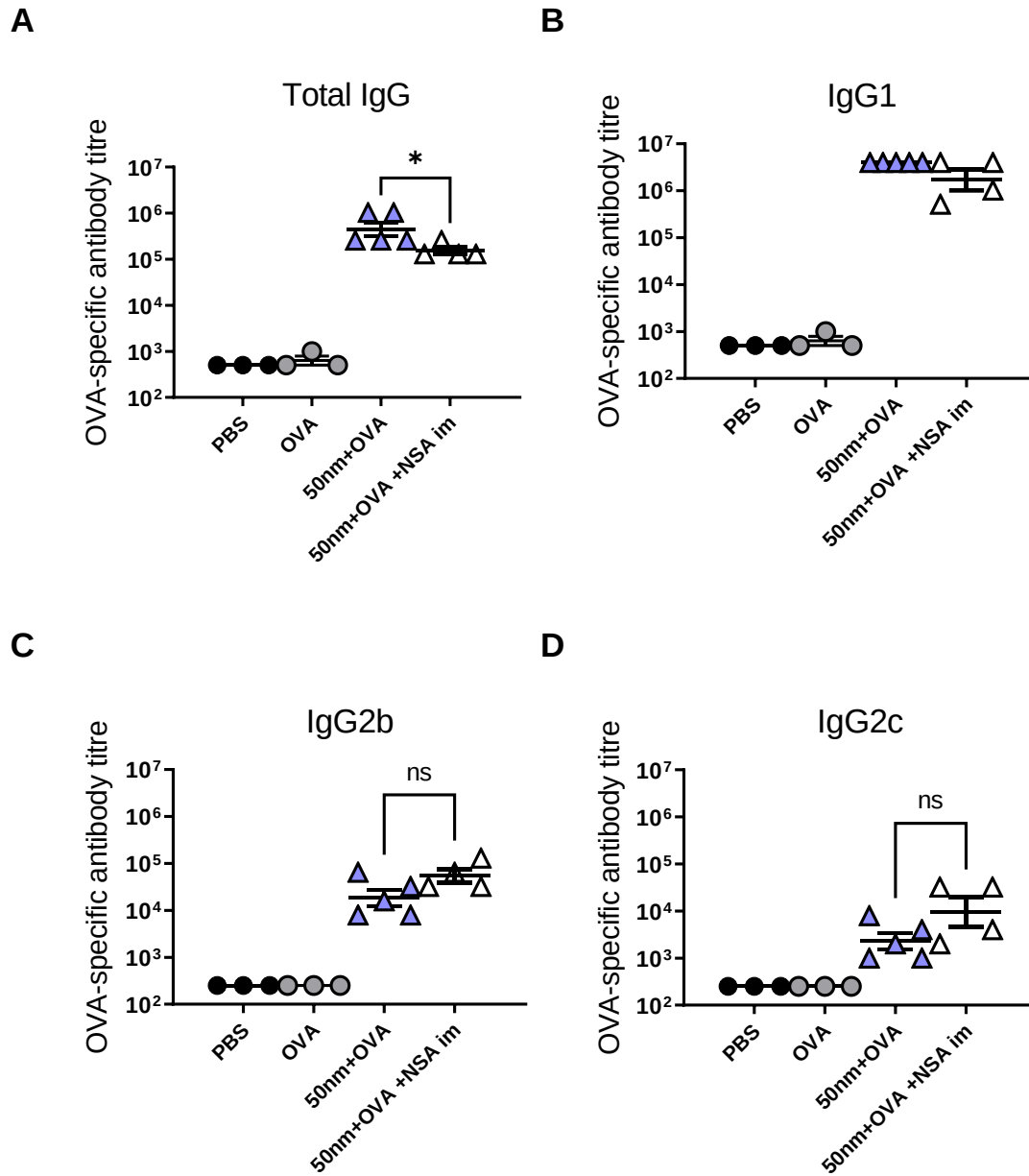


Figure 3.9.4 Inhibition of pyroptosis with NSA slightly reduced antigen-specific serum IgG titres

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g) + 0.25mg of the GSDMD inhibitor, NSAD via the intramuscular route according to Fig.3.7.1. On day 21, mice were sacrificed, and antigen-specific A) Total IgG, B) IgG1, C) IgG2b and D) IgG2c titres were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

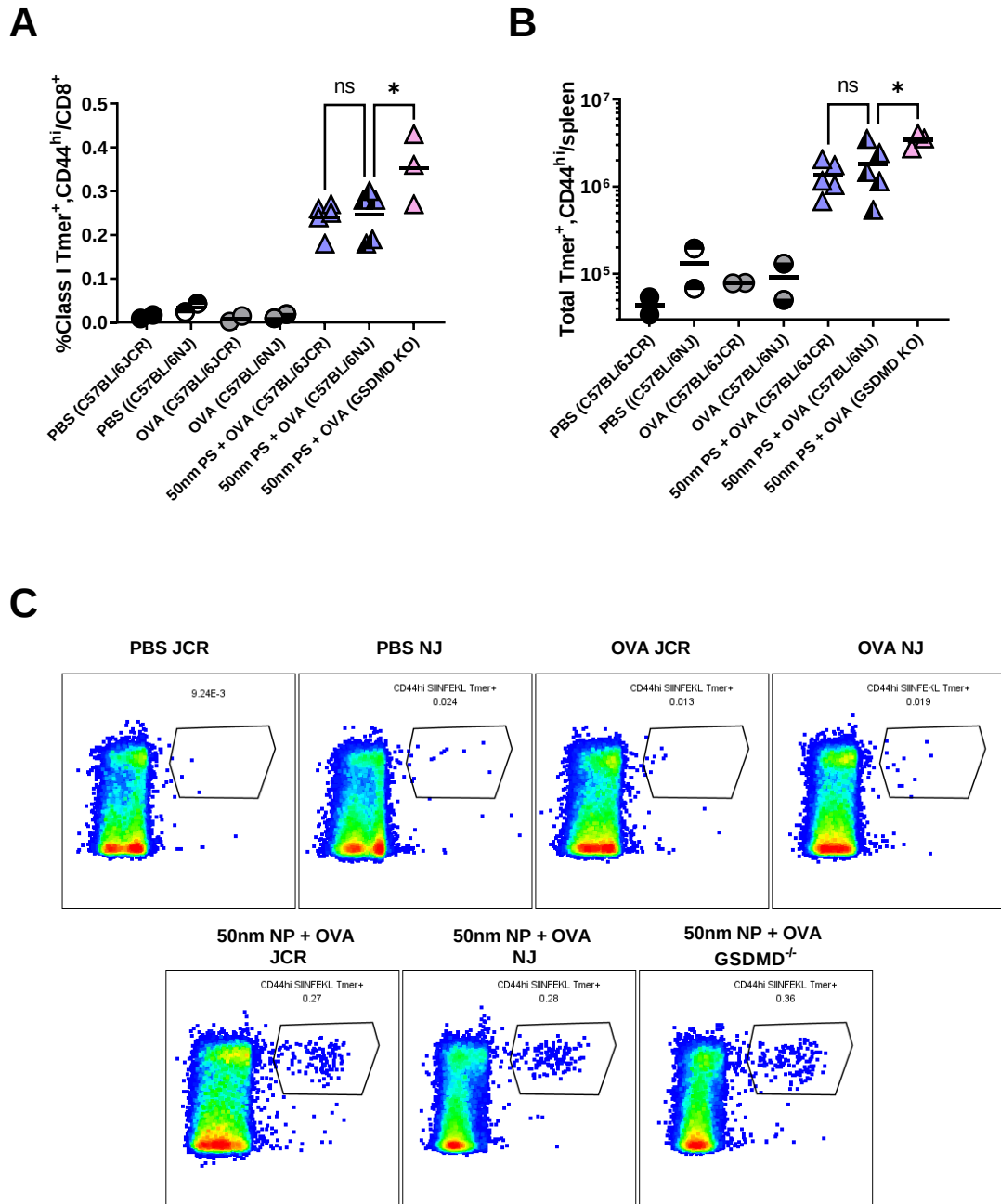


Figure 3.9.5 GSDMD KO mice do not exhibit lower induction of antigen specific CD8⁺ T cells following vaccination with 50nm NPs + OVA when compared to WT mice.

C57BL/6JCR, C57BL/6NJ and GSDMD KO (NJ background) mice were vaccinated with PBS, OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g), via the intramuscular route according to Fig.3.7.1. On day 21, antigen-specific CD8⁺ T cell responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen only controls. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen. (C) Representative FACS plots gated on CD44^{hi} Class I tetramer⁺ CD8⁺ T cells. Statistical differences as per one-way ANOVA and Dunnett's

post-hoc test. Asterisks indicate significant differences, Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

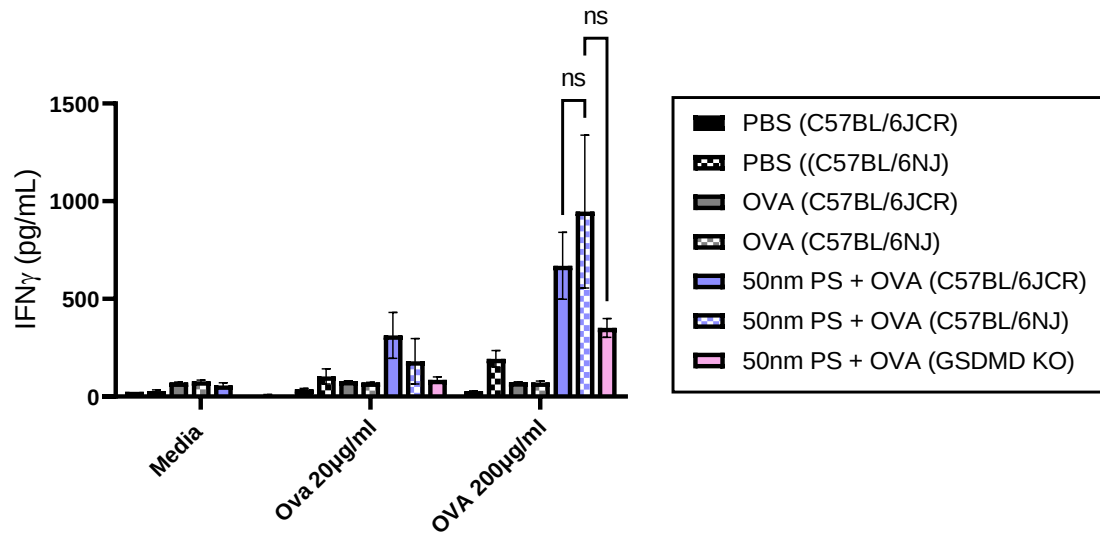


Figure 3.9.6 Splenocyte antigen-specific IFN γ production was lower in GSDMD KO mice vaccinated with 50nm PS NP and OVA when compared to WT mice.

C57BL/6JCR, C57BL/6NJ and GSDMD KO (NJ background) mice were vaccinated with PBS, OVA (10 μ g), or 50nm NP (1mg) + OVA (10 μ g), via the intramuscular route according to Fig.3.7.1. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media or ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

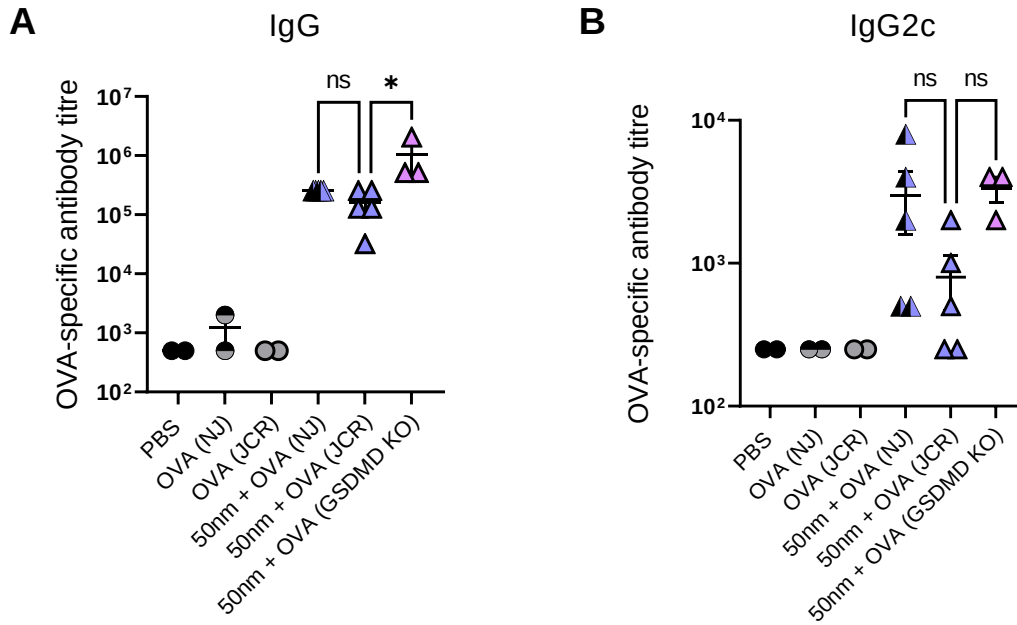


Figure 3.9.7 GSDMD KO mice do not exhibit lower induction of antigen specific antibody titres following intramuscular vaccination with 50nm PS NP and OVA when compared to WT mice.

C57BL/6JCR, C57BL/6NJ and GSDMD KO (NJ background) mice were vaccinated with PBS, OVA (10µg), or 50nm NP (1mg) + OVA (10µg), via the intramuscular route according to Fig.3.7.1. On day 21, mice were sacrificed, and antigen-specific total A) IgG titres and B) IgG2c titres were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns, P value > 0.05; *P < 0.05; **P < 0.01; ***P 0.001 and ****P < 0.0001.

3.10 50nm PS NPs can outperform alternative adjuvants and the clinically relevant ChAdOx1-OVA vector vaccine in eliciting antigen specific CD8⁺ T cell responses

In the rational design of novel vaccine adjuvants, it is crucial to benchmark adjuvanticity against other vaccine adjuvant candidates and alternative vaccine platforms. To address this, the adjuvanticity of 50nm PS NPs was compared to the alternative adjuvants: CpG 1826, 2'3'cGAMP and Alum. CpG 1826 is a 20-mer Class B CpG oligonucleotide (ODNs), designed to specifically bind murine TLR9. CpG ODN adjuvants have been extensively trialled through both pre-clinical and clinical studies where they have been demonstrated to promote Th1 and CD8⁺ T cell responses in mice³⁰⁶ and humans³⁰⁷. A class B CpG ODN – CpG 1018, is included in the FDA approved hepatitis B vaccine – Heplisav-B. 2'3'cGAMP, a STING activating cyclic dinucleotide, has been characterised as an effective adjuvant in promoting both human and mouse CD8⁺ T cells³⁰⁸ and IFN γ responses. Furthermore, OVA adjuvanted with 50nm PS NPs was compared to a clinically relevant ChAdOx1-OVA vectored vaccine. The ChAdOx1 vaccine platform has been shown to promote IFN γ and CD8⁺ T cell responses in mice and human recipients^{309,310}, and is used in the ChAdOx1 nCoV-19 vaccine developed and produced in collaboration between the University of Oxford and AstraZeneca.

Mice vaccinated with 50nm PS NPs + OVA displayed significantly higher induction of splenic antigen-specific CD8⁺ T cells on day 21 post initial priming immunisation, when compared to mice immunised with CpG, cGAMP or alum as an adjuvant. This is displayed as % of CD44^{hi} Class I Tetramer⁺ /CD8⁺ CD3⁺ T cells (Fig.3.10.1A) and in terms of the total number of these cells (Fig.3.10.1B). Similarly, splenocyte supernatants from mice vaccinated with OVA and 50nm PS NPs exhibited significantly higher concentrations of antigen specific IFN γ than those from mice vaccinated with the other formulations (Fig.3.10.2). This contrasts with the induction of antigen specific IgG which was comparable between all adjuvants examined (Fig.3.10.3A). However, mice immunised with CpG had significantly higher serum antigen specific IgG2C titres when compared to those vaccinated with 50nm NP. Altogether this demonstrates that 50nm NPs display a higher degree of adjuvanticity in terms of CD8⁺ T cell induction than the panel of adjuvants selected.

Furthermore, vaccination with 50nm NPs and OVA promoted a significantly higher magnitude of antigen specific CD8⁺ T cells than a ChAdOx1-OVA vectored vaccine. This is displayed as % of CD44^{hi} Class I Tetramer⁺ /CD8⁺ CD3⁺ T cells (Fig.3.10.4A) and in terms of the total number of

these cells (Fig.3.10.4B). However, mice vaccinated with ChAdOx1-OVA exhibited a higher splenocyte antigen specific IFN γ response than those vaccinated with 50nm PS particles and OVA (Fig.3.10.5). No significant differences were observed between the titres of antigen specific IgG or IgG2c induced in the sera following vaccination with ChAdOx1-OVA or 50nm PS NP and OVA (Fig.3.10.6).

These data effectively characterise the superior adjuvanticity of 50nm NPs in terms of induction of antigen specific CD8 $^{+}$ T cells when compared to CpG, cGAMP and Alum, providing rationale for their inclusion over these adjuvants in vaccines aiming to elicit such responses. Additionally, 50nm NPs were shown to outperform a ChAdOx1-OVA vector vaccine in terms of the induction of antigen specific CD8 $^{+}$ T cell responses.

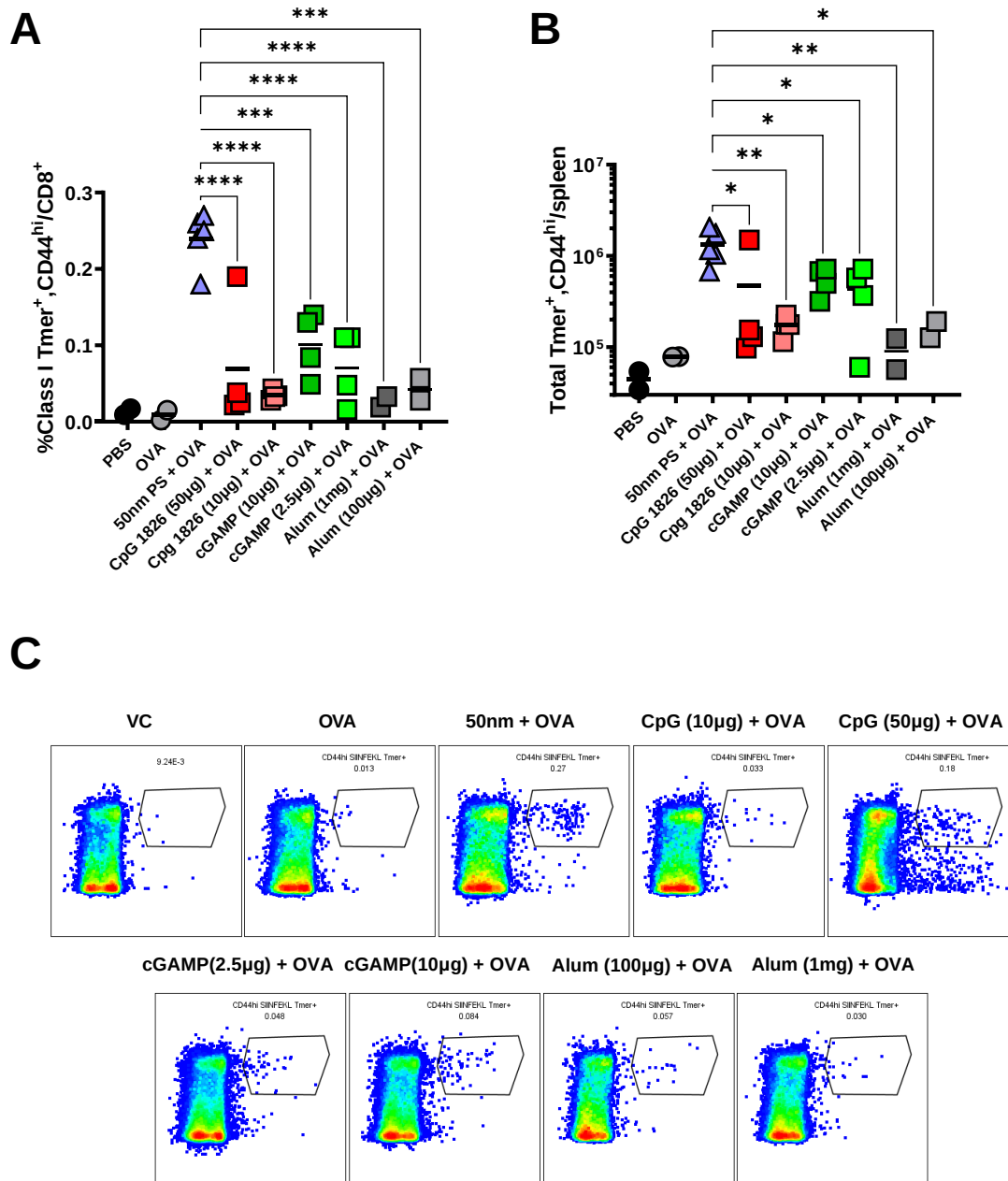


Figure 3.10.1 50nm PS NPs outperform established adjuvants in eliciting CD8⁺ T cells following vaccination

C57BL/6 mice were vaccinated with PBS, OVA (10µg), 50nm NP (1mg) + OVA (10µg), CpG 1826 (50µg) + OVA (10µg), CpG 1826 (10µg) + OVA (10µg), cGAMP (10µg) + OVA (10µg), cGAMP (2.5µg) + OVA (10µg), Alum (1mg) + OVA (10µg), or Alum (100µg) + OVA (10µg) via the intramuscular route on days 0 and 14. On day 21, antigen-specific CD8⁺ T cell responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen only controls. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen. (C) Representative FACS plots gated on CD44^{hi} Class I Tetramer⁺ CD8⁺ T cells. Statistical differences as per one-

way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences, Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

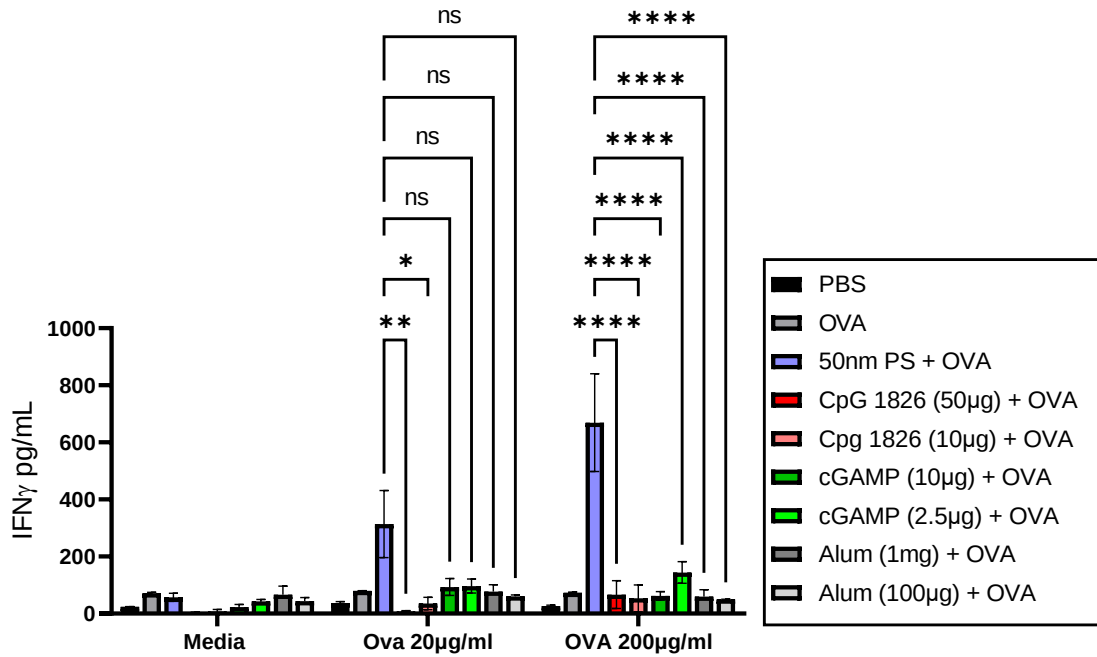


Figure 3.10.2 50nm NPs outperform alternative adjuvants in eliciting antigen specific IFN γ responses in restimulated splenocytes following vaccination

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), CpG 1826 (50 μ g) + OVA (10 μ g), CpG 1826 (10 μ g) + OVA (10 μ g), cGAMP (10 μ g) + OVA (10 μ g), cGAMP (2.5 μ g) + OVA (10 μ g), Alum (1mg) + OVA (10 μ g), or Alum (100 μ g) + OVA (10 μ g) via intramuscular route on day 0 and 14. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media, ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001. ** P<0.01, * P<0.05.

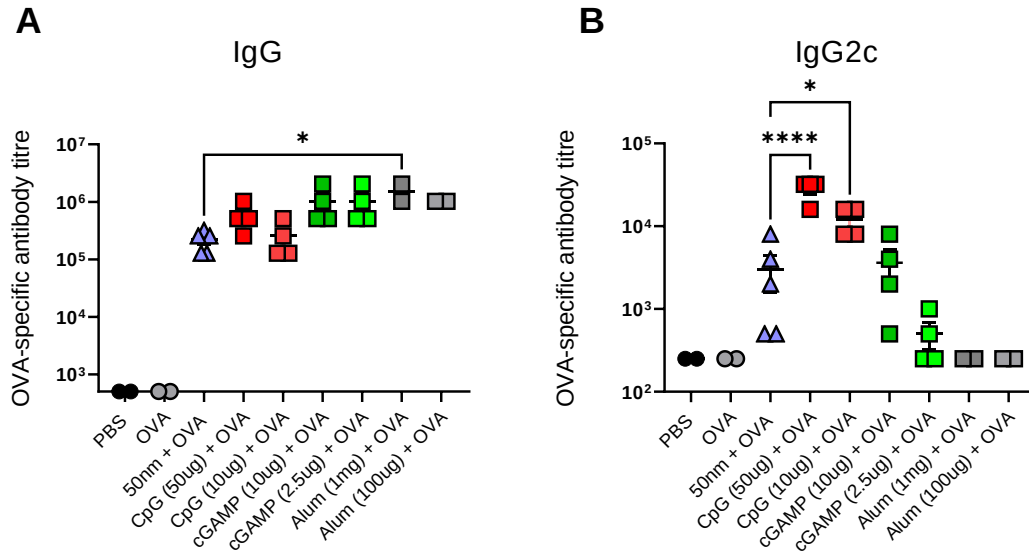


Figure 3.10.3 Vaccination with OVA and 50nm PS NPs elicits comparable titres of serum antigen specific antibody responses to established vaccine adjuvants

C57BL/6 mice were vaccinated with PBS, OVA (10µg), 50nm NP (1mg) + OVA (10µg), CpG 1826 (50µg) + OVA (10µg), CpG 1826 (10µg) + OVA (10µg), cGAMP (10µg) + OVA (10µg), cGAMP (2.5µg) + OVA (10µg), Alum (1mg) + OVA (10µg), or Alum (100µg) + OVA (10µg) via the intramuscular route on days 0 and 14. On day 21, mice were sacrificed, and antigen-specific total A) IgG titres and B) IgG2c were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

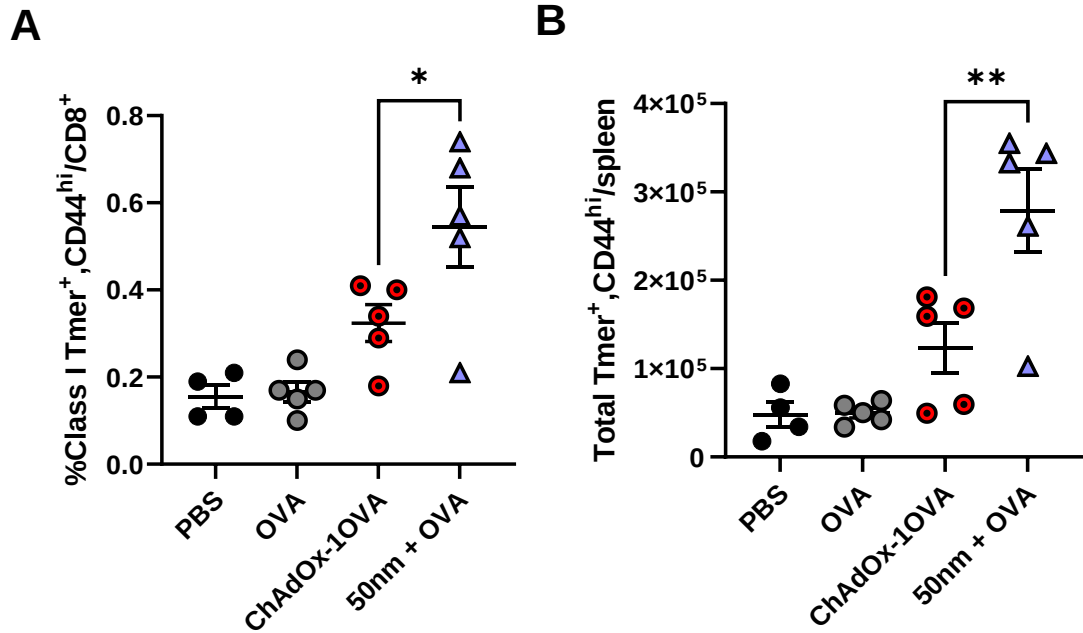


Figure 3.10.4 A 50nm NP adjuvanted vaccine outperformed a ChAdOx1-OVA vector vaccine in eliciting CD8⁺ T cells following vaccination

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), or 10⁸ i.u. of ChAdOx1-OVA via the intramuscular route on days 0 and 14. On day 21, antigen-specific CD8⁺ T cell responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen only controls. (A) Percentage of OVA-specific CD8⁺ over total CD8⁺ T cells; (B) Total number of OVA-specific CD8⁺ T cells in the spleen. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences, not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

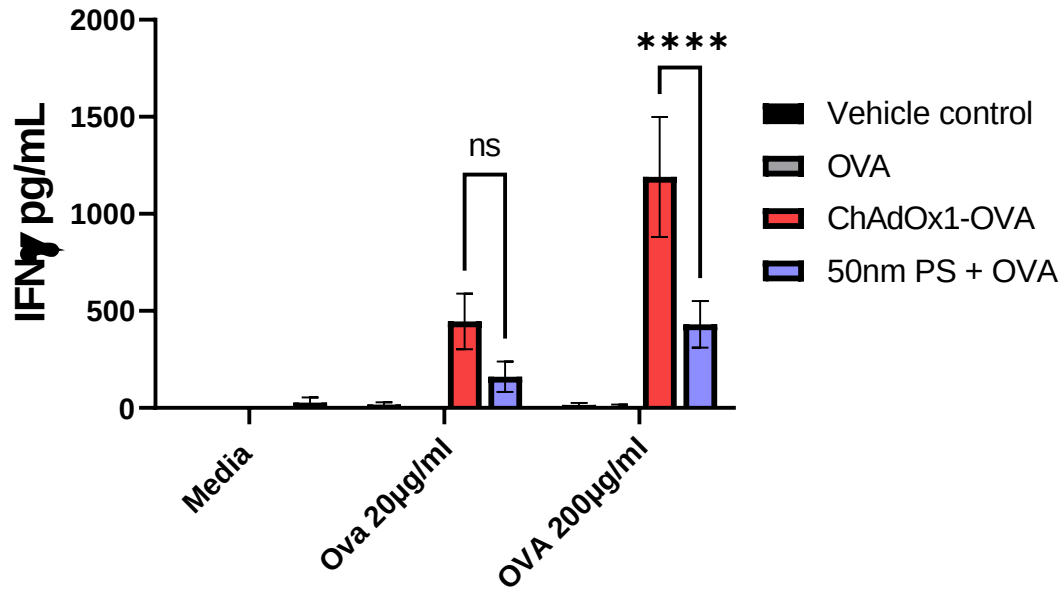


Figure 3.10.5 A ChAdOx1-OVA vaccine significantly outperforms a 50nm PS NP adjuvanted vaccine in eliciting antigen specific IFN γ responses following vaccination

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), or 10⁸ i.u. of ChAdOx1-OVA via the intramuscular route on days 0 and 14. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with cRPMI media or ova (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine levels were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

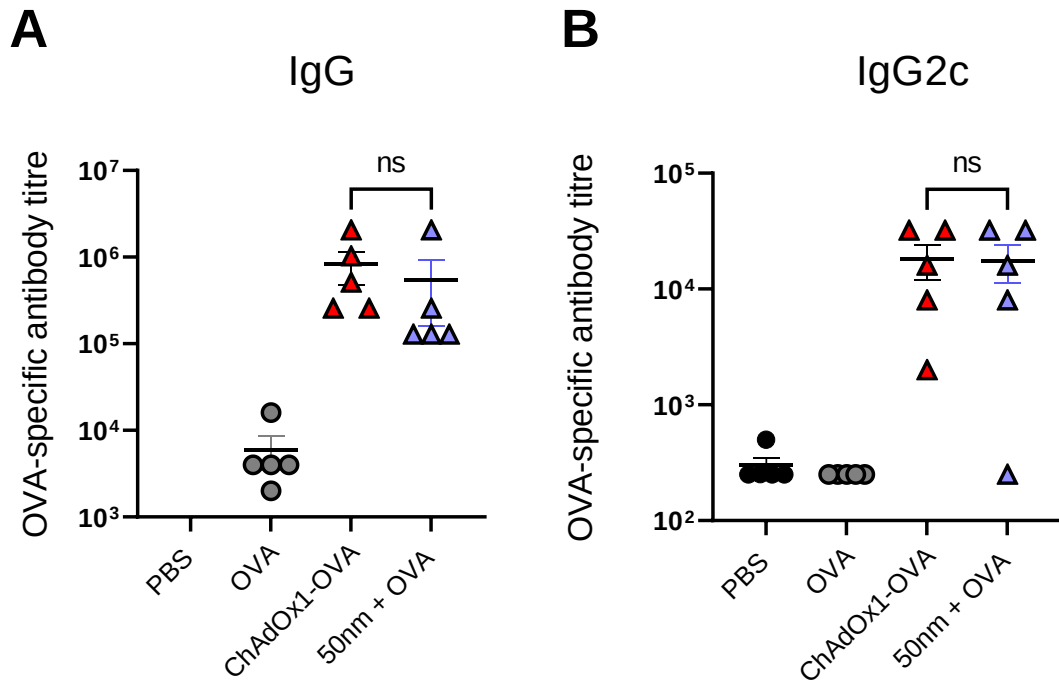


Figure 3.10.6 A 50nm NPs adjuvanted vaccine and the ChAdOx1-OVA vector vaccine elicit comparable titres of antigen specific antibody responses in the sera of mice following vaccination

C57BL/6 mice were vaccinated with PBS, OVA (10 μ g), 50nm NP (1mg) + OVA (10 μ g), or 10⁸ i.u. of ChAdOx1-OVA via the intramuscular route on day 0 and 14. On day 21, mice were sacrificed, and antigen-specific total A) IgG titres and B) IgG2c were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

3.11 Discussion

Firstly, this project has successfully characterised the unique adjuvanticity of 50nm NP, made from PS which are used here mostly for experimentation, and importantly in PLGA:DOPs, a polymer with clinical translatability. Stabilisation of 50nm NP with DOPS, a phospholipid similar in properties and structure to phosphatidylserine (PhS), confers a negative charge on the PLGA particles. PLGA:DOPs outperformed neutrally charged PLGA, this may be due to an increased susceptibility of neutrally charged particles to form clumps during and after injection, or perhaps a lower capacity to interact with the cellular membrane of target cells. In addition, PLGA stabilised with the lipid DDA, a cationic lipid, did not display adjuvant properties. This indicates that the choice of charge on the 50nm PLGA NP is crucial to their adjuvanticity. The charge of these NPs may be dictating their behaviour *in vivo* not only with the cell types present at the injection site but also in modulating the binding of free antigen, and this may explain the differences in responses observed. Unfortunately, it was not possible to compare all three 50nm PLGA NP candidates in one vaccination study due to logistical issues surrounding their production and freeze-drying. While the adjuvanticity of 50nm PLGA:DOPs NP was lower than 50nm PS NP, this may be due to the increased biodegradability of PLGA. As a result, in future experiments the dose of 50nm PLGA:DOPS will be increased to compensate and PLGA/PLA polymers with a slower degradation rate will be used.

In addition, this work has demonstrated that vaccination with antigen and 50nm NP can successfully induce systemic cellular immunity, alongside the accumulation of antigen-specific CD8⁺ T cells at the intramuscular injection site. Preliminary experiments indicate that the majority of these muscle-resident cells are non-tissue resident and are likely as a result to be just predisposed for homing to the muscle, but this pilot study warrants further investigation/confirmation. This should include analysis of different organs e.g., the lungs, for the presence of these antigen specific CD8⁺ T cells, with comparisons to proportions found in the muscle, to confirm muscle specific homing of these cells. In addition, the relative contribution of adjuvant vs antigen to this preferential homing will be examined, with experiments utilising booster vaccinations with adjuvant alone. Furthermore, experiments involving intramuscular vaccination of one leg, and subsequent analysis of the uninjected leg will indicate whether this is an injection site dependant effect vs general preferential homing of these cells to the muscle. Questions remain regarding the phenotype (e.g., effector/memory characteristics) and functionality of these T cells and in-depth sequencing of these cells should be carried out going forward.

Additionally, this project was also able to successfully benchmark the adjuvanticity of our 50nm NP against alternative adjuvants and the clinically relevant ChAdOx1 vector encoding OVA. 50nm NP were shown to elicit higher antigen specific CD8⁺ and IFN γ responses than CpG 1826, 2'3'cGAMP and alum. This crucially identifies 50nm NPs as a more potent adjuvant in driving cellular responses than these alternative adjuvants. While this superiority over alum is not surprising given alums documented lack of adjuvanticity in terms of inducing cellular immune responses²¹⁷, both CpG and 2'3'cGAMP have been characterised as inducers of cellular immune responses^{306–308}. The ChAdOx1 vector, developed at the University of Oxford, similarly has been shown to elicit cellular immune responses^{310,311}, which has been incorporated in EMA approved and globally distributed Vaxzervria vaccine with an inserted recombinant spike encoding construct targeting SARS-CoV-2³¹². In addition, the ChAdOx1 vector has been trialled in vaccines for Zika virus³¹³, MERS³¹⁴, *Mycobacterium tuberculosis*³¹⁵, Nipah virus³¹⁶ and even prostate cancer³¹⁷. As a result, this is a gold standard vaccine platform to be used as a control. In comparison to ChAdOx1, 50nm NP performed favourably, eliciting similar titres of antigen specific antibody titres, significantly higher magnitude of antigen specific CD8⁺ T cells, albeit with significantly less induction of antigen specific IFN γ responses. This is a very promising result as although the ChAdOx1 vector is an optimal formulation used clinically, our 50nm NPs are still early in development, with many future optimisations and improvements possible.

However, to infer rational improvements and developments to the 50nm NPs, it is crucial to decipher the mechanism of action of these adjuvants. Indeed, there is a distinct lack of precise mechanistic data illustrated in studies utilising nanoparticle adjuvants, which is not conducive to rational adjuvant design. Therefore, the identification of functioning pyroptotic signalling as a necessity for 50nm PS NP adjuvanticity represents a major breakthrough that may potentially be applied to multiple adjuvants in similar size ranges. The mechanistic insights into 50nm NP adjuvanticity from *in vivo* experimentation in this chapter are summarised in Table 3.11.1 and 3.11.2. Pyroptosis has been demonstrated to be an immunogenic form of cell death that can be utilised to initiate potent adaptive immune responses against tumours^{245,246}, this capacity to propagate cellular immunity provides foundational basis for our observations of immune responses *in vivo* following vaccination with antigen and 50nm PS NP. Pyroptosis culminates in the recruitment of immune cells to the site of insult via release of IL-1 family cytokines and other DAMPs alongside chemokines such as CCL2. Previous work conducted in the Lavelle lab has shown that there is an influx of innate immune cells to the injection site following injection of 50nm PS particles, composed predominantly of neutrophils & monocytes, followed by DCs and

macrophages²⁹¹. *In vitro* experiments uncovered the capacity for 50nm PS NP to drive pyroptotic cell death in BMDCs and C2C12 cells through use of inhibitors blocking caspase activation and others converging on inhibition of GSDMD. These cell types are representative of cells found at the intramuscular injection sites following immunisation, and DCs and skeletal muscle cells can function as professional and non-professional APCs following vaccination respectively^{318,319}. In addition, a particle size dependent induction of cellular ROS and GSDMD cleavage is demonstrated. These observations are potentially intricately tied together as ROS have been implicated in the generation of oxidised endogenous ligands for caspase 11²⁴² and also in the direct oxidation of GSDMD²³⁷, sensitising the protein for subsequent activation/oligomerisation. Indeed it has been suggested that GSDMD acts a cellular redox sensor²³⁷.

This *in vitro* discovery of pyroptosis elicitation has been successfully linked to an *in vivo* vaccination setting as the *in vivo* results clearly demonstrate the dependence of 50nm PS NP adjuvanticity on local components of the pyroptotic signalling cascade, with co-injection of NSA and vaccination in caspase 11 KO mice completely diminishing the adjuvanticity of 50nm PS NPs, measured via the induction of local and systemic antigen-specific CD8⁺ T cells and antigen-specific IFN γ production, which has been attributed to CD4⁺ T cells²⁹⁰. Additionally, pre-treatment of the intramuscular injection site with the ROS scavenger TEMPOL impaired antigen specific CD8⁺ T cell responses. Altogether these results implicate the non-canonical pyroptotic pathway in 50nm PS NP mediated induction of antigen-specific cellular immunity. Disrupting the pyroptotic response may result in impaired immune cell recruitment to the site of injection arising from the release of IL-1 family cytokines and other DAMPs, thus impairing the efficacy of adjuvant/antigen transport to dLNs which may be essential for adjuvanticity. To investigate this, immune cell infiltration to the injection site following co-injection of NSA and 50nm NPs should be examined alongside tracking of fluorescently labelled 50nm PS NPs *in vivo*. However, experiments inferring a role for GSDMD using NSA have been somewhat confounded by 50nm NP vaccination results in GSDMD KO mice, as the antigen-specific CD8⁺ T cell response remained intact, with only a slight impact on antigen specific IFN γ responses observed. These results may potentially be explained through the elicitation of alternative or compensatory cell death pathways in these KO mice which will be discussed below.

There was a significant reduction in antigen-specific IFN γ responses in mice vaccinated with 50nm NP and OVA in IL-1R1 and NLRP3 KO mice, as there was in mice that received a co-injection of the caspase 1 inhibitor, Ac-YVAD-cmk. To better understand these results, shared pathways between these molecules should be considered. Particularly relevant here is the canonical

pyroptotic signalling pathway, characterised by inflammasome activation and assembly, caspase 1 activation and subsequent processing of GSDMD and IL-1 family cytokines^{239,320}. The canonical and non-canonical pyroptotic pathways are intricately linked to each other, and activation of the non-canonical pathway often leads to subsequent activation of its canonical counterpart via perturbations of cellular homeostasis and NLRP3 activation²³⁸. These data may suggest a role for NLRP3 mediated activation of caspase 1, with release of active IL-1 via GSDMD pores for the optimum 50nm PS NP mediated induction of IFN γ ⁺ CD4⁺ T cells. While the data point to a partial dependence on IL-1R1, it has not been shown which IL-1 is responsible. IL-1 α does not have the same dependence as IL-1 β on caspase 1 mediated cleavage and has some bioactivity in its immature form³²¹. This is dependent on cell type however, with certain cell types expressing cytosolic IL-1R2 that sequesters and inactivates IL-1 α , requiring caspase 1 mediated cleavage to dissociate the complex and restore cytokine activity³²². Vascular smooth muscle cells do not express IL-1R2³²² and it remains to be determined whether skeletal muscle cells, that would be at the site of injection, express this cytosolic inhibitory receptor. It is a distinct possibility that IL-1 α is being released from GSDMD pores, leading to subsequent immune cell recruitment and activation. IL-1 α has been shown to promote the recruitment of neutrophils to sites of insult³²³ and to potentiate the ability of moDCs to activate IFN γ ⁺ T cells³²⁴. IL-1 β has been shown to support the expansion, and survival of Th1 cells²⁶². Without the non-canonical activation of canonical signalling pathways, such processing and release of IL-1 cytokines would not be seen, potentially contributing to the lack of 50nm NP adjuvanticity observed in caspase 11 KO mice and mice injected with NSA. Additionally, NSA has been shown by some groups to inhibit the formation of the NLRP3 inflammasome and caspase 1³²⁵.

There are certain caveats to the results presented here however, predominantly the intact antigen specific CD8⁺ T cell response observed in GSDMD KO mice vaccinated with 50nm PS NP and OVA, the promiscuity of NSA as a cysteine targeting inhibitor, the possibility of cellular hyperactivation and the nature of the ligand for caspase 11 activation in this setting.

The elicitation of alternative or compensatory cell death pathways is a phenomenon that has been observed in GSDMD KO cells, whereby secondary pyroptosis is induced following GSDME activation via caspase 3 mediated cleavage²⁴⁷. Indeed, it has been shown that optimum IL-1 release and cell lysis following pyroptotic stimuli is not only dependant on GSDMD activation, but also GSDME in both murine and human myeloid cell lines²⁴⁸. It is possible that in our vaccination system that GSDMD KO mice could have impaired cell lysis in cell types locally at the injection site, but could still possess intact GSDME pores at a sub lytic concentration which may act as conduits for IL-1 family cytokine release²⁴⁸. This could be akin to hyperactivated cells,

whereby GSDMD pores allow for sustained release of IL-1 family cytokines in viable cells, which have been documented to possess adjuvant properties²⁴². The kinetics of the elicitation of alternative cell death pathways in KO and chemically inhibited cells are likely different as NSA mediated GSDMD inhibition does not inhibit GSDMD cleavage and dimerisation but inhibits oligomerisation and subsequent pore formation.

Necrosulfonamide, like many cysteine residue targeting inhibitors, is somewhat non-specific in its binding, raising concerns over specificity of action. While it can be concluded that caspase 11 is playing a role in 50nm NP adjuvanticity, the exact role of GSDMD is not as clear. NSA has been shown to inhibit ASC speck formation, inflammasome assembly, caspase 1 cleavage, and surprisingly LPS induced IL-1 β transcription³²⁵. These are red flags over specificity seen also in similar acting inhibitors BAY 11-7082 and Disulfiram, leading to caution over use *in vivo*. However *in vitro* cytotoxicity has been validated using DMF and NSA, and while both are broadly acting inhibitors, they do overlap in the inhibition of GSDMD via two distinct mechanisms. DMF has additionally been shown to inhibit GSDME cleavage³⁰² but to our knowledge no groups have demonstrated that NSA has this capacity. Owing to its inhibitory properties on inflammasome assembly and caspase cleavage it is possible that NSA could inhibit the assembly of the caspase 11 inflammasome and subsequent cleavage (Fig.3.11.1) – although this effect on caspase 11 has not been investigated here or in the literature. Hypothetically, this could explain observed results in NSA treated vaccinated mice where the comprehensive reduction of nanoparticle adjuvanticity could be due to inhibition of the canonical and non-canonical inflammasome pathways. This would mean a lack of IL-1 family cytokine processing and cleavage culminating in a lack of release regardless of whether GSDME pores are formed. Additional experiments will be required to dissect the role of GSDMD and GSDME in 50nm NP adjuvanticity, ideally double KO mice would be vaccinated but these currently are not available. Instead, a panel of caspase 3 inhibitors have been provided by a collaborator. Experiments are planned to treat GSDMD KO mice with the inhibitors followed by subsequent vaccination with antigen and 50nm NP to probe the potential contribution of GSDME to the intact adjuvanticity observed in GSDMD KO mice

In a broader context, the relative importance of cell death for nanoparticle induced T cell responses is unknown as induction of cellular hyperactivation is also a plausible explanation for the observed results, Zanoni et al²⁴² first described this state in DCs stimulated with oxPAPCs, where activation of caspase 11 can lead to the sustained release of IL-1 β through GSDMD, but critically not pyroptosis. They also demonstrated that this effect had adjuvant properties, enhancing antigen-specific CD4⁺T cells. Hyperactivation has been described in multiple cell types

including macrophages, DCs and neutrophils^{258,259}. Higher cytotoxicity in 50nm NP treated cells is seen when using live dead staining and FACS analysis compared to the cytotoxicity determined when utilising an LDH assay. This potentially could be explained through maintenance of membrane integrity despite GSDMD pore formation as LDH is too large to pass through GSDMD pores²⁵⁹, while live dead stains like PI can enter through them. Experiments are therefore required to determine if oxPAPCs are generated in response to 50nm NP explaining caspase 11 activation, and if there is subsequent hyperactivation of cells in response to 50nm NPs *in vitro* & *in vivo*. However, 50nm PS NP mediated cytotoxicity has been validated here via MTT and LDH assays *in vitro*, which may be instructive on how responses are elicited *in vivo*.

Overall, mechanistically the current data show that components of the non-canonical pyroptotic signalling cascade at the site of injection are essential for 50nm NP induced cellular immune responses. It remains to be determined how important the execution of cell death is in this process, what cytokines/chemokines are fundamental to this response *in vivo*, and the relative contribution of GSDME to 50nm NP adjuvanticity. Future work will aim to further build on these mechanistic insights.

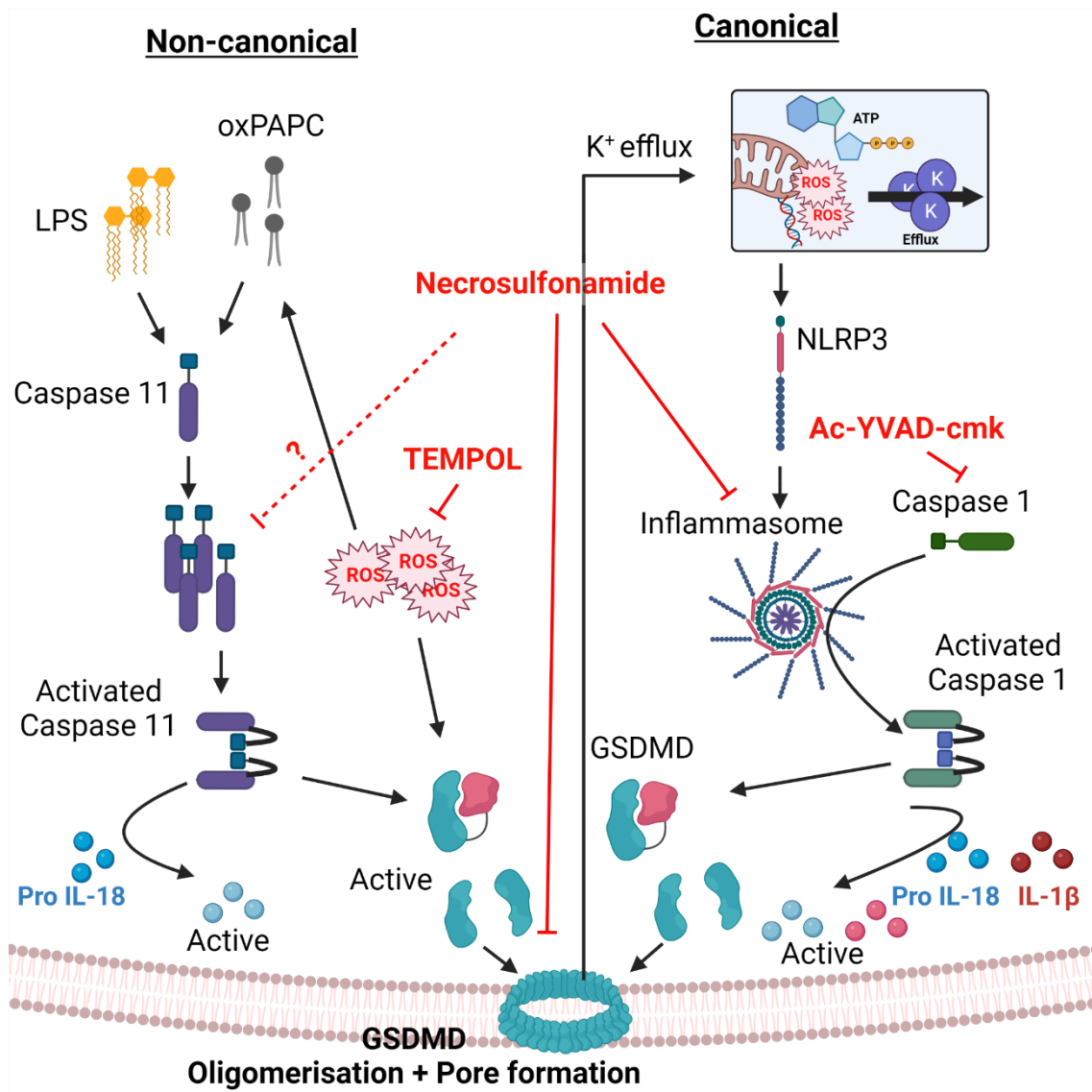


Figure 3.12.1 Non-canonical and canonical inflammasome pathways with components targeted by inhibitors used in 50nm NP *in vivo* studies

Treatment/Genotype	Impact on antigen-specific CD8⁺ T cell induction	Impact on antigen-specific IFNγ responses
IL-1R1 KO	No impact/Possible increase	Significantly reduced
NLRP3 KO	No impact	Significantly reduced
GSDMD KO	No impact	Significantly reduced
IL-18 KO*	Significantly reduced	No impact
Caspase 11	Significantly reduced	Significantly reduced
Y-VAD-cmk treated	No impact	Significantly reduced
Necrosulfonamide treated	Significantly reduced	Significantly reduced
TEMPOL treated	Significantly reduced	No impact

Table 3.12.1 Summary of effects of mouse genotype or treatment on 50nm NP induced cellular immunity.

*Data not shown in this thesis – previously conducted by Dr Natalia Muñoz-Wolf

Treatment	Functional Caspase 11	Functional Caspase 1	IL-1 cleavage + signalling	IL-18 cleavage + signalling	Functional GSDMD
Y-VAD-cmk	√	X	X	√*	√
Necrosulfonamide	?	X	X	?	X
Tempol	√**	√	√	√	√**

Table 3.12.2 Summary of effects of inhibitors/treatments on pathways and molecules relevant to 50nm NP adjuvanticity

√ indicates that treatment has no effect on functionality/process

X indicates that treatment results in impaired functionality/inhibits process

*Caspase 11 can mediate the cleavage of IL-18

**Tempol treatment may result in the scavenging of ROS that can generate oxidised ligands for caspase 11 activation or directly oxidise GSDMD

Chapter Four - Investigating the anti-tumour potential of 50nm NP adjuvants

4.1 Introduction

Anti-tumour immunity relies on the detection and elimination of transformed cells via immune surveillance, mediated by cellular components of the innate and adaptive immune system. While cytotoxic innate cells, including NKs and macrophage subtypes, are crucial in initial clearance and surveillance, the adaptive immune response is required for robust and long-lived anti-tumour immunity.

Cytotoxic CD8⁺ and CD4⁺T cells play crucial roles in tumour immune surveillance and elimination, alongside their role in production of anti-tumorigenic cytokines, including IFN γ . Similarly, certain antibody subtypes can have anti-tumourigenic roles, such as the role of (human) IgG1 in antibody-dependent cellular cytotoxicity³²⁶ (ADCC) via engagement of Fc γ Rs on immune cells such as NK cells, frequently exploited in the development of novel anti-tumour therapeutics³²⁶.

Parallels can be drawn between the ideal components of adaptive immunity involved in tumour surveillance and elimination detailed above, and the adaptive immune responses elicited following vaccination with 50nm NP and antigen detailed throughout Chapter Three. Therefore, 50nm NP may be candidates for inclusion in cancer vaccine approaches as vaccine adjuvants. Cancer vaccines have applicability in prophylactic and therapeutic approaches, and as standalone treatments or in combinatorial approaches, particularly suitable for the treatment of immunologically cold tumours⁶⁶.

However, while the phenotype of 50nm PS NP mediated immune responses has been characterised, the functionality of these immune responses remains to be determined, and this is a crucial step in characterising their suitability as cancer vaccine adjuvants. To investigate the prophylactic and therapeutic anti-tumour functionality of 50nm PS NP induced immune responses both as a monotherapy and in combination with checkpoint blockade, a pre-clinical subcutaneous tumour model was utilised *in vivo*. This involves the use of B16 melanoma cells, derived from C57BL/6J mice³²⁷, which have been genetically engineered to constitutively express OVA protein (B16-OVA). Furthermore, the relative contribution of components of these 50nm NP adjuvant-mediated immune responses to the overall anti-tumour immunity was addressed with a focus on characterising the phenotype of antigen specific CD8⁺ T cells elicited following vaccination.

4.2 Hypothesis

The hypothesis underlying the research in this chapter was that immune responses generated following vaccination with 50nm PS NP possess anti-tumour functionality.

4.3 Aims

- To characterise the efficacy of 50nm PS NP as cancer vaccine adjuvants using pre-clinical tumour models, and to benchmark this anti-tumour functionality against other vaccine adjuvants and platforms.
- To mechanistically dissect the importance of components of 50nm PS NP elicited adaptive immune responses towards overall anti-tumour immunity.
- To investigate therapeutic synergy with checkpoint blockade.
- To characterise the phenotype of 50nm NP induced antigen specific CD8⁺ T cells, with a focus on markers relative to anti-tumour effector function.

4.4 Vaccination using 50nm PS particles and protein antigen induces robust anti-tumour immunity in pre-clinical murine tumour models

To investigate the potential anti-tumour capacity of 50nm PS NP induced immunity, a subcutaneous B16 OVA expressing model was utilised. This involves the *in vitro* culture and harvesting of cells, resuspension in HBSS, with subsequent injection into the flank of the animal as described in Overwijk et al., 2001³²⁷. Prior to tumour challenge, mice were given an intramuscular prime on day 0, followed by boosting on day 14, with tumour challenge on day 28.

Mice immunised with 50nm PS NP and OVA protein exhibited complete protection from s.c B16-OVA challenge (Fig.4.4.1A+B) and as a result had complete survival for the course of study (Fig.4.4.1C). In comparison, vaccination with OVA protein alone led to roughly 30% protection (Fig.4.4.1A+B). These results demonstrate that 50nm NP can confer robust anti-tumour immunity when combined with a protein antigen.

Additionally, the durability of this anti-tumour immunity was assessed. The experimental design for vaccination remained the same as did the challenge with 3.5×10^5 B16-OVA cells, however, these mice were left for observation for 120 days. 92% of 50nm PS NP + OVA immunised mice remained tumour free at day 120, compared to 25% of mice given OVA alone (Fig.4.4.3). This suggests that the anti-tumour immunity generated following immunisation with 50nm PS NP is durable and long-lasting.

While these results highlight the potential of 50nm NP adjuvants in cancer vaccines incorporating protein antigens, it is of note that most adjuvanted cancer vaccines have been trialled utilising peptide antigens, with some success observed when combined with checkpoint blockade therapeutics⁸⁴. As such and to investigate the potential of 50nm NP induced anti-tumour immunity in another phenotypically distinct tumour model, the MC38 adenocarcinoma tumour cell line was utilised with peptides uniquely expressed by MC38 utilised as vaccine antigens. These peptides were designed following on from work conducted by Hos et al³²⁸ where neoantigen candidates were identified in MC38 cells through extensive screening. These neoantigens contained neoepitopes that can be presented via the MHC Class I pathway and recognised by the TCRs of CD8⁺ T cells, in particular, the antigens Rpl18 and Adpgk had been documented to elicit IFN γ ⁺ TNF α ⁺ CD8⁺ T cell responses with some anti-tumour functionality³²⁸. In addition, another peptide that had been designed through collaboration with researchers in

Queens University Belfast was utilised 'Rpl18 scr'. These peptides described were synthesised, ranging in size from 18-21 amino acids in length and are listed below with the mutated amino acid presented in bold:

Peptide name	Amino acid sequence
Aatf1	SFMAPIDHT T MSDDARTE
Adpgk	HLELASMTN M ELMSSIVHQ
Cpne1	GSNGDPSSP Y SLHYLSPTGVNE
Rpl18	KAGGKILTFD R LALESPK
Rpl18 scr	KGFGRLRSAPAITKLKEDL

Peptides were mixed in a 'peptide cocktail' in two concentrations: 10µg or 50µg, prior to vaccination and were formulated alone or in combination with 50nm NP. Vaccination with both doses of peptide cocktail alone resulted in delayed tumour growth (Fig.4.4.3A+B) and enhanced survival (Fig.4.4.4) compared to mice vaccinated with vehicle control, with the lower of dose of 10µg outperforming the higher concentration of the peptide cocktail. When formulated with 50nm NP, adjuvanticity was only observed with the 10µg peptide cocktail dose, slightly improving tumour growth inhibition (Fig.4.4.3) and median survival time (Fig.4.4.4) compared with peptide alone. However, these effects are modest and require further studies to confirm. Altogether, these results indicate that vaccination strategies utilising 50nm NP and peptide antigens may not perform as efficaciously as 50nm NP adjuvanted whole protein vaccines. This may warrant optimisation of peptide vaccination strategies using carrier proteins, longer peptides, or different formulation strategies to encourage NP and peptide interactions.

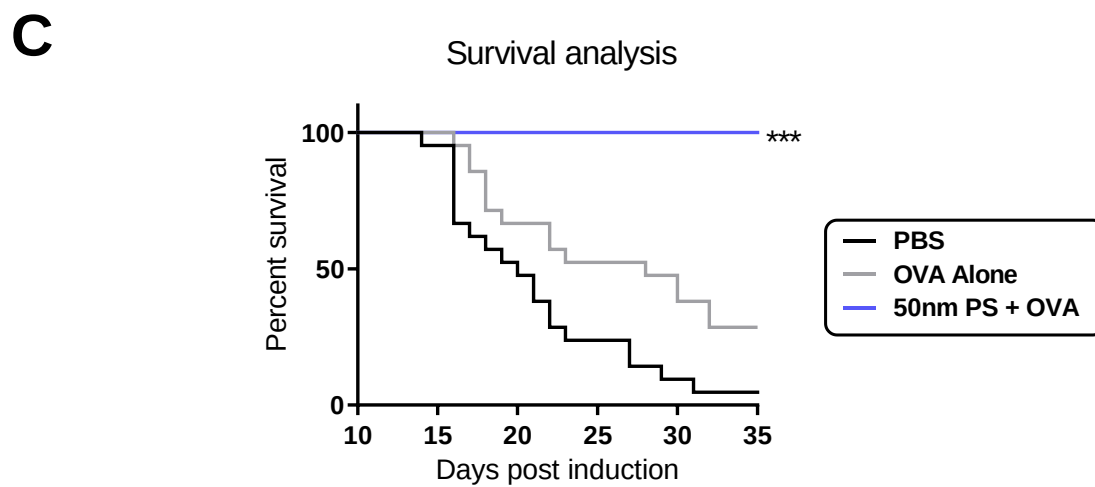
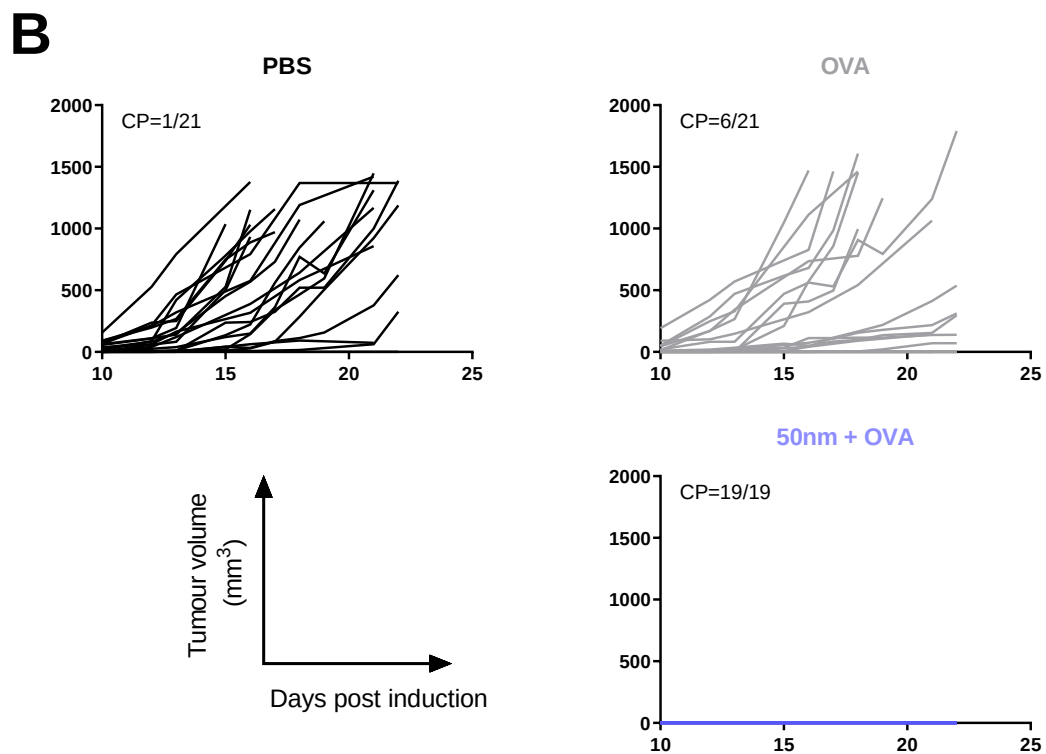
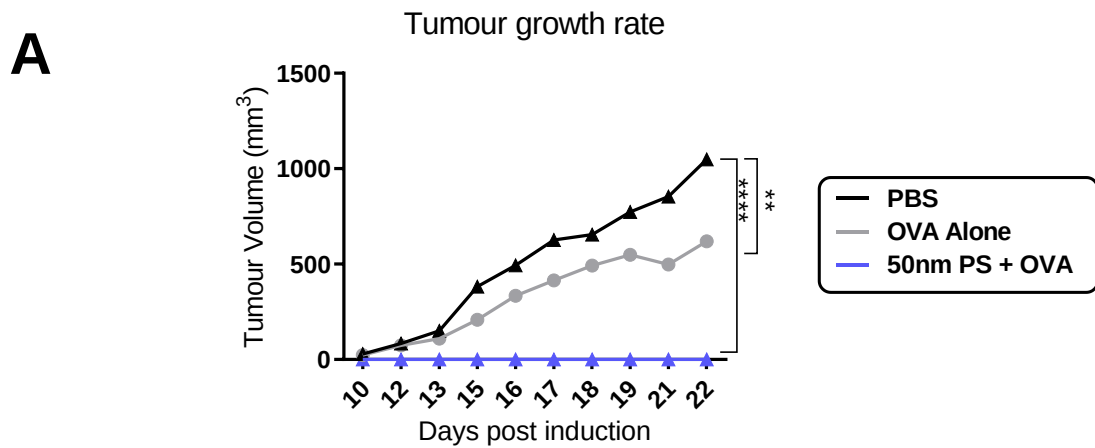


Figure 4.4.1 Vaccination with 50nm PS NPs + OVA protein provides robust anti-tumour immunity against B16-OVA melanoma.

Mice were vaccinated on days 0 and 14 with PBS, OVA (10µg) alone or 50nm PS NPs (1mg) plus OVA (10µg) followed by s.c challenge with 3.5×10^5 B16-OVA tumour cells on day 28. (PBS n=21, OVA n=21, 50nm PS + OVA n=19). (A) Initial tumour growth rate at challenge site of mice bearing tumours prior to a humane endpoint, displayed as mean tumour volume. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of differences between the tumour growth curves of 50nm PS + OVA vaccinated mice and control groups where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Spider plots of individual tumour growth. Proportions of mice displaying complete protection (CP) indicated on plots. (C) Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between PBS and 50nm PS NP + OVA immunised mice where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

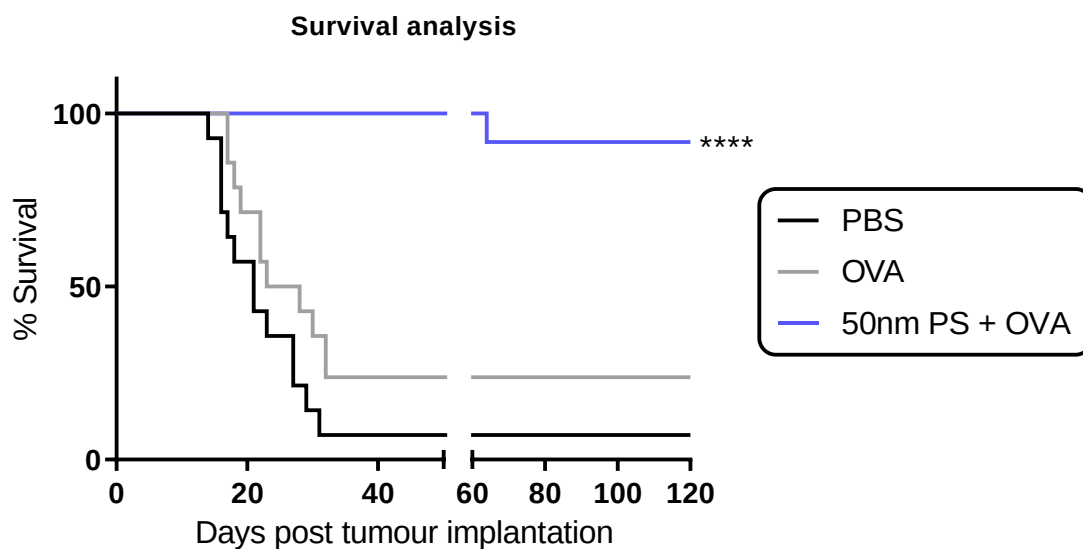


Figure 4.4.2 Vaccination with 50nm PS NP and OVA protein provides long term robust anti-tumour immunity against B16-OVA melanoma.

Mice were vaccinated on days 0 and 14 with PBS (n=14), OVA (10 μ g) alone (n=14) or 50nm PS NPs (1mg) plus OVA (10 μ g) (n=12) followed by s.c challenge with 3.5x10⁵ B16-OVA tumour cells on day 28. Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between PBS and 50nm PS NP + OVA immunised mice where *p<0.05, **p<0.01, and ***p<0.001.

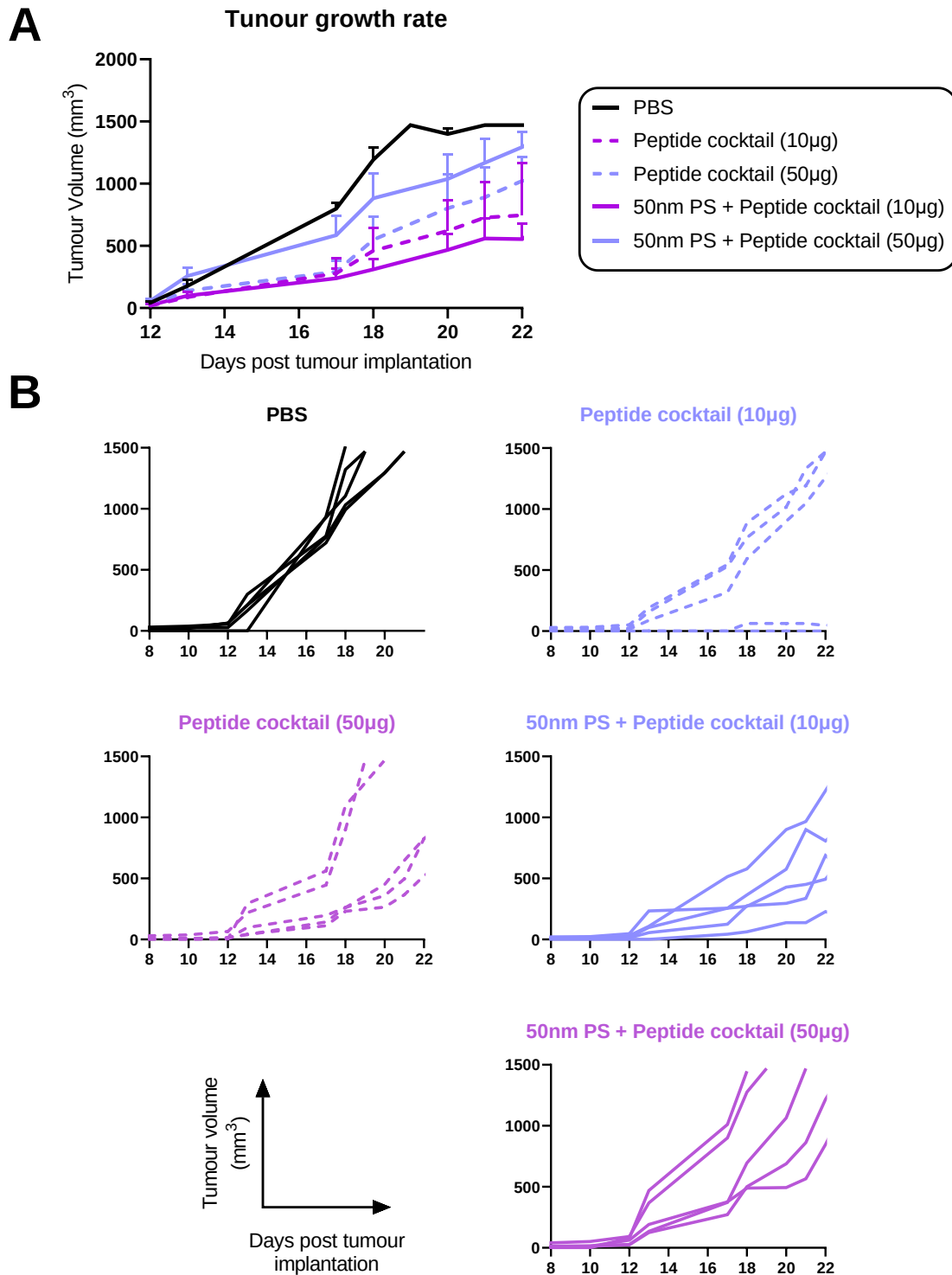
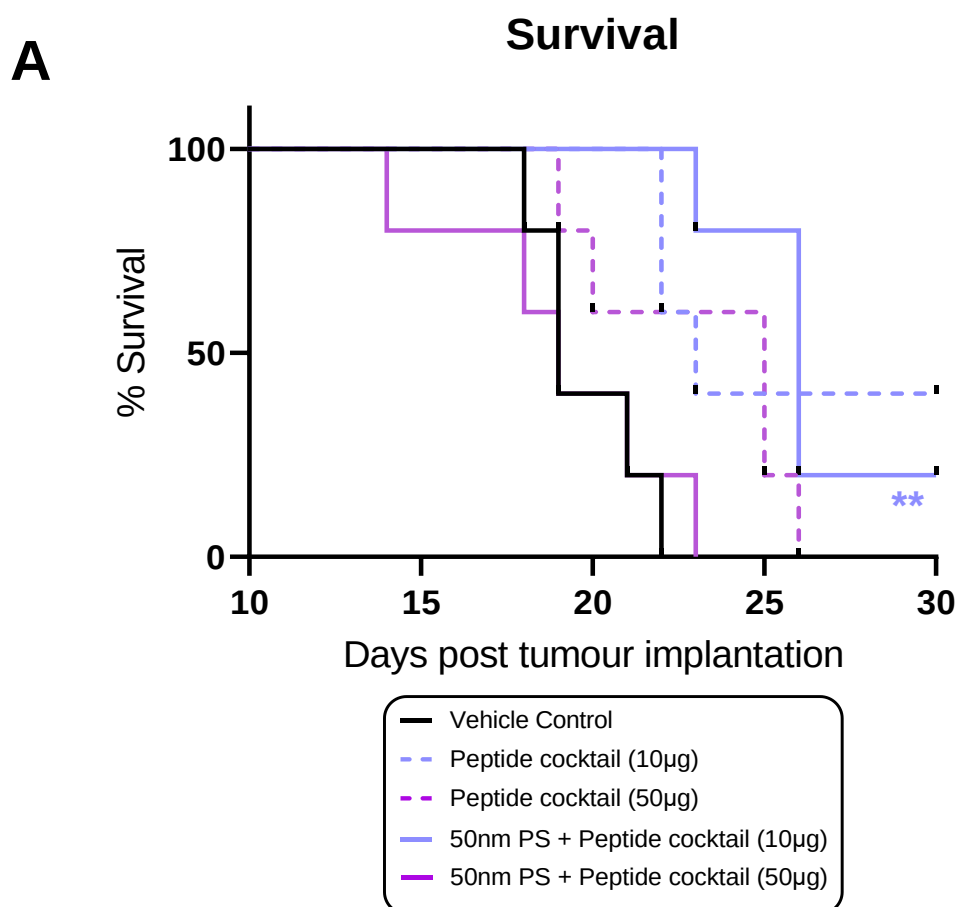


Figure 4.4.3 Vaccination with 50nm PS NPs and MC38 peptide antigens provides a lower degree of anti-tumour immunity

Mice were vaccinated on days 0 and 14 with PBS, MC38 peptide cocktail (10µg) alone, MC38 peptide cocktail (50µg), 50nm PS NPs (1mg) plus MC38 peptide cocktail (10µg) or 50nm PS NPs (1mg) plus MC38 peptide cocktail (50µg) followed by s.c challenge with 5×10^5 MC38 tumour cells. (All groups n=5). (A) Initial

tumour growth rate at challenge site of mice bearing tumours prior to a humane endpoint, displayed as mean tumour volume. B) Spider plots of individual tumour growth.



B

Median survival time (Days post tumour implantation)	
Vehicle Control	19
Peptide cocktail (10µg)	23
Peptide cocktail (50µg)	25
50nm PS + Peptide cocktail (10µg)	26
50nm PS + Peptide cocktail (50µg)	19

Figure 4.4.4 Vaccination with 50nm PS NPs and MC38 peptide antigens may enhance survival of MC38 challenged mice

Mice were vaccinated on days 0 and 14 with PBS, MC38 peptide cocktail (10µg) alone, MC38 peptide cocktail (50µg), 50nm PS NPs (1mg) plus MC38 peptide cocktail (10µg) or 50nm PS NPs (1mg) plus MC38 peptide cocktail (50µg) followed by s.c challenge with 5×10^5 MC38 tumour cells. (All groups n=5). Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between PBS and 50nm PS NP + Peptide cocktail (10µg) immunised mice where *p<.05, **p<0.01, and ***p<0.001. (B) Median survival time of mice.

4.5 Optimal 50nm PS NP mediated anti-tumour immunity is reliant on elicitation of CD8⁺ T cells

As previously shown in chapter 3, prime boost vaccination utilising 50nm PS NP adjuvants results in the induction of antigen-specific CD8⁺ T cell, IFN γ and antibody responses. While robust 50nm PS NP + OVA vaccine-induced anti-tumour protection was demonstrated in section 4.4, the correlates of protective immunity remained to be determined. To investigate the importance of the vaccine-induced CD8⁺ T cells, monoclonal antibody mediated cell depletion was utilised.

An anti-CD8 α monoclonal antibody was injected i.p according to the schematic detailed in Fig. 4.5.1. This antibody is highly specific and binds to CD8 α , following which bound cells are targeted for removal via ADCC. However, a relatively high level of cellular expression is required for cell clearance³²⁹. Mice were injected i.p with an initial dose of 250 μ g and subsequent 100 μ g doses, of either the α -CD8 α or isotype control monoclonal antibodies. This was an optimised regimen as when a 200 μ g dose was trialled, residual CD8⁺ T cells were detected in the blood and spleen of recipient mice. CD8⁺ T cell depletion was successful in the experimental mice groups compared to mice receiving the isotype control antibody, as determined through analysis of peripheral blood sampled on days 31 and 35 post initial priming immunisation (Fig 4.5.2). This depletion analysis was done utilising a different, non-competing clone antibody (KT15) for CD8⁺ detection. The importance of using a non-competing clone has been demonstrated by Jung et al, 2018³²⁹.

In PBS recipient mice, CD8⁺ T cell depletion results in an increased susceptibility to tumour challenge when compared to those given the isotype control, mean survival time was reduced from 23 to 19 days post tumour implantation (Fig. 4.5.4). The isotype control did not impact 50nm PS NP OVA induced protection from tumour challenge, all mice were protected, as indicated in the survival analysis and lack of tumour establishment at the challenge site (Fig. 4.5.3). Crucially, when CD8⁺ T cells were depleted in mice vaccinated with 50nm PS NP and OVA, this protection was significantly reduced from 100% to 30% , reflected in survival analysis and tumour establishment at the challenge site (Fig. 4.5.3 and 4.5.4). These data support the hypothesis that antigen-specific CD8⁺ T cells are essential mediators of the protective anti-tumour immunity induced by vaccination with 50nm PS NP and OVA.

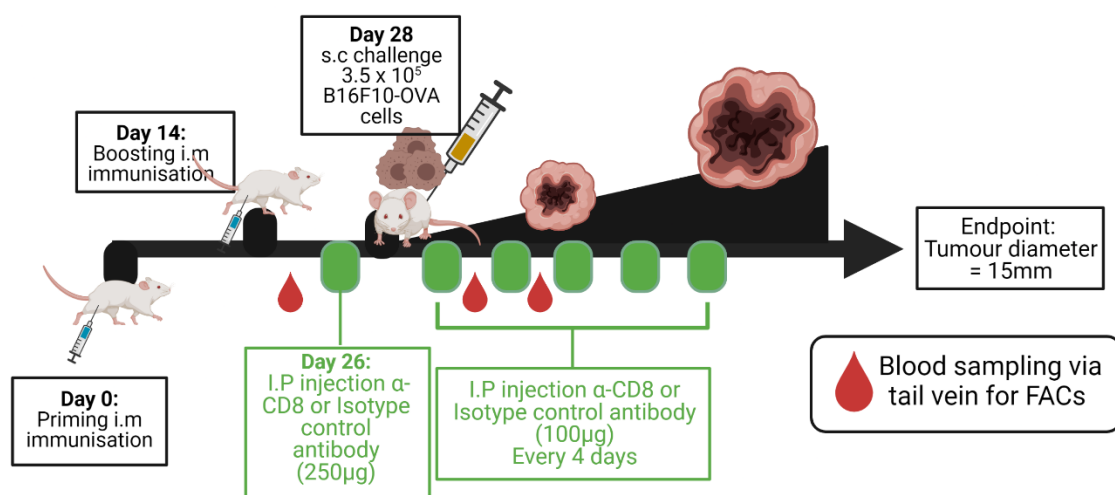


Fig. 4.5.1 Schematic of vaccine-induced anti- tumour protection study with CD8⁺ T cell depletion.

Mice were vaccinated on days 0 and 14 with PBS, OVA (10µg) alone, or 50nm PS NPs (1mg) plus OVA (10µg). On day 26, mice were injected i.p with 250µg of either a monoclonal α-CD8α antibody (Clone 2.43) or a monoclonal isotype control antibody (Clone LTF-2). Mice received subsequent injections of 100µg of respective monoclonal antibody every 4 days for the duration of the study. Blood was sampled from mice via the tail vein on days 23, 31 and 35 to detect presence of peripheral CD8⁺ T cells. On day 28, mice were challenged s.c with 3.5x10⁵ B16-OVA tumour cells, and tumour growth was monitored daily until clinical endpoint for growth curve and survival analysis.

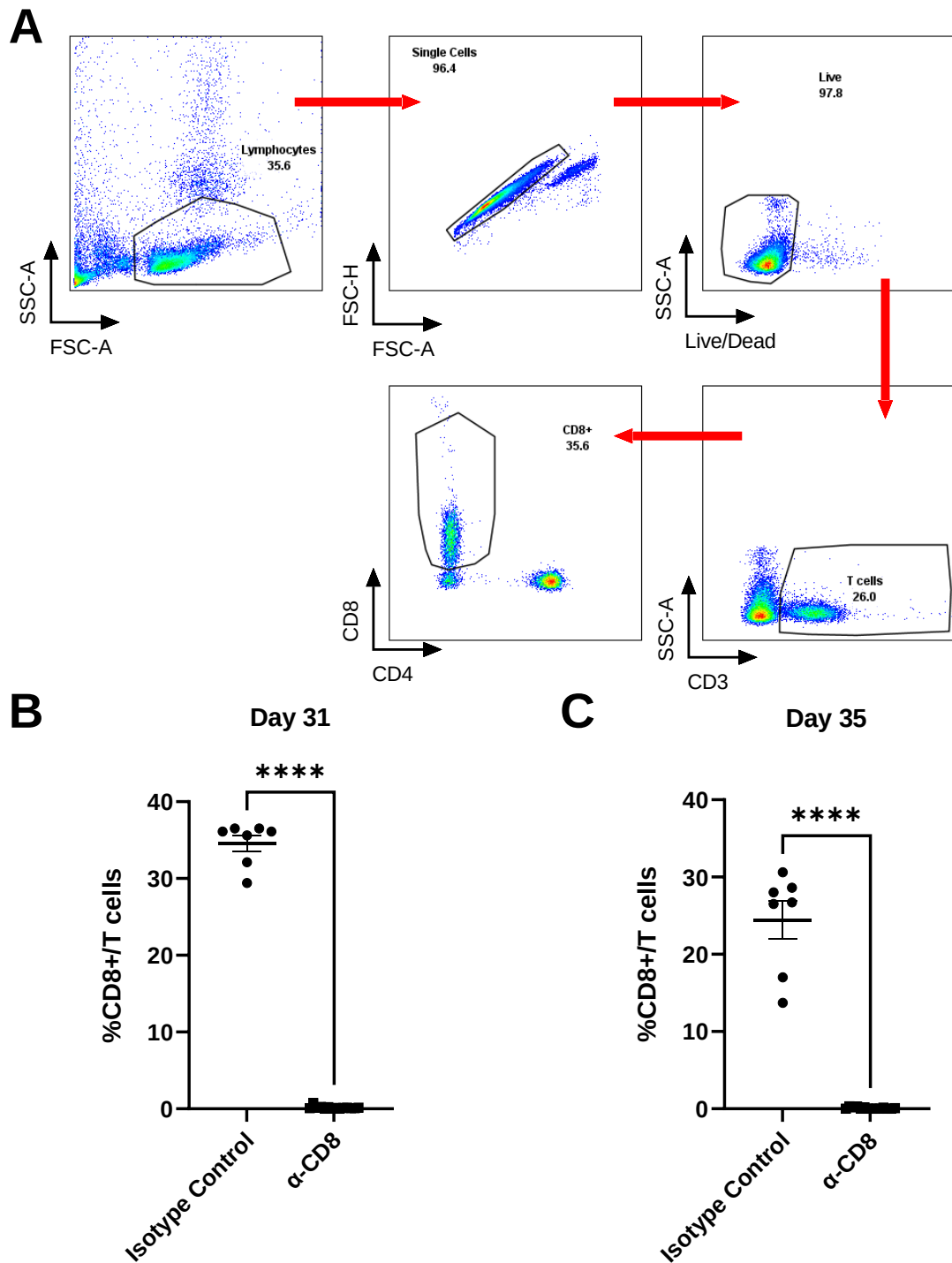


Fig. 4.5.2 CD8⁺ T cells were successfully depleted in experimental mice

Mice were treated as described in Fig.4.5.1. A) Gating strategy used to characterise the presence of CD8⁺ T cells in peripheral blood of experimental mice. Cells were acquired using BD FACSCanto™. B) Frequency of CD8⁺ T cell over total CD3⁺ T cells on day 31 and 35 post initial priming vaccination. An unpaired t test was used to determine statistical significance where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

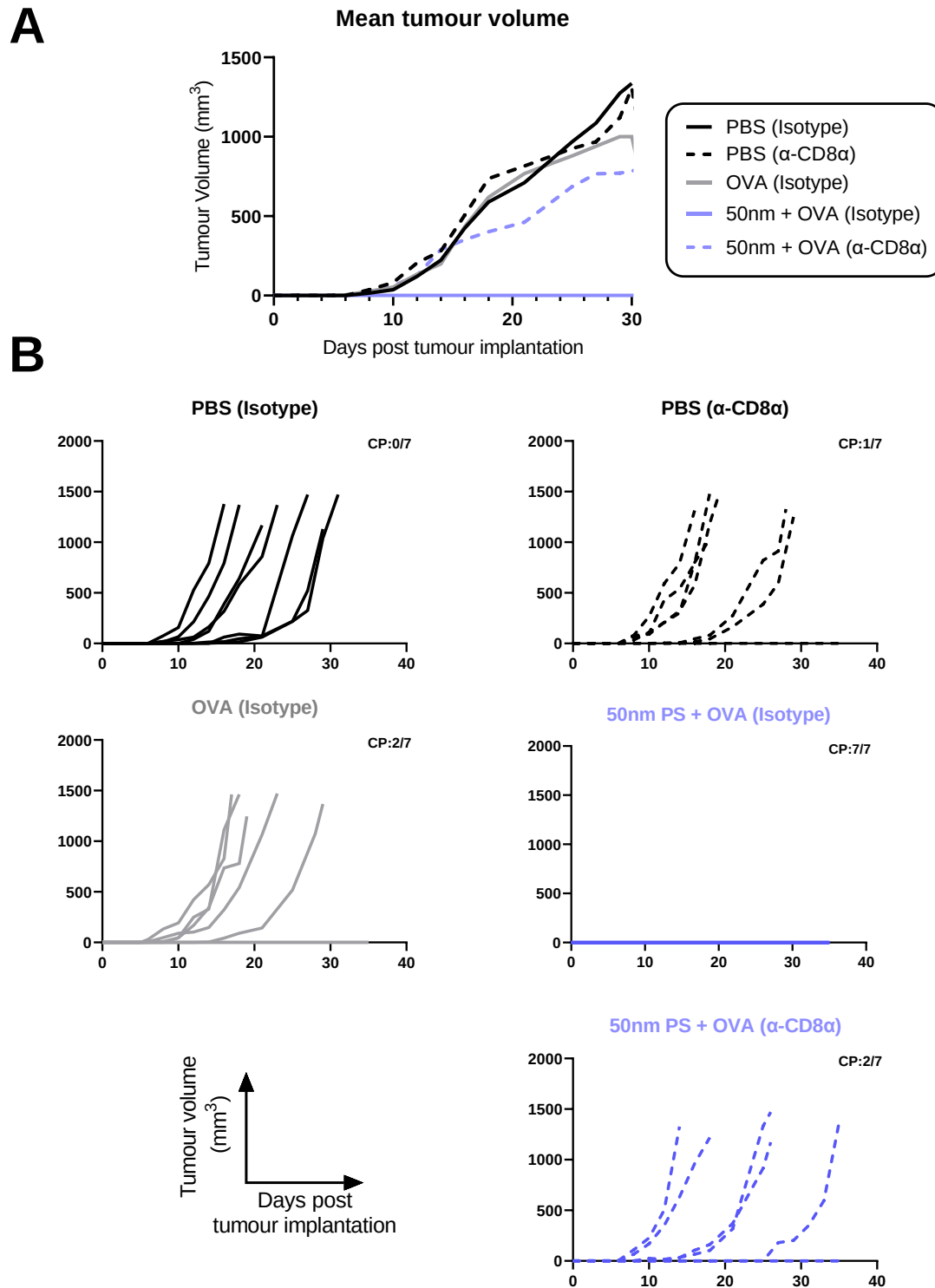


Fig. 4.5.3 CD8⁺ T cells are required for optimal protection from B16-OVA challenge following vaccination with 50nm PS NP and OVA.

Mice were treated as described in Fig. 4.5.1, with 7 mice per group. A) Mean tumour growth B) Individual tumour growth rate displayed as spider growth plots. CP denotes complete protection from tumour establishment.

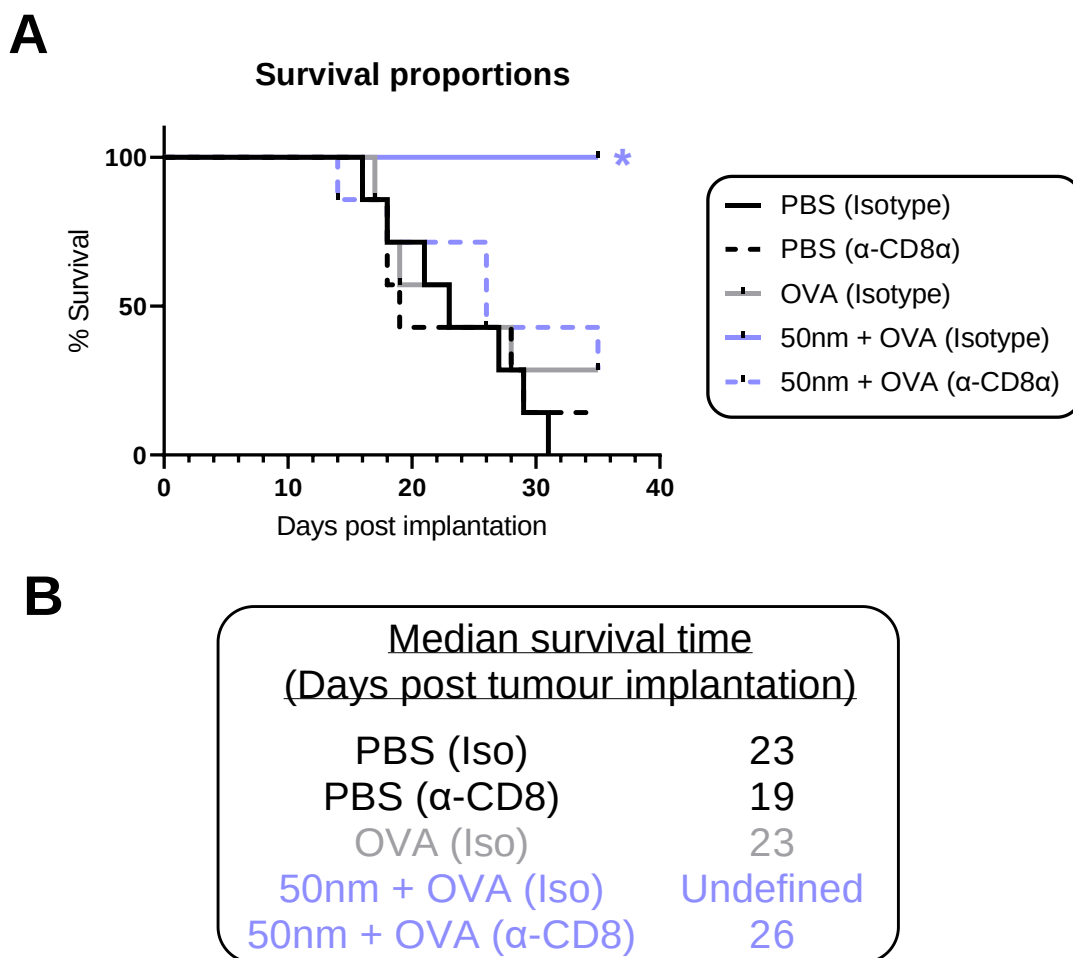


Fig. 4.5.4 CD8⁺ T cells are required for optimal protection from B16-OVA challenge following vaccination with 50nm PS NP and OVA.

Mice were treated as described in Fig. 4.5.1. Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between the survival curves of CD8 depleted and non-depleted 50nm PS NP + OVA vaccinated mice where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Median survival time of mice.

4.6 Therapeutic vaccination with 50nm PS NPs + OVA can inhibit tumour growth and prolong survival in tumour challenged mice

While the efficacy of 50nm PS NPs as adjuvants in prophylactic cancer vaccines had been demonstrated, it was important to address efficacy in more clinically relevant therapeutic vaccination approaches. Therapeutic cancer vaccines have been trialled clinically utilising tumour associated antigens, or more recently, tumour specific antigens, including tumour neoantigens arising through mutation⁸⁴. Many of these vaccines have utilised Poly IC:LC as a vaccine adjuvant, or in some cases, combinations of poly IC:LC and emulsion adjuvants like montanide¹³⁸. The therapeutic efficacy of these cancer vaccines has remained modest, correlating with low magnitudes of antigen specific CD8⁺ T cell responses elicited. While this may be due to the choice/nature of neoantigens identified, adjuvant choice could also play a role. As such, novel adjuvants that can effectively drive functional anti-tumour CD8⁺ T cell responses would be a welcome addition to the cancer vaccinologists repertoire.

To evaluate the functionality of 50nm PS NPs in a therapeutic cancer vaccine setting, a B16F10-OVA model was utilised where mice were vaccinated intramuscularly when tumour volume reached 10-20mm³, receiving a booster a week later (Fig.4.6.1). This vaccination schedule was utilised due to the rapid kinetics of B16 tumour growth, and the reliance of protection on vaccine induced T cells and subsequent migration to the tumour following intramuscular injection. In addition, a smaller number of mice were vaccinated with the alternative adjuvants; CpG 1826 and 2'3'cGAMP for benchmarking purposes.

Crucially, therapeutic vaccination with 50nm PS NPs + OVA led to significant inhibition of tumour growth when compared to vehicle control immunised mice, while vaccination with antigen alone did not have any impact on tumour growth (Fig.4.6.2). Likewise, therapeutic vaccination with 50nm PS NPs + OVA led to significantly enhanced survival when compared to vehicle control immunised mice, while vaccination with antigen alone did not confer any enhanced survival (Fig.4.6.3). While only a small number of mice were included in the groups trialling CpG 1826 and 2'3'cGAMP adjuvanted therapeutic vaccines, therapeutic benefit in terms of tumour growth inhibition and enhanced survival was observed both groups (Fig.4.6.2 and 4.6.3). However, this therapeutic benefit was to a lesser extent than that observed with the nanoparticle adjuvanted formulation.

Altogether these results identify 50nm PS NPs as suitable adjuvants for use in therapeutic cancer vaccination approaches, resulting in a significant delay in tumour progression. This warrants further investigation in different tumour models to confirm efficacy.

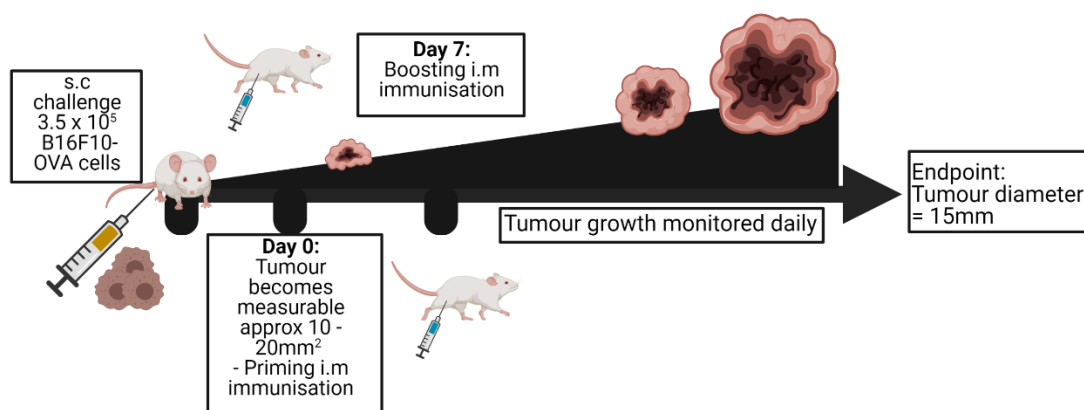


Fig. 4.6.1 Schematic of intramuscular therapeutic vaccination model using B16-OVA tumour challenge.

C57BL6/J mice were vaccinated intramuscularly when tumours reached 10-20mm² and received an intramuscular booster vaccination 7 days later. Mice were vaccinated with either PBS, OVA (10µg) alone, 50nm PS NPs (1mg) plus OVA (10µg), CpG 1826 (50µg) plus OVA (10µg) or cGAMP (10µg) plus OVA (10µg). Tumour growth was monitored daily until clinical endpoint for growth curve and survival analysis.

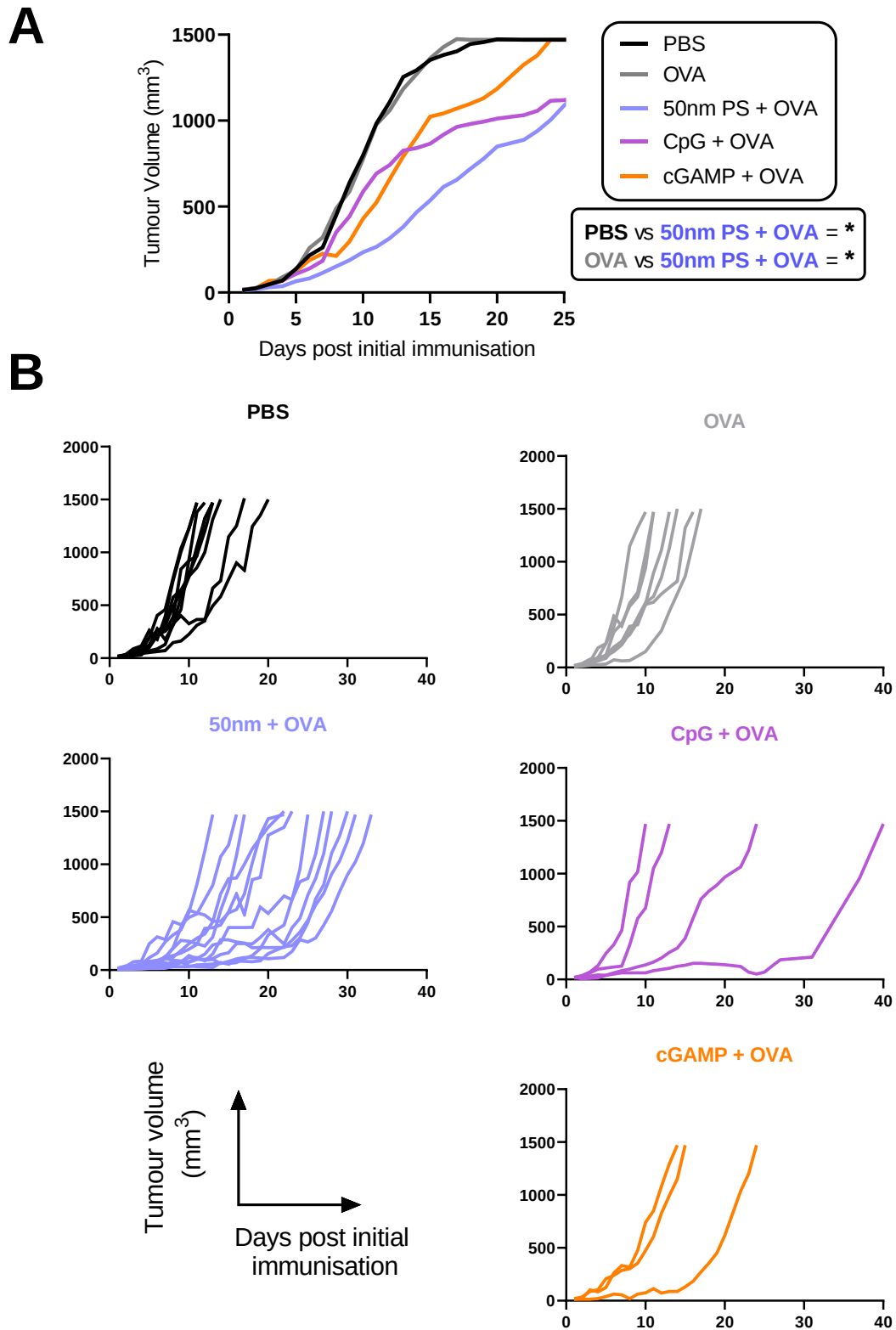
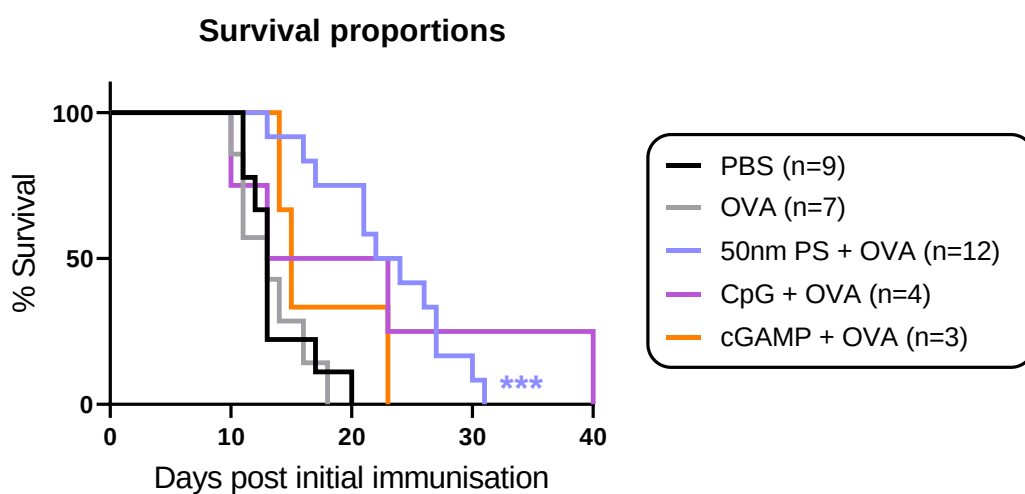


Fig. 4.6.2 Therapeutic vaccination with 50nm PS NPs and OVA inhibits B16-OVA growth *in vivo*.

Mice were treated as described in Fig. 4.6.1. A) Mean tumour growth B) Individual tumour growth rate displayed as spider growth plots. B) Percentage of mice bearing tumours at challenge site post tumour implantation. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine

statistical significance of differences between the tumour growth curves of 50nm PS + OVA vaccinated mice and control groups. Asterisks indicate significant differences. Not significant – ns (not displayed on graph), P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

A**B**

Median survival time (Days post initial immunisation)	
PBS	13
OVA	13
50nm PS + OVA	23
CpG + OVA	18
cGAMP + OVA	15

Fig. 4.6.3 Therapeutic vaccination with 50nm PS NPs and OVA prolongs survival in B16-OVA challenged mice

Mice were treated as described in Fig. 4.6.1. (A) Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between the survival curves of PBA and 50nm PS NPs + OVA vaccinated mice where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Median survival time of mice.

4.7 Therapeutic vaccination with 50nm PS NPs can synergise with checkpoint blockade

A key application of cancer vaccines is their inclusion in existing immunotherapeutic strategies, where they have the potential to sensitise patients to respond clinically to and synergise with checkpoint blockade and even to other immunotherapeutic approaches including CAR therapies^{66,84}. As such, a therapeutic tumour model was designed whereby mice were vaccinated intramuscularly as before when tumour volume reached 10-20mm³, but additionally mice were treated systemically with a PD1 targeted monoclonal antibody (Clone RMP1-14). It is worth noting that B16 tumours do not respond optimally to checkpoint blockade strategies^{104,330}, owing to poor immunogenicity and low immune tumour infiltrate of CD8⁺ T cells. This provides a good model for investigating synergy between vaccination and checkpoint blockade strategies.

Unfortunately, the therapeutic efficacy of vaccination with 50nm NP + OVA alone was less than had been observed previously, still inhibiting tumour growth (Fig.4.7.2) and significantly prolonging survival (Fig.4.7.3) but to a lesser extent than results described in section 4.6. This may be due to issues surrounding the concentration of particles used for vaccination, which can arise from issues arising from the washing/centrifugation of NPs which has been previously observed in the lab (Oral Communication, Natalia Muñoz-Wolf) or improper immunisations in some mice. However, despite these issues, strong synergy between 50nm NP + OVA vaccination and systemic α -PD1 therapy was observed as mice displayed significantly improved inhibition of tumour growth (Fig.4.7.2) and significantly enhanced survival (Fig.4.7.3) when compared to mice receiving α -PD1 therapy alone.

In addition, the therapeutic efficacy of vaccination with 50nm NPs + OVA was compared to the ChAdOx1-OVA vector. Compared head-to-head, mice vaccinated with ChAdOx1-OVA had significantly higher inhibition of tumour growth rate (Fig.4.7.2) and had increased survival time (Fig.4.7.3), albeit not statistically significant, when compared to mice vaccinated with 50nm NP + OVA. Despite the superiority of ChAdOx1-OVA over 50nm PS NPs + OVA in exerting therapeutic efficacy, this is a promising result in that 50nm NPs compared relatively well to a clinically relevant vaccine platform.

Altogether these results demonstrate that therapeutic vaccination with 50nm PS NPs and tumour antigen can sensitise tumours with normally poor response rates to respond to checkpoint blockade strategies. This warrants further investigation with different tumour

models and checkpoint blockade therapies. Additionally, the mechanisms underlying this synergy should be investigated.

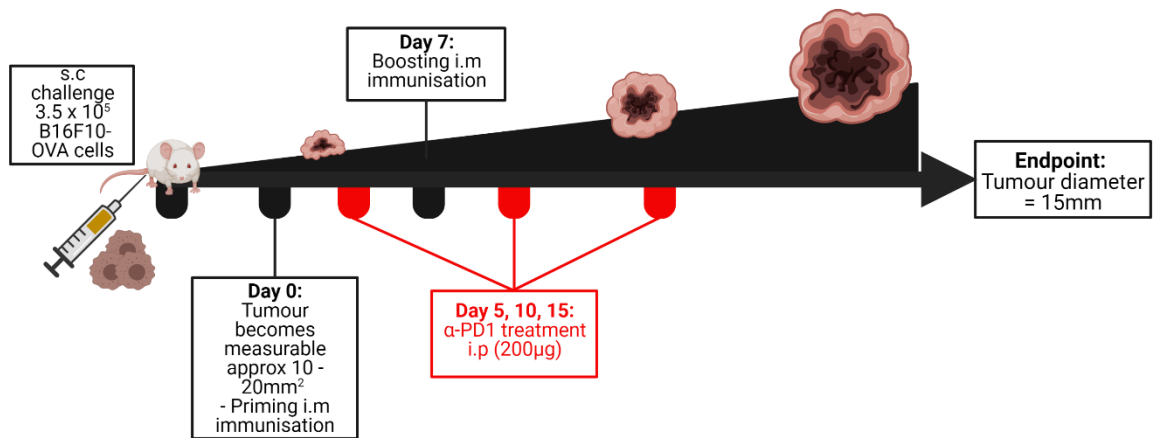


Fig. 4.7.1 Schematic of intramuscular therapeutic vaccination and checkpoint blockade model using B16-OVA tumour challenge.

C57BL6/J mice were vaccinated intramuscularly when tumours reached 10-20mm² and received an intramuscular booster vaccination 7 days later. Mice were vaccinated with either PBS, OVA (10µg) alone, 50nm PS NPs (1mg) plus OVA (10µg), or ChAdOx1-OVA (10⁸ i.u.). On days 5, 10, and 15 mice received 200µg of a monoclonal α-PD-1 antibody or PBS. Tumour growth was monitored daily until clinical endpoint for growth curve and survival analysis.

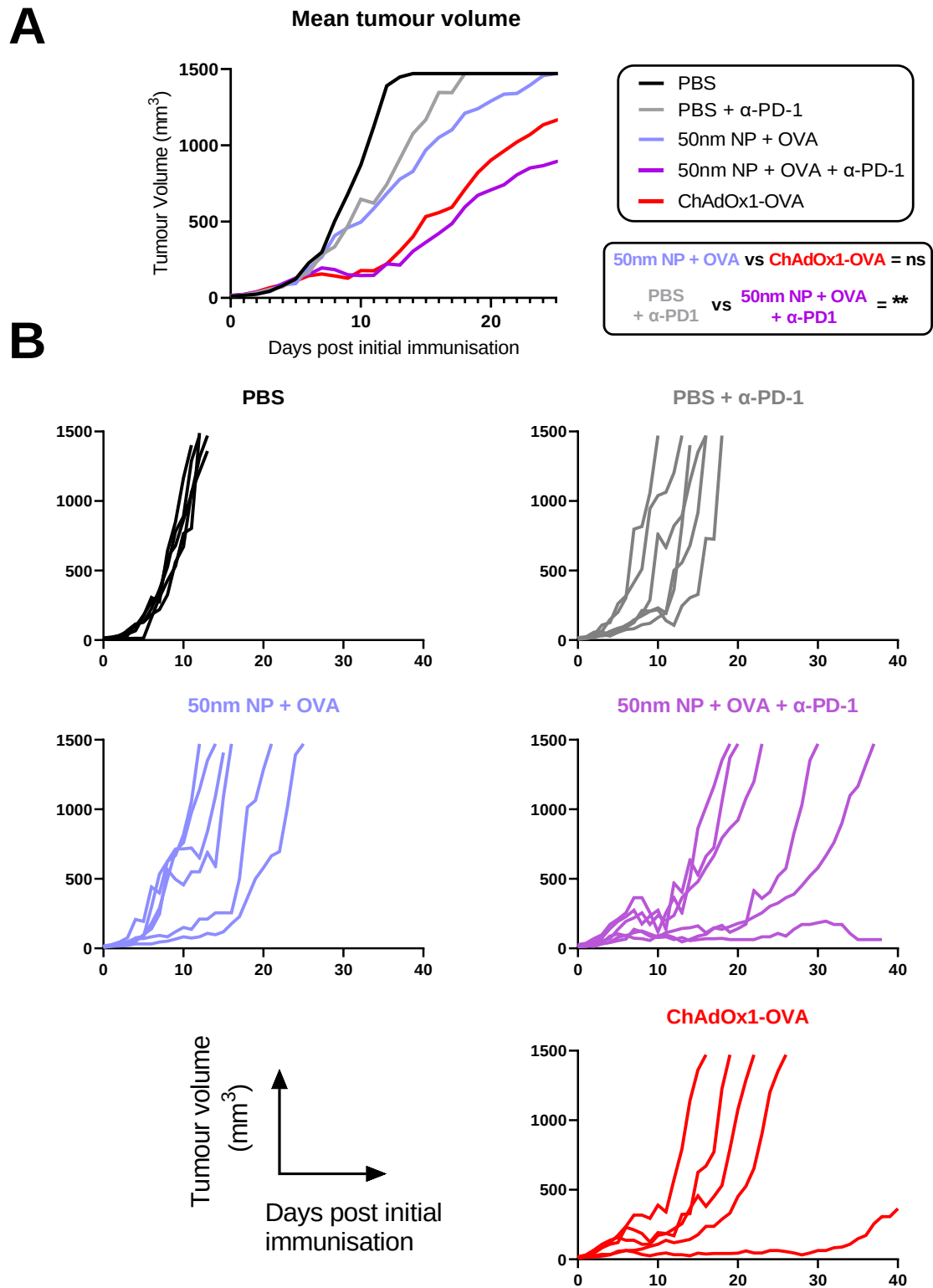


Fig. 4.7.2 Therapeutic vaccination with 50nm PS NPs can synergise with systemic α -PD-1 therapy to inhibit B16-OVA growth *in vivo*.

Mice were treated as described in Fig. 4.7.1. A) Mean tumour growth B) Individual tumour growth rate displayed as spider growth plots. B) Percentage of mice bearing tumours at challenge site post tumour

implantation. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of differences between the tumour growth curves of 50nm PS + OVA vaccinated mice and control groups. Asterisks indicate significant differences. Not significant – ns (not displayed on graph), P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

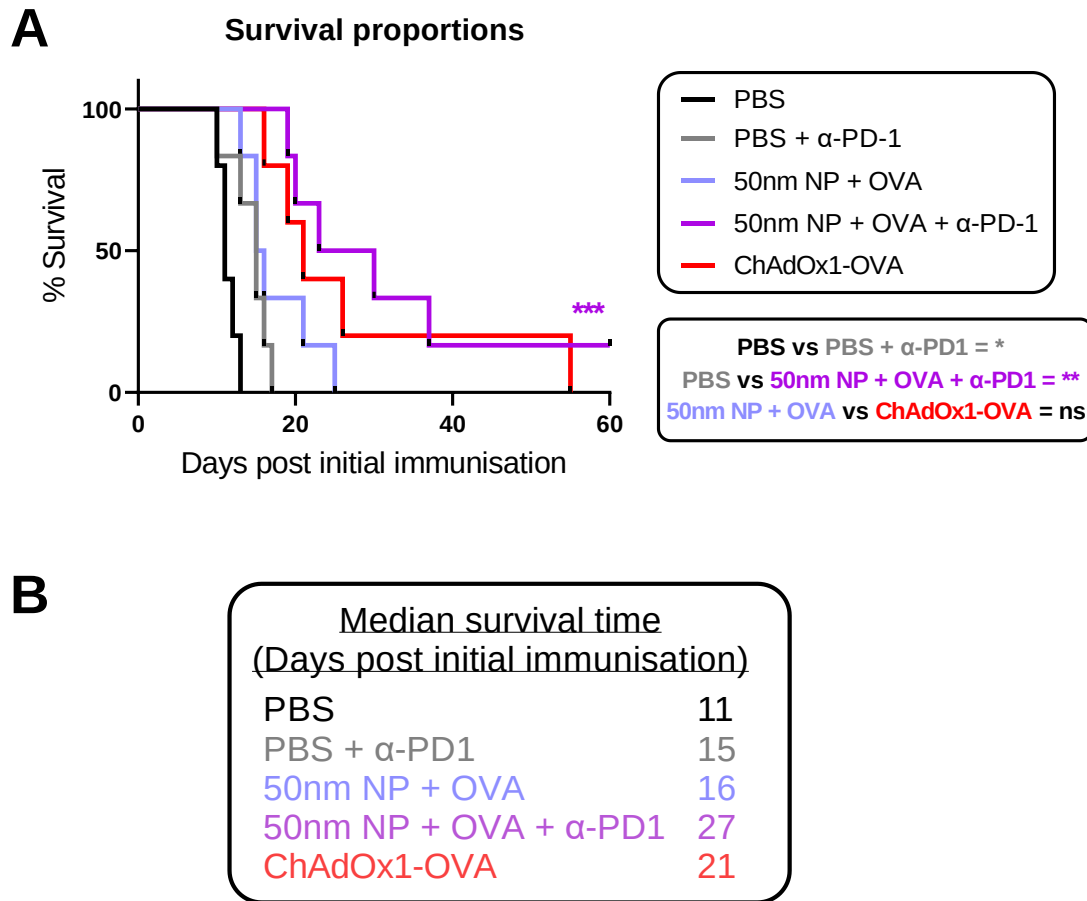


Fig. 4.7.3 Therapeutic vaccination with OVA and 50nm PS NPs synergises with α -PD1 therapy to prolong survival in B16-OVA challenged mice

Mice were treated as described in Fig. 4.7.1. (A) Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between the survival curves of PBS + α -PD1 treated vs 50nm PS NP + OVA + α -PD1 treated mice where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Median survival time of mice.

4.8 Bulk transcriptional analysis uncovers a unique ‘stem-like’ phenotype of antigen specific CD8⁺ T cells induced following vaccination with antigen and 50nm NPs

Having demonstrated that vaccination with antigen and 50nm PS NPs could induce functional CD8⁺ T cell dependent anti-tumour immune responses, experiments were designed to uncover the phenotype of these antigen specific T cells. To do so, bulk RNAseq was performed on CD44⁺ antigen specific CD8⁺ T cells a week following completion of prime boost vaccination, as displayed in Fig.4.8.1. CD8⁺ T cells elicited following vaccination with ChAdOx1-OVA were used as a control, as it was shown in Figure.3.10.4 that this clinically relevant vaccine platform drove antigen specific CD8⁺ T cells and anti-tumour immunity

PCA plots display tight clustering of the CD8⁺ T cells isolated from spleens of mice vaccinated with 50nm PS NP + OVA, unfortunately more variability (predominantly along PC1) was observed in cells isolated from ChAdOx1-OVA immunised mice (Fig.4.8.2A). Differential gene expression analysis uncovered key differences between these groups (Fig.4.8.2B + Fig.4.8.3), with subsequent GO enrichment analysis of these differentially expressed genes highlighting several gene sets where significant differences were observed (Fig.4.8.4). Of particular interest was the differential expression noted in the ‘T cell activation/involved in immune response’, ‘positive regulation of cell migration/motility’, and in the ‘cytokine receptor activity’ gene sets. As noted in the volcano plot displaying differential expression, many of the differentially expressed genes were downregulated in CD8⁺ T cells from mice vaccinated with antigen and 50nm PS NP compared to those elicited by ChAdOx1-OVA (Fig.4.8.5A).

Further analysis revealed a unique phenotype of CD8⁺ T cells from mice vaccinated with antigen and 50nm PS NP when compared to those elicited by ChAdOx1-OVA, with reduced expression of genes involved in mediating the cytotoxic effects of CTLs (*Gzma*, *Gzmb*, *Prf1*) and effector cytokines (*Ifng*, *Tnf*) (Fig.4.8.5B). However, intriguingly ‘stem’ markers such as *Tcf7*, *Sell* and *Lef1*³³¹ were elevated alongside *Cxcr5* which has been noted to be expressed on stem-like CD8⁺ T cells³³². Likewise, elevated levels of the checkpoint-encoding *Pdcd1* (PD-1) were observed. This was accompanied by increased levels of the activatory markers *Tnfrsf4* (OX40), *Icos* and *Cd27*. This is of particular interest as this data points to a ‘stem-like’ phenotype of CD8⁺ T cell that has been linked to increased response to checkpoint blockade approaches and anti-tumour functionality^{74,84}.

Altogether these data highlight the unique phenotype of antigen specific CD8⁺ T cells elicited following vaccination with 50nm PS NP + OVA when benchmarked/compared against those induced following ChAdOx1-OVA vaccination. A unique phenotype was uncovered, displaying elevated levels of canonical CD8⁺ T cell stem markers and co-stimulatory molecules expression which may provide insights into the functionality of these cells in an anti-tumour context. Moving forward, this phenotype should be confirmed in CD8⁺ T cells present in the tumour and in the tumour draining lymph nodes following therapeutic vaccination with OVA and 50nm PS NP.

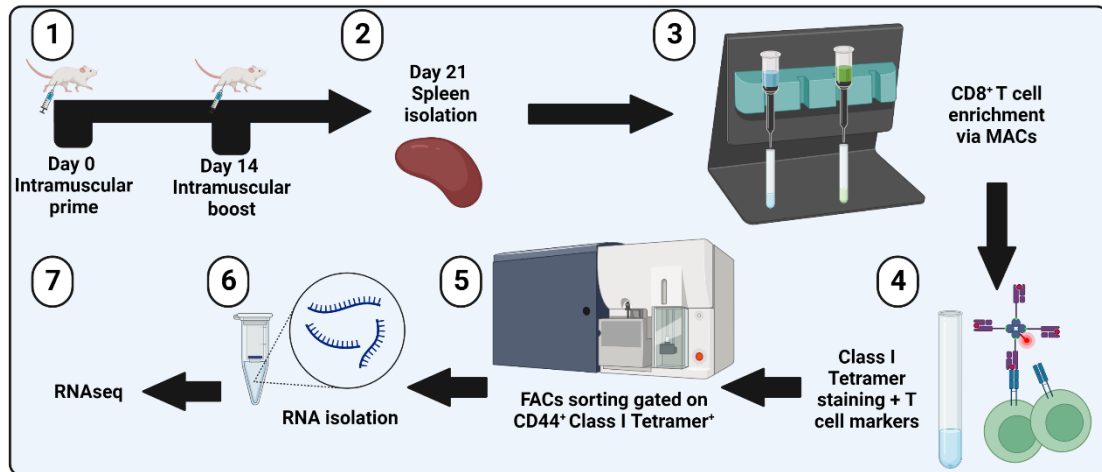


Figure 4.8.1 – Schematic of experimental design comparing the phenotype of antigen specific CD8⁺ T cells induced following vaccination with 50nm PS NP + OVA or ChAdOx1-OVA

C57BL/6 mice were vaccinated with 50nm PS NP (1mg) + OVA (10µg), or 10⁸ i.u. of ChAdOx1-OVA via the intramuscular route on days 0 and 14. On day 21, CD8⁺ T cells were sorted from the spleens of vaccinated mice via MACs. Antigen-specific CD8⁺ T cell responses were determined utilising a H-2Kb/OVA (SIINFEKL) MHC Tetramer. CD44⁺ Class I SIINFEKL Tmer⁺ CD8⁺ T cells were sorted via acquisition on the FACS ARIA. RNA was subsequently isolated from these cells, and bulk transcriptional analysis was performed through RNAseq. (50nm + OVA n=3, ChAdOx1-OVA n=4).

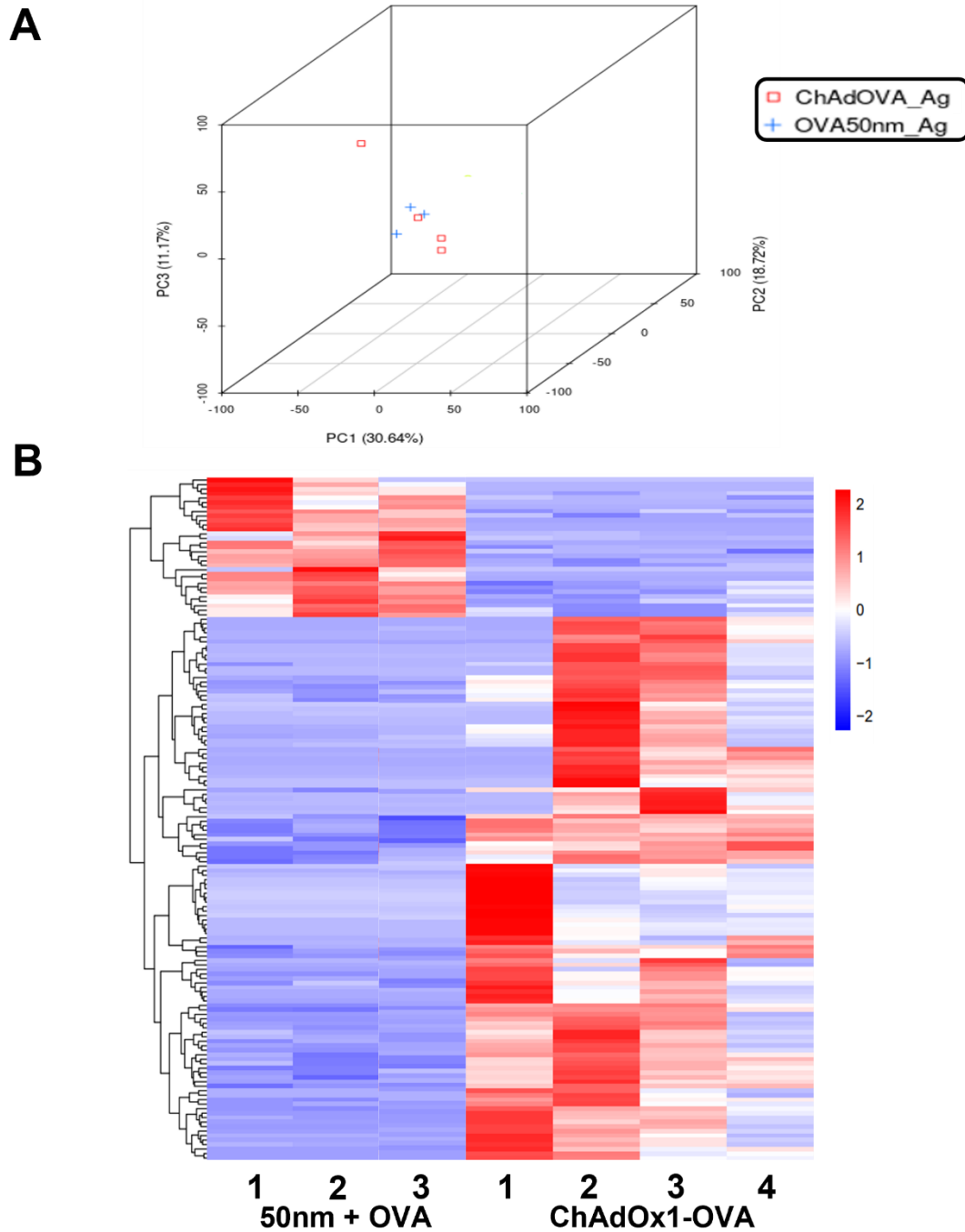


Figure 4.8.2 – Bulk transcriptional analysis of antigen specific CD8⁺ T cells following vaccination

(A) A 3D PCA plot of antigen specific CD8⁺ T cells following vaccination with 50nm NPs + OVA or ChAdOx1-OVA. (B) Heatmap displaying differential gene expression analysis of antigen specific CD8⁺ T cells following vaccination with 50nm PS NPs + OVA or ChAdOx1-OVA.

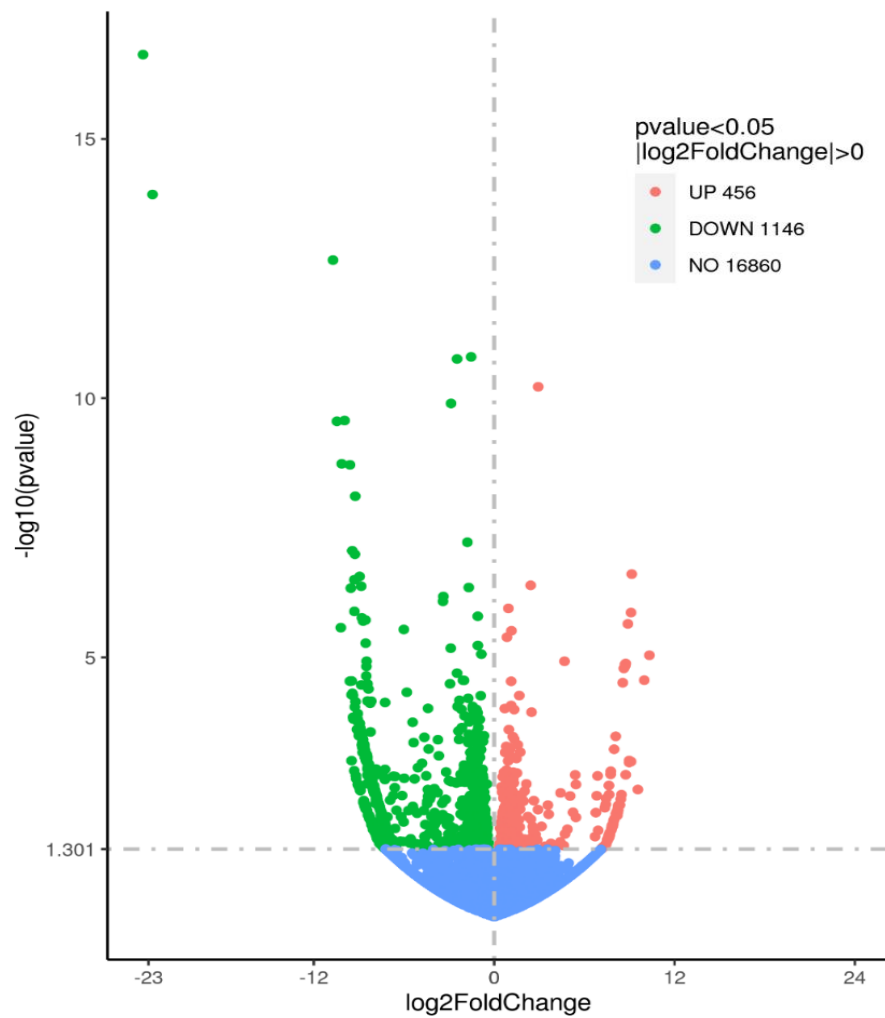


Figure 4.8.3 – Global volcano plot of differentially expressed genes

Volcano plot of DEGs in antigen specific CD8⁺ T cells following vaccination with 50nm PS NP + OVA when compared to those elicited following ChAdOx1-OVA vaccination.

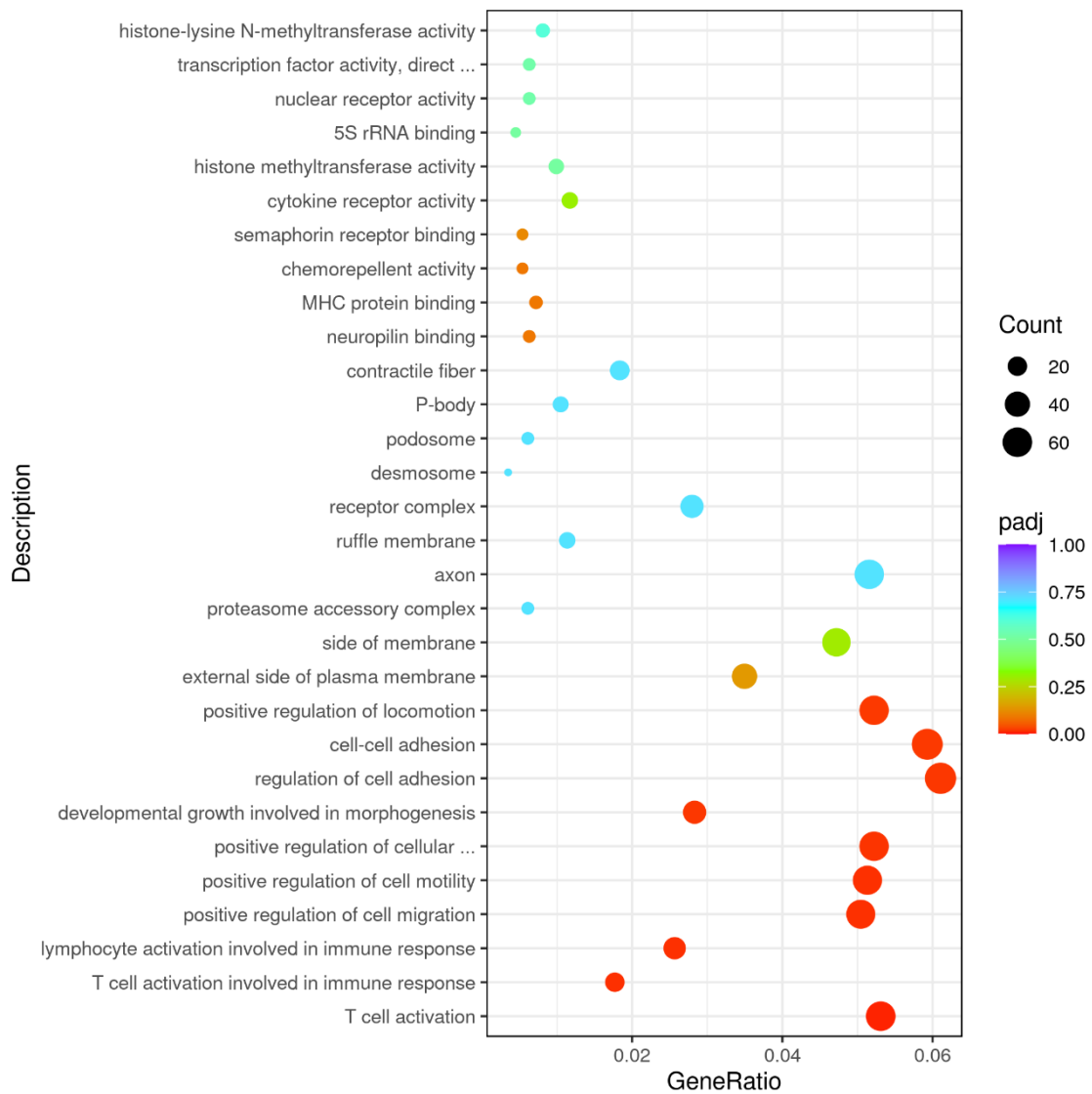
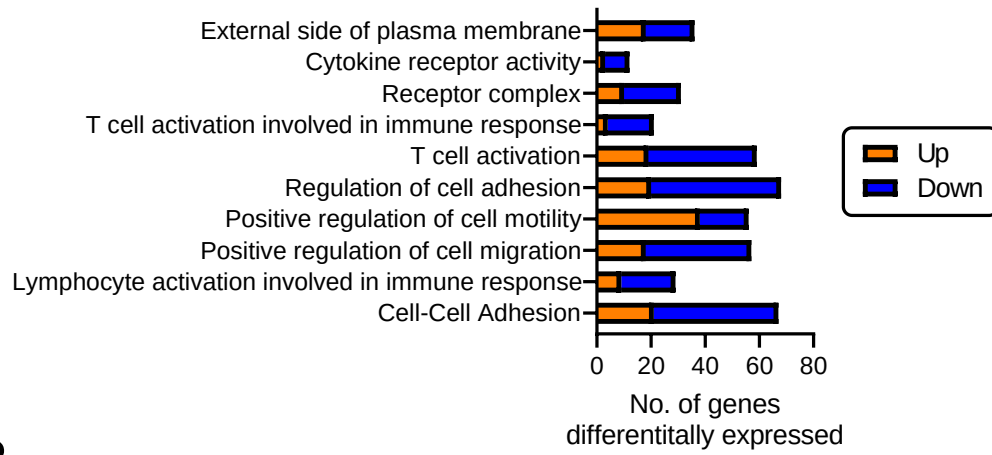


Figure 4.8.4 – GO enrichment analysis of differentially expressed genes

Summary of results from GO enrichment analysis on differentially expressed genes in antigen specific CD8⁺ T cells following vaccination with 50nm PS NPs + OVA when compared to those elicited following vaccination with ChAdOx1-OVA.

A

GO: Differentially expressed genes



B

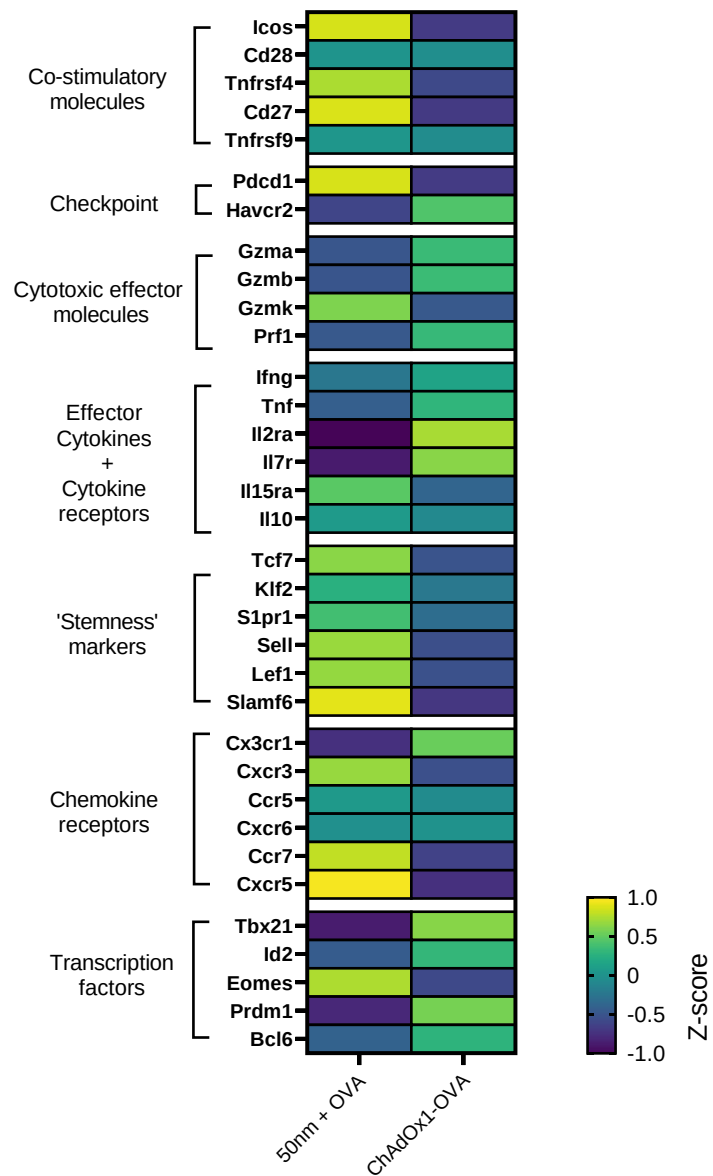


Figure 4.8.5 – Differentially expressed genes in antigen specific CD8⁺ T cells elicited following vaccination with 50nm NP + OVA or ChAdOx1-OVA

(A) Number of genes up and down regulated within GO enrichment analysis gene sets when comparing antigen specific CD8⁺ T cells elicited following vaccination with 50nm NP + OVA to those elicited following ChAdOx1-OVA vaccination. (B) Scaled average heatmap displaying differential expression of selected genes.

4.9 Discussion

These data clearly demonstrate the robust functionality and durability of anti-tumour immune responses induced by vaccination with antigen and 50nm PS NP, in both prophylactic and therapeutic tumour vaccination models.

Prophylactic vaccination with 50nm PS NPs + OVA induced complete protection from tumour challenge up until roughly 35 days, and 93% protection when immunised mice were left for 120 days post tumour implantation. Therapeutically, immunisation with 50nm PS NP + OVA significantly inhibited tumour growth and enhanced survival, with synergistic therapeutic efficacy demonstrated with systemic checkpoint blockade. It is worth mentioning that the B16-F10 cell line from which the genetically engineered B16-OVA line is derived, is a particularly aggressive mouse tumour model with low immunogenicity^{333–335}. These results, therefore, indicate an impressive degree of induced anti-tumour immunity in a challenging tumour model and provide experimental foundations for potential future use as cancer vaccine adjuvants.

When the mediators of this anti-tumour immunity were dissected in a prophylactic vaccine setting, CD8⁺ T cells were identified as crucial for vaccine induced protection, as anti-tumour immunity was significantly impaired in their absence through depletion. This is perhaps not surprising, given the robust antigen-specific CD8⁺ T cell responses observed following immunisation with antigen and 50nm PS NP, and the fundamental role that CD8⁺ CTLs play in tumour surveillance and elimination⁵⁶. However, this functional validation was crucial as CD8⁺ T cell comprise a wide range of subsets, somewhat analogous to those documented for CD4⁺ T helper cells. Some of these subsets possess more anti-tumour functionality than others⁶¹, including CD8⁺ T regulatory cells, which may exert pro-tumourigenic effects. This insight into their importance to the anti-tumour immunity induced by vaccination with antigen and 50nm PS NP may identify these CD8⁺ T cells phenotypically as Tc1, which correlated with relatively high levels of *Eomes*^{61,336} detected through RNAseq analysis. While it may be inferred that CD8⁺ T cells are likely playing a crucial role in the therapeutic anti-tumour efficacy of vaccination with OVA and 50nm PS NP, this will have to be confirmed using a cell depletion experiment in the therapeutic vaccination model.

While CD8⁺ T cell depletion was appropriately validated in the experimental mice via blood sampling and FACs analysis, it is important to consider the off-target effects of this depletion protocol. The antibody used for depletion targets CD8 α , and this cell surface molecule is expressed on multiple cell types apart from CD8⁺ T cells including some pDCs, DCs, and NK cells. It is of note however that the magnitude of expression is crucial to depletion, and only cell types

expressing high levels of CD8 α will be depleted³²⁹. While this means that NKs are not likely to be depleted, pDCs and CD8 α ⁺ DCs are likely depleted³²⁹. This is an important consideration, as both cell types can contribute to anti-tumour immunity through presentation of antigens, production of cytokines/chemokines and promotion of cancer cell killing³³⁷. However, CD8 α ⁺ DCs are functionally adapted to the promotion of CD8⁺ T cell responses, and therefore depletion of these DCs will in part impair CD8⁺ T cell responses. Therefore, this off-target effect should not impact our ability to draw conclusions on the importance of CD8⁺ T cells in vaccine induced anti-tumour immunity.

While some protection was observed despite CD8⁺ T cell depletion, it is possibly due to intact antigen-specific IFN γ responses, which our lab has previously shown to be mediated by CD4⁺ T cells downstream of vaccination with antigen and 50nm PS NP²⁹⁰. IFN γ has been shown to have anti-tumour effects not only through promoting anti-tumour immunity via immune cell signalling but also through direct activity on tumour cells, promoting immunogenicity and sensitivity to death stimuli^{338–346}. In addition, CD4⁺ T cells can acquire a cytotoxic phenotype and can kill via tumour antigen sensing through MHC-II recognition^{81–83}, and this phenotype has been observed when CD8⁺ T cells are depleted experimentally. To address the contribution of CD4⁺ T cells to this anti-tumour immunity, a cell depletion experiment like that performed for CD8⁺ T cells would be required, however interpretations following this technique may be somewhat confounded by the resultant depletion of both anti-tumour CD4⁺ effector and pro-tumour regulatory T cells^{347,348}. However, the results shown clearly highlight the importance of CD8⁺ T cells in 50nm PS NP-mediated anti-tumour immunity. Apart from T cell responses, it is possible that antigen-specific antibodies may be playing a role in the vaccine induced protection. It was shown that 50nm NP adjuvanted vaccines drive potent antigen-specific IgG responses, including the IgG1, IgG2b and IgG2c subclasses (Figure 3.7.5). These antibodies have been implicated in anti-tumour immunity via ADCC, ADCP and complement dependant cellular cytotoxicity (CDCC) and could exert anti-tumourigenic effects in the TME⁸⁵.

As mentioned previously, B16 cell lines are poorly immunogenic, partly due to the presence of low mutational burden and downregulation of components of the MHC-I pathway^{334,335}, resulting in a tumour cell line particularly insensitive to CD8⁺ T cell mediated killing. This makes these observations more impressive and even more robust anti-tumour immunity may be expected in other murine tumour cell line models with enhanced CD8⁺ sensitivity, such as MC38 or CT26 models. To investigate this, a prophylactic vaccination experiment utilising MC38 derived peptides and subsequent MC38 challenge was carried out. However, this study yielded underwhelming results compared to the observations in the B16-OVA model. Vaccination with

the peptide cocktails alone promoted some tumour growth inhibition, in line with data presented by *Hos et al*³²⁸ despite different vaccination routes used in this study, and this was only enhanced through formulation with 50nm PS NPs for the 10µg peptide cocktail dose. These underwhelming results may be explained through the lack of inclusion of distinct CD4⁺ epitopes in the MC38 derived peptides utilised³²⁸. CD4⁺ T cells can not only have direct anti-tumour effects but are required for the optimum priming, expansion and activation of antigen specific CD8⁺ T cells⁵⁷ in a tumour setting (Fig.1.2). In addition, all previous vaccination studies using 50nm NPs adjuvants had utilised whole protein antigens, and as a result the substitution of peptide antigens for use could impact adjuvanticity. Peptides, due to their lower size and molecular weight may have a reduced capacity to associate with the 50nm PS NP. Effective formulation of 50nm NP with peptide antigens may require surface conjugation to ensure interaction rather than reliance on surface adsorption, or the use of a carrier protein to facilitate CD4⁺ T cell help may be warranted. This will have to be examined in more detail moving forward if 50nm NP are to be used in cancer vaccination strategies utilising peptides as antigens, which has been an area of focus in completed and ongoing clinical trials^{84,349}. This differential adjuvanticity when using protein vs peptide antigens could be investigated in more detail utilising our standard vaccination experimental set up replacing OVA protein for SIINFEKL or a longer peptide containing the SIINFEKL sequence as an antigen.

Importantly, the 50nm PS NP were successfully benchmarked against a number of leading adjuvants and the ChAdOx1-OVA vector that were described in Chapter Three. 50nm NPs were shown to outperform both CpG and cGAMP in inducing therapeutic anti-tumour immunity in the B16-OVA model, however, it is worth noting that the number of animals used in the CpG and cGAMP groups were small and as a result additional repeats are required to draw significant conclusions on adjuvant superiority. Nonetheless, these observations are promising as CpG (as SD-101)^{153,154} has been extensively trialled in both preclinical and clinical studies in cancer vaccination approaches, while cGAMP has been investigated pre-clinically in adjuvanted cancer vaccines^{350,351}. Likewise, the ChAdOx1 vaccine platform has been trialled both preclinically and clinically in cancer vaccine approaches^{317,352}, and is being further trialled by Vaccitech™ in heterologous prime boost strategies with a modified Vaccinia Ankara vector (MVA) in prostate cancer and HPV associated cancers (NCT04908111, NCT05617040 & NCT04607850). While mice immunised with ChAdOx1-OVA had a higher degree of tumour growth inhibition and enhanced survival compared to mice vaccinated with 50nm PS NP + OVA, there was no statistically significant difference between the survival or growth curves of these groups (p=0.0992). Unfortunately, in this experiment there was a lesser therapeutic benefit observed in mice

vaccinated with antigen and 50nm NP when compared to previous therapeutic experiments. This may have been due to technical issues surrounding the centrifuge used for washing the 50nm NP prior to experimentation, which can affect the yield and subsequent concentration of 50nm NP used for study. This has been previously documented in our lab (Oral Communication, Dr Natalia Muñoz-Wolf). However, despite this challenge, these results again highlight the potential of 50nm PS NPs as cancer adjuvants and provide a key benchmark against a clinically relevant cancer vaccine platform, and as previously discussed in chapter three further optimisations are envisaged to be made to improve 50nm NP induced immune responses. Going forward it will be essential to validate therapeutic efficacy in different, phenotypically distinct tumour models. Likewise, experiments using more 'canonical' tumour antigens will be essential to confirm the current observations in the B16-OVA model, including the use of vaccination strategies targeting neoantigens exclusively expressed in these tumour models.

Interrogation of the phenotype of the antigen specific CD8⁺ T cells induced by vaccination with 50nm NP + OVA and comparison to those induced following ChAdOx1-OVA vaccination via bulk RNA sequencing analysis, provides insights into their anti-tumour functionality as a unique 'stem-cell' like phenotype was uncovered. Vaccination with 50nm PS NP + OVA induced antigen specific CD8⁺ T cells that had elevated expression of *Tcf7* (encoding TCF1), *Sell* (encoding CD62L), *Lef1*, *Ccr7* and *Cxcr5* which have all been documented as markers of stem cell like CD8⁺ T cells^{74,76,353}. These T cells also had upregulation of the co-stimulatory molecules, *Tnfrsf4* and *Cd27* and upregulation of the co-inhibitory molecule *Pdcd1* (PD-1), indicating the elevated activatory status of these cells. These insights are interesting in a tumour context as 'stemness' has emerged as a critical parameter correlating with favourable responses to immunotherapeutic approaches. PD-1⁺ 'stem-like' CD8⁺ T cells have been documented to expand in response to α-PD1 therapy and correlate with improved response rates^{74,84,332}. Similarly the efficacy of adoptive cell transfer therapies has been demonstrated to be dependent on these 'stem-like' CD8⁺ T cells⁷⁶. Crucially in line with these data, therapeutic vaccination with 50nm PS NP + OVA was observed to synergise with systemic PD-1 treatment. This is particularly promising as B16 tumour models have been documented to have a low response to checkpoint blockade strategies^{104,330}, indicating a potential for sensitising immunologically cold tumours to immunotherapeutic approaches. It is possible that more impressive synergistic results could be observed in a more immunogenic and immunoresponsive tumour like MC38. It will be crucial to determine the phenotype of intratumoral antigen specific CD8⁺ T cells following therapeutic vaccination with 50nm NPs, with a focus on the expression of the stem markers mentioned above.

Furthermore, it remains to be determined whether 50nm NP induced immune responses could synergise with other immunotherapeutic approaches such as those targeting alternative checkpoint molecules or even adoptive cell/CAR therapies. This may help to address shortcomings in CAR T cell therapy in solid tumours^{84,354}, and strategies have been utilised to 'decorate' the surfaces of APCs with CAR T cell ligands to encourage optimal CAR T cell expansion and activation in preclinical mouse tumour models³⁵⁵. In line with this, Pfizer recently reported synergistic effects clinically when combining mRNA vaccination aiming to promote claudin-6 expression in DCs and claudin-6 targeting CAR T cells in patients with testicular/ovarian tumours³⁵⁶.

Therapeutically, our standard prime boost regimen had to be shortened from 14 days to 7 days due to the aggressive growth of the B16 tumour, it has noted in previous experiments that the induction of antigen specific CD8⁺ T cells and IFN γ responses is blunted by this shortened window between vaccinations (data not shown). Clinically, due to the slower kinetics of relative tumour growth in patients, a longer window between vaccinations could be utilised which may enhance immunity, this elevated immunogenicity/efficacy with longer interval between vaccinations was observed in several vaccines developed over during the course of the COVID pandemic including BNT162b2 and mRNA-1273³⁵⁷, and ChAdOx1-nCoV19³⁵⁸. This will be an important consideration as cancer vaccination approaches are often trialled in patients with a high degree of disease burden, so an optimum vaccination schedule will be fundamental to efficacy.

There are several additional areas of potential application of these adjuvants in a therapeutic cancer setting, these include vaccination post tumour regression or resection to reduce rates of recurrence or use in neoadjuvant therapeutic strategies. Neoadjuvant strategies using ICB have had impressive results clinically¹¹², improving the pathologic response rates in patients associated with enhanced expansion and activation of anti-tumour T cell responses. This likely hinges on the reinvigoration of pre-existing anti-tumour immune response in the tumour prior to surgical resection, which may be disrupted following surgery. The use of cancer vaccines alone or in combination with ICB in neoadjuvant settings is an interesting area of focus in the field moving forward.

In summary, 50nm PS NPs have been characterised as potent cancer vaccine adjuvants, which can be used in both prophylactic and therapeutic cancer vaccination approaches. This anti-tumour immunity was demonstrated to be largely dependent on the induction of CD8⁺ T lymphocytes with a high degree of stemness. Crucially, a synergistic effect between therapeutic vaccination with 50nm NPs and systemic α -PD1 therapy was demonstrated.

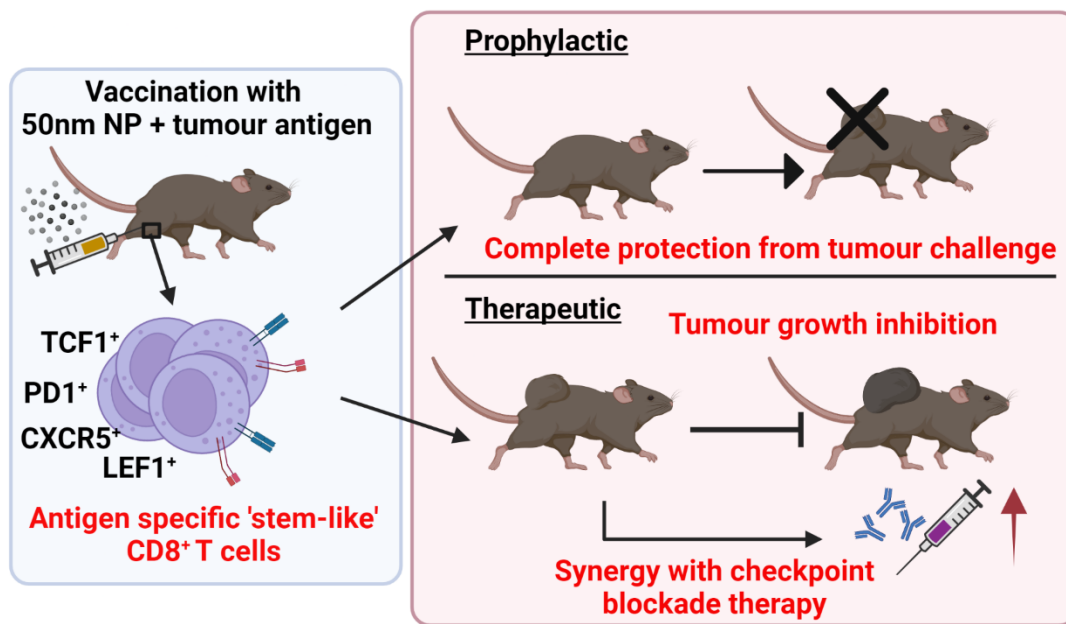


Figure 4.9.1 – Chapter 4 summary – Vaccination with 50nm NPs and antigen leads to induction of antigen specific 'stem-like' CD8⁺ T cells and robust anti-tumour immunity.

**Chapter Five - Investigating the anti-tumour
functionality of highly deacetylated chitin-derived
polymers**

5.1 Introduction

The Lavelle Lab has previously characterised Protasan CL213 as an effective vaccine adjuvant when delivered via the intraperitoneal and intranasal routes, eliciting antigen-specific IFN γ and antibody responses (in particular IgG2c)²¹⁸. Furthermore, C100 and C90, chitin-derived polymers with degree of deacetylation (DD) of 100% and 90% respectively, have yielded promising results both *in vitro* and *in vivo* via the s.c route^{219,222}, both proving to possess more potent adjuvant properties than Protasan CL213^{219,224}. However, the i.p and s.c route is not commonly used clinically for immunisations, and while mucosal vaccination routes are an important consideration for future vaccination strategies³⁵⁹, it is important to validate such findings via the intramuscular immunisation route, as it constitutes the most commonly used and effective route for parenteral vaccination in humans³⁶⁰. Likewise, the functionality of these Protasan CL213, C100, and C90- mediated immune responses, remained to be validated. As such C100, C90 and Protasan CL213 were evaluated for their capacity to induce prophylactic anti-tumour immune responses using the B16-F10 OVA model discussed previously.

Aside from prophylactic approaches, cancer vaccine adjuvants also have potential applications in therapeutic settings, with intratumoral adjuvant delivery one such strategy. These 'off-the-shelf' *in situ* vaccinations are advantageous as instead of a reliance on co-administered antigen, they rely on the release of antigen locally following injection³⁶¹, which can be effectively achieved through induction of cell death^{227,234,362} and promotion of immune cell recruitment and activation⁸⁴. Intratumoral therapies may change the immune landscape of tumours, increasing the immunogenicity of the tumour itself, promoting an increased immune infiltrate and priming for combinatorial treatment approaches⁶⁶. Additionally, intratumoral strategies may reduce adverse side effects which can plague therapeutic strategies administered systemically.

Extensive research conducted in the Lavelle Lab has provided excellent rationale for the use of chitin-derived adjuvants intratumorally, characterising their potent activation of STING signalling, which has proved essential for their adjuvanticity²¹⁸. This activation results in the secretion of type I IFNs, CXCL10, the NLRP3 mediated release of IL-1 β , and the maturation of APCs as measured by upregulation of CD40, and CD80/86²¹⁸⁻²²⁰. Both STING signalling and type I IFN production have been shown to be crucial for the spontaneous priming of cellular anti-tumour immunity mediated by the innate immune system^{275,276,278} and endothelial cells²⁷⁴. As mentioned in Section 1.4.1 this has led to the identification of STING as a potential target for cancer therapies, and as a result there has been a sharp increase in STING activating molecules being

trialled as cancer therapeutics. However these therapeutics have yielded underwhelming results clinically with issues such as poor mouse-human translatability, poor bioavailability and stability²⁸⁴ and in some cases potentially the downregulation of STING pathway signalling components by tumour cells.

This project aims to characterise highly deacetylated chitin derived polymers as alternative cGAS-STING targeting therapeutic adjuvants, which possess distinct advantages over conventional STING agonists. To determine the applicability of C100, C90 and Protasan CL213 as adjuvants for therapeutic vaccines, B16F10 and MC38 tumour *in vivo* models were utilised incorporating intratumoral delivery of therapeutic adjuvants.

5.2 Hypothesis

The hypothesis underling the research in this chapter was that highly deacetylated chitin-derived adjuvants may have application for incorporation into prophylactic and therapeutic intratumoral cancer vaccine strategies .

5.3 Aims

- To investigate the ability of highly deacetylated chitin-derived adjuvants to promote prophylactic anti-tumour immunity via parenteral vaccination.
- To investigate the therapeutic efficacy of these highly deacetylated chitin-derived adjuvants when delivered intratumorally
- To determine the signalling components and cellular mediators underlying this anti-tumour immunity and investigate the efficacy of therapeutic combination approaches with checkpoint blockade

5.4 C100 and C90 can promote protective prophylactic anti-tumour immune responses

To investigate the capacity of C100, C90 and Protasan CL213 to drive cellular immune responses when injected intramuscularly, and furthermore to interrogate anti-tumour functionality, a prime boost (day 0,14) vaccination model was used with a B16-F10 OVA tumour challenge on day 28 as previously described.

C57BL6/J mice immunised intramuscularly with C100 (200µg) + OVA and C90 (200µg) + OVA exhibited significant protection from tumour challenge when compared to naïve challenged mice. This is displayed in terms of mean tumour volume (Fig.5.4.1A) and individual tumour growth plots (Fig.5.4.2B). Survival of these tumour-challenged mice was also analysed using Kaplan-Meier survival curve analysis (Fig.5.4.2). 66% of C90 + OVA and 50% of C100 + OVA immunised mice exhibited complete protection from tumour challenge compared to just 16% of mice immunised with OVA alone. There was no observed enhancement in survival when Protasan CL213 (200µg) was used as an adjuvant compared to OVA immunisation alone (Fig.5.4.2), only a delay in tumour growth/ establishment was observed (Fig.5.4.1). These results characterise C100 and C90 as effective adjuvants for use via the intramuscular vaccination route, eliciting a protective anti-tumour immune response and outperforming Protasan CL213.

While similar efficacy was observed here between C100 and C90, C100 has been determined to be a more promising vaccine adjuvant from previous *in vitro* and *in vivo* experiments in the Lavelle Lab conducted by Dr Joanna Turley and Dr Hannah Moran^{219,222}. As a result, C100 was focused on to dissect its induction of immune responses to better understand the anti-tumour immunity elicited. C57BL6/J mice were immunised using the standard intramuscular prime boost regimen, with the spleens and muscle isolated on day 21 post priming vaccination. Mice immunised with 50nm PS NP + OVA were included as positive control for induction of CD8⁺ T cells and validation of MHC-I SIINFEKL tetramer staining.

There was no induction of antigen-specific CD8⁺ T cells observed in mice immunised with C100 + OVA in the spleens (Fig.5.4.3A+C) or locally in the muscle (Fig.5.4.B+C). Crucially, high concentrations of antigen-specific IFN γ were observed in the supernatants of splenocytes from C100 + OVA immunised mice, upon 72-hour antigen restimulation (Fig.5.4.4). Serum antigen-specific antibody titres were determined using ELISA, and C100 + OVA immunised mice display robust induction of antigen-specific IgG (Fig.5.4.5A), including the IgG2b and IgG2c subclasses (Fig.5.4.5C+D).

These results indicate that the prophylactic anti-tumour immunity observed in C100 + OVA immunised mice is unlikely to be mediated by antigen-specific CD8⁺ T cells. This immunity may be reliant on the induction of antigen-specific IFN γ ⁺ Th1 cells and possibly antigen-specific IgG responses. Future work should aim to validate these results utilising larger group sizes.

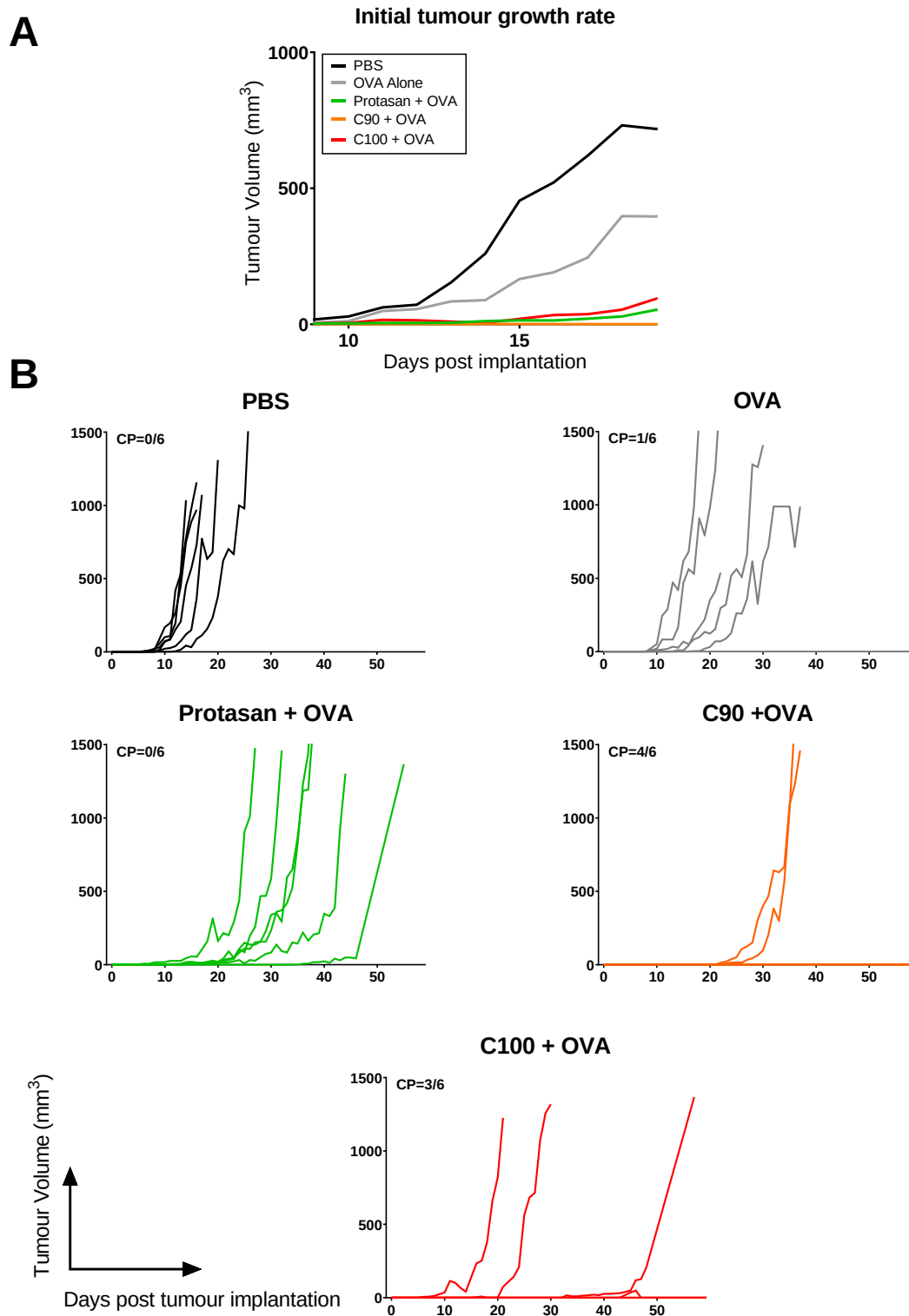


Fig. 5.4.1 Highly deacetylated chitin-derived adjuvants delay tumour growth in vaccinated mice

C57BL6/J mice were vaccinated on days 0 and 7 with PBS (n=6), OVA (10µg) alone (n=6), C100 (200µg) plus OVA (10µg) (n=6), C90 (200µg) plus OVA (10µg) (n=6) or Protasan CL213 (200µg) plus OVA (10µg) (n=6), followed by s.c challenge with 3.5×10^5 B16-OVA tumour cells. (A) Mean tumour volume (B) Spider plots illustrating individual tumour growth. CP denotes complete protection from tumour establishment.

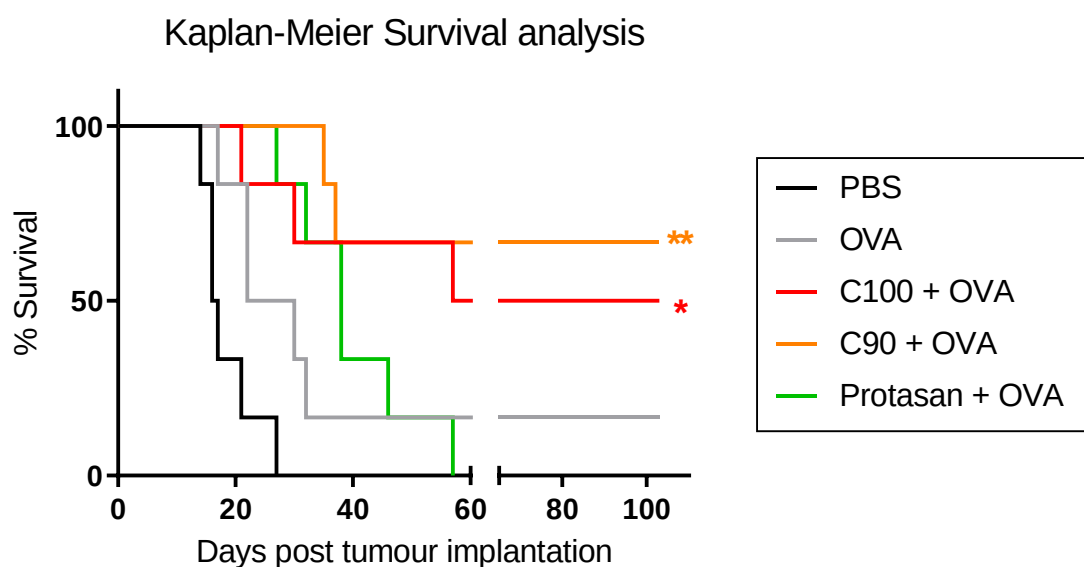


Fig. 5.4.2 Highly deacetylated chitin-derived adjuvants enhance survival from tumour challenge in vaccinated mice

C57BL6/J mice were vaccinated on days 0 and 7 with PBS (n=6), OVA (10µg) alone (n=6), C100 (200µg) plus OVA (10µg) (n=6), C90 (200µg) plus OVA (10µg) (n=6) or Protasan CL213 (200µg) plus OVA (10µg) (n=6), followed by s.c challenge with 3.5×10^5 B16-OVA tumour cells. A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

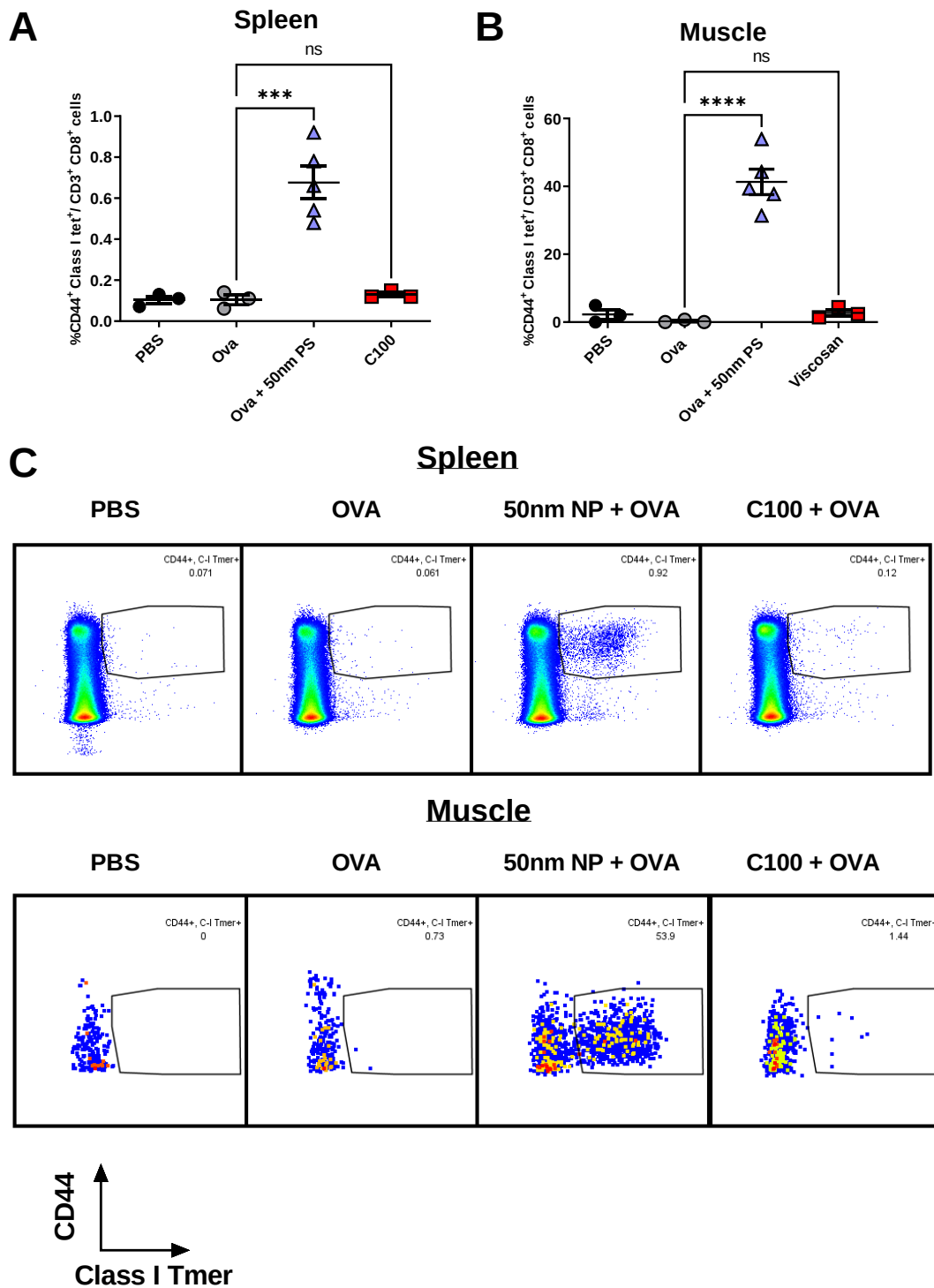


Fig. 5.4.3 C100 does not promote antigen-specific CD8⁺ T cells.

C57BL6/J mice were vaccinated on days 0 and 7 with PBS (n=3), OVA (10 μ g) alone (n=3), 50nm PS NPs (1mg) plus OVA (10 μ g) (n=5), or C100 (200 μ g) plus OVA (10 μ g) (n=3) intramuscularly. On day 21, antigen-specific responses were quantified in the spleen using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS, OVA alone and 50nm PS + OVA groups were included as vehicle control, antigen alone and positive control groups respectively. (A) Percentage of OVA-specific CD44⁺ CD8⁺ over total CD8⁺ T cells; (B) Percentage of

OVA-specific CD44⁺ CD8⁺ over total CD8⁺ T cells in the muscle. . Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001 (C) Representative FACS plots gated on CD44⁺ Class I SIINFEKL Tetramer⁺ CD8⁺ T cells.

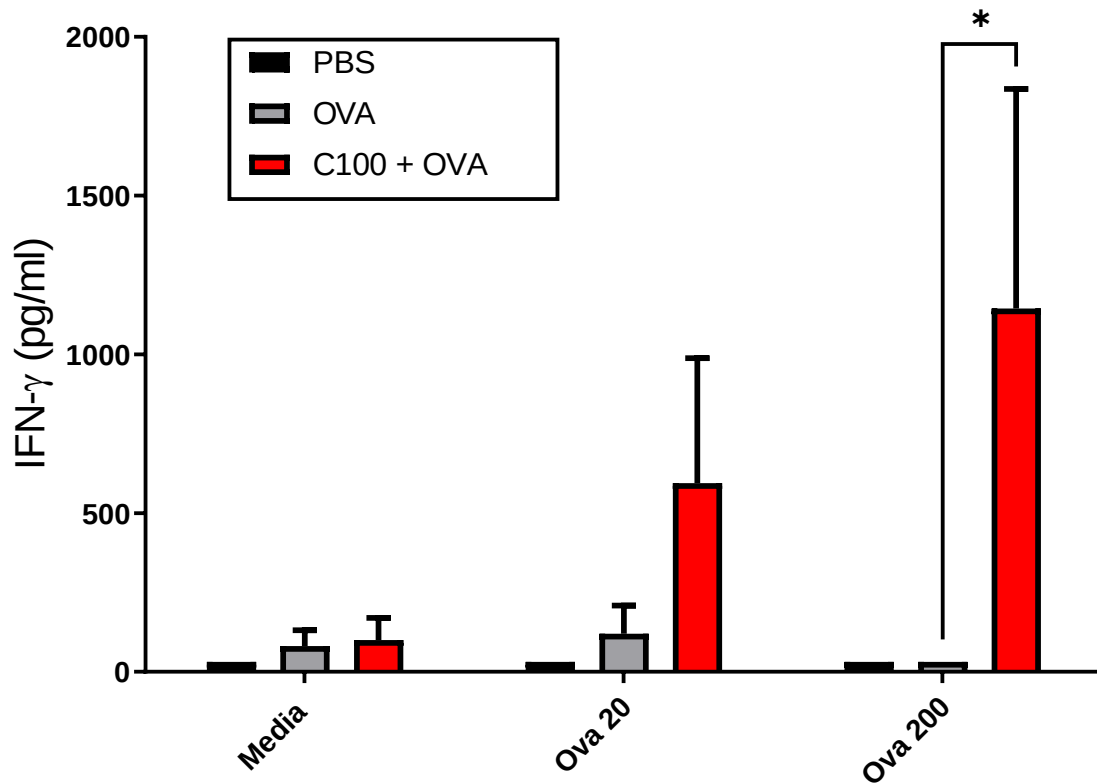


Fig 5.4.4 C100 promotes a robust antigen-specific IFN γ response.

C57BL6/J mice were vaccinated on days 0 and 7 with PBS (n=3), OVA (10 μ g) alone (n=3), or C100 (200 μ g) plus OVA (10 μ g) (n=3) intramuscularly. On day 21, mice were sacrificed, spleens were isolated, cells were plated and re-stimulated *ex vivo* with media alone, or OVA protein (20 μ g/mL or 200 μ g/mL) for 72 hours. Supernatants were collected and cytokine concentrations were determined by ELISA. Data are presented as the mean $\pm$ SEM. Statistical differences as per two-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

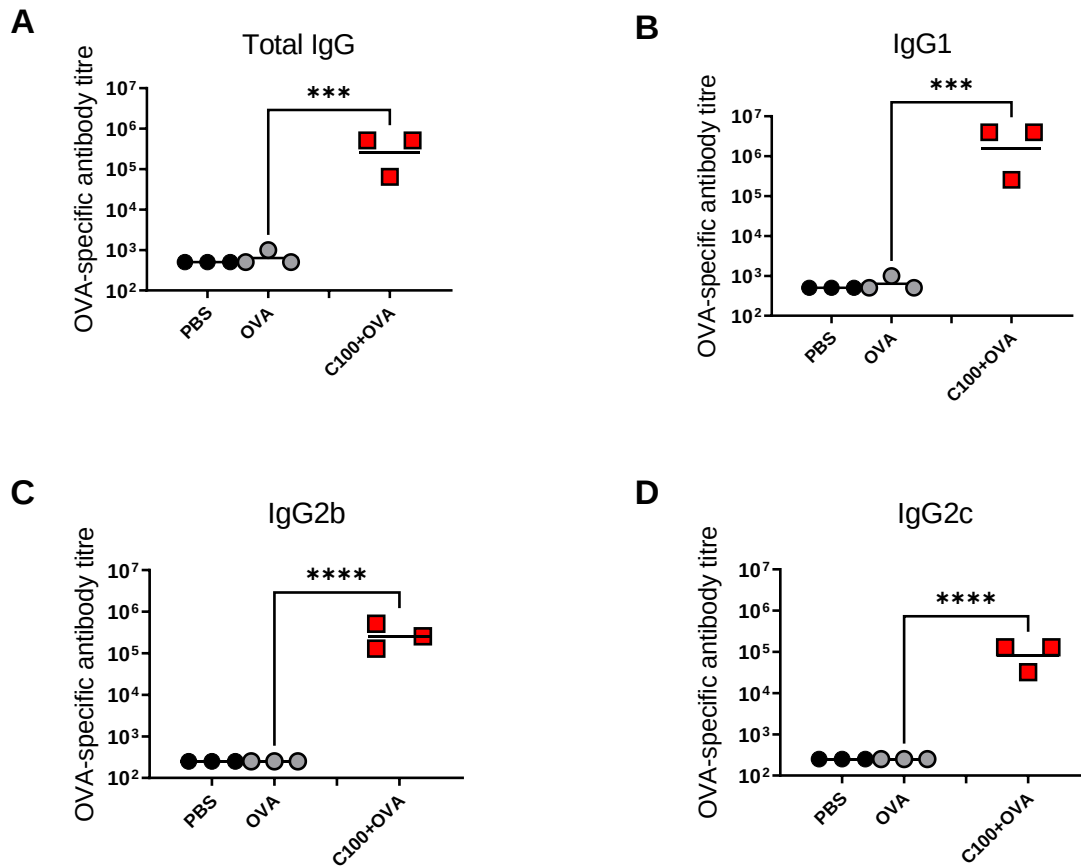


Fig. 5.4.5 C100 promotes induction of antigen-specific IgG, including IgG2b and IgG2c subclasses.

C57BL6/J mice were vaccinated on days 0 and 7 with PBS (n=3), OVA (10 μ g) alone (n=3), or C100 (200 μ g) plus OVA (10 μ g) (n=3) intramuscularly. On day 21, mice were sacrificed, and antigen-specific A) Total IgG, B) IgG1, C) IgG2b and D) IgG2c titres were determined via ELISA. Statistical differences as per one-way ANOVA and Dunnett's post-hoc test. Asterisks indicate significant differences. Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

5.5 Intratumoral delivery of C100 stunts tumour growth and prolongs survival in both an B16F10 and MC38 therapeutic tumour challenge model

Previous work conducted in the Lavelle Lab has characterised the immunomodulatory effects that chitin-derived adjuvants exert on APCs, promoting maturation, cytokine/ chemokine secretion and in some cases cytotoxicity^{218,219,224}. These immunomodulatory and cytotoxic effects led to an interest in the effect that chitin-derived adjuvants may exert on tumour cells. Traditionally agents that elicit tumour cell death have been trialled as chemotherapeutics, and more recently with the advent of immunotherapy, increasing the immunogenicity of a tumour is an attractive concept⁶⁶.

As such, the direct effects of Protasan CL213, C90 and C100 on B16F10 cells *in vitro* was examined. All three chitin-derived polymers were found to exert a cytotoxic effect when compared to unstimulated cells as measured through live/ dead staining and FACs analysis (Fig.5.5.1) C100 was observed to elicit the most cell death, in a dose dependant manner. CpG, which was included as control, failed to elicit any cell death (Fig.5.5.1A). Melanoma and adenocarcinoma cell lines can render the cGAS-STING pathway defective by blocking STING trafficking or by hypomethylating STING promoter regions and decreasing STING expression²⁶⁸. As such, Dr Joanna Turley previously assessed the expression and functionality of STING in the B16F10 and MC38 cell lines by monitoring levels by Immunoblot (Fig.5.5.1B). Both cell lines expressed STING. Signifying functionality, DMXAA treatment drove complete STING degradation, with very faint phosphorylation bands appearing for both cell lines.

Findings that C100, C90 and Protasan CL213 exert cytotoxic and immunomodulatory effects on B16F10 cells *in vitro*, and their known activation of STING and IFN signalling in APCs provided a rationale to investigate their potential as therapeutic intratumoral adjuvants.

A therapeutic B16F10 model was designed and optimised, based somewhat on the methodology employed by Sagiv-Barfi et al¹⁵⁰. Mice received two intratumoral injections, the first when tumour volume reached 40-60mm³ (as measured by $(\text{Length} \times \text{Width}^2)/2$), and the second 4 days later as illustrated in Fig.5.5.2A. CpG was included as a control as it has shown promise as an intratumoral therapeutic^{363,364}, eliciting systemic anti-tumour immune responses when combined with a monoclonal antibody targeting OX-40¹⁵⁰. SD-101, a CpG compound, has recently had success in clinical trials in patients with lymphoma when combined with radiation therapy³⁶⁵ and is to be trialled alongside nivolumab and radiation therapy in metastatic pancreatic adenocarcinoma patients with liver metastases (NCT04050085)³⁶⁶.

Only CpG significantly impacted on initial tumour growth rate following intratumoral delivery (Fig.5.5.2). Of the chitosan polymers tested C100 exerted the most inhibition on initial tumour growth when compared to the initial tumour growth of Protasan CL213 and C90 treated mice. In some mice there was an observed initial increase in tumour size following intratumoral injection with the chitin-derived polymers. This is likely due to an initial inflammatory response following injection and was characterised by red swelling around the tumour area which resolves 2-3 days later. It is particularly evident in the initial growth of mice injected with Protasan CL213 (Fig.5.5.2A).

Only mice intratumorally injected with CpG or C100 had significantly prolonged survival compared to the PBS treated group (Fig.5.5.3) and there was no significant difference between the CpG and C100 survival curves. C90 and Protasan CL213 exerted a modest, non-significant impact on survival when compared to the PBS treated group (Fig.5.5.3). This study crucially identified C100 as the most suitable chitin-derived polymer for use in intratumoral vaccination strategies, outperforming C90 and Protasan CL213 in terms of delaying tumour growth and prolonging survival, with efficacy comparable to the positive control CpG.

Moving forward it was essential to determine the optimum dose for intratumoural delivery of C100. This is an important consideration as the magnitude of STING activation mediated by cGAS-STING targeted therapeutics has been shown to dictate subsequent anti-tumour responses and overall clinical benefit³⁶⁷. As such, another therapeutic B16F10 tumour challenge study was designed as before, this time with doses of 200µg and 300µg of C100 included alongside the 100µg dose used previously. While all three doses delayed tumour growth to a certain extent as displayed as mean tumour growth rate (Fig.3.5.4A) and individual growth spider plots (Fig.3.5.4B), the most profound growth inhibition was observed with the 100µg dose of C100. This is reflected in the survival analysis where only the 100µg dose was shown to provide a statistically significant prolonging of survival as determined via a Mantel-Cox test of the survival curves (Fig.3.5.4C). The median survival times post initial treatment day were as follows: PBS = 12.5 days, C100 (100µg) = 17 days, C100 (200µg) = 14 days and C100 (300µg) = 14 days.

The therapeutic efficacy of C100 in the B16F10 model was a promising result as B16 is a difficult tumour to treat with an aggressive growth rate and poor immunogenicity compared to other murine tumour models^{334,335,368,369}. It was next sought to confirm therapeutic efficacy in another tumour cell line, this time utilising the MC38 adenocarcinoma cell line. This cell line has higher

immunogenicity and slower growth rate compared to B16F10, and therefore responds better to therapeutic strategies aiming to harness anti-tumour immune responses.

A similar therapeutic tumour model and treatment regimen was established to that of the B16F10 model described earlier using MC38, as displayed in Fig.5.5.5A. This time, a 25µg dose of C100 was included alongside the standard 100µg to additionally determine if a dose lower than 100ug could be more effective. As expected, the therapeutic benefit mediated by C100 and the control CpG was more impressive in the MC38 model when compared to the B16F10 model, likely attributed to an increased tumour immunogenicity. This can be observed in the inhibition of tumour growth rate as displayed in mean initial tumour growth (Fig.5.5.5B), individual tumour growth (Fig.5.5.C) and in the survival analysis (Fig.5.5.6). In addition, the 100µg dose of C100 outperformed the lower 25µg with a 2 day increase in median survival time in the 100µg treated mice compared to those receiving the 25µg dose.

Overall, these data characterise C100 as the most effective highly deacylated chitin-derived polymer for use as an intratumoral therapeutic and provide a key benchmarked of C100 therapeutic efficacy against a clinically relevant intratumoral therapy. 100µg was also identified as the optimum dose for intratumoural use of C100, with higher or lower doses yielding lower therapeutic benefit.

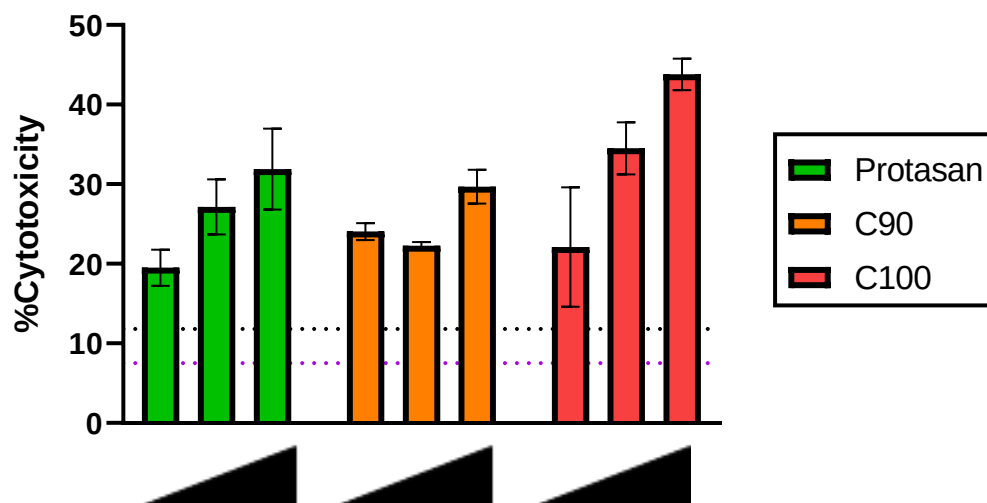
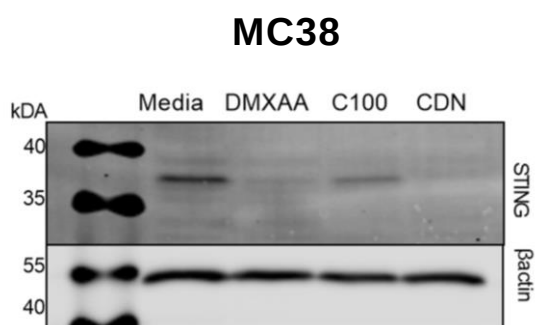
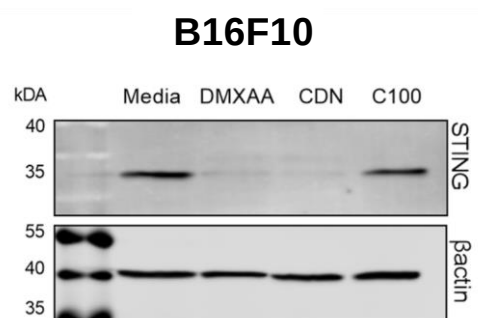
A**B****C**

Fig.5.5.1 C100, C90 and Protasan CL213 exert cytotoxic effects on B16F10 cells *in vitro* and the MC38 and B16F10 cell lines used to study retained functional STING signalling

(A) 2×10^5 B16 melanoma cells were stimulated with media, 5 $\mu\text{g}/\text{ml}$ CpG or 10, 20 and 40 $\mu\text{g}/\text{ml}$ of C90, C100 or Protasan CL213 for 24 hours in 24 well plates. Cells were then removed from wells and cell death was analysed via live dead staining and FACS. Data are displayed in duplicates $\pm$ SD, with the dotted black y-intercept indicating mean cytotoxicity observed in media alone. X-axis triangles indicate an increase in concentration from left to right. (B) 1×10^6 B16F10 or MC38 cells were treated with media, 5 $\mu\text{g}/\text{ml}$ of Protasan CL213, C90 or C100, 10 $\mu\text{g}/\text{ml}$ of DMXAA or 10 μM of 2'3'cGAMP for 6 hours. Cells were lysed and STING protein levels were determined by Immunoblot with β -actin used as a loading control. *Blots performed by Dr Joanna Turley

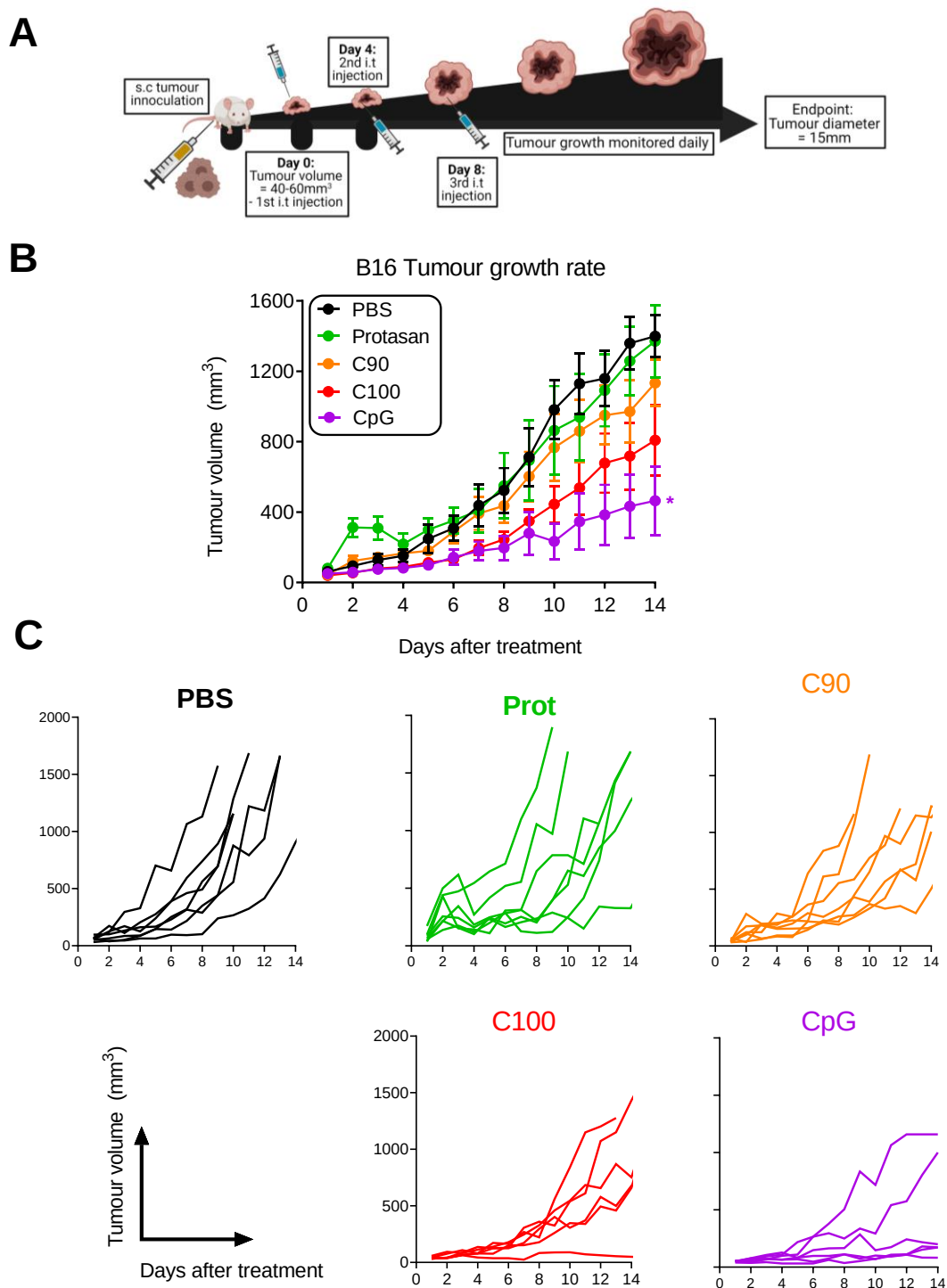


Fig.5.5.2 Intratumoral treatment with C100 and the CpG control stunt initial tumour growth rate in a therapeutic B16F10 model

(A) C57BL/6 mice were injected s.c. with 3.5×10^5 B16 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS, C100 (100µg), C90 (100µg), Protasan CL213 (100µg) or CpG (50µg) in a volume of 30µl with injections repeated twice more

4 days apart. Tumour growth was monitored daily until a clinical humane endpoint of $\geq 15\text{mm}$ in diameter was reached. Check this (B) Mean initial tumour growth rate data. C) Spider plots displaying individual tumour growth rates. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of differences between the tumour growth curves of treated mice and the PBS control group. Asterisks indicate significant differences. Not significant – ns (not displayed on graph), P value > 0.05 , *P < 0.05 , **P < 0.01 , ***P 0.001 and ****P < 0.0001 .

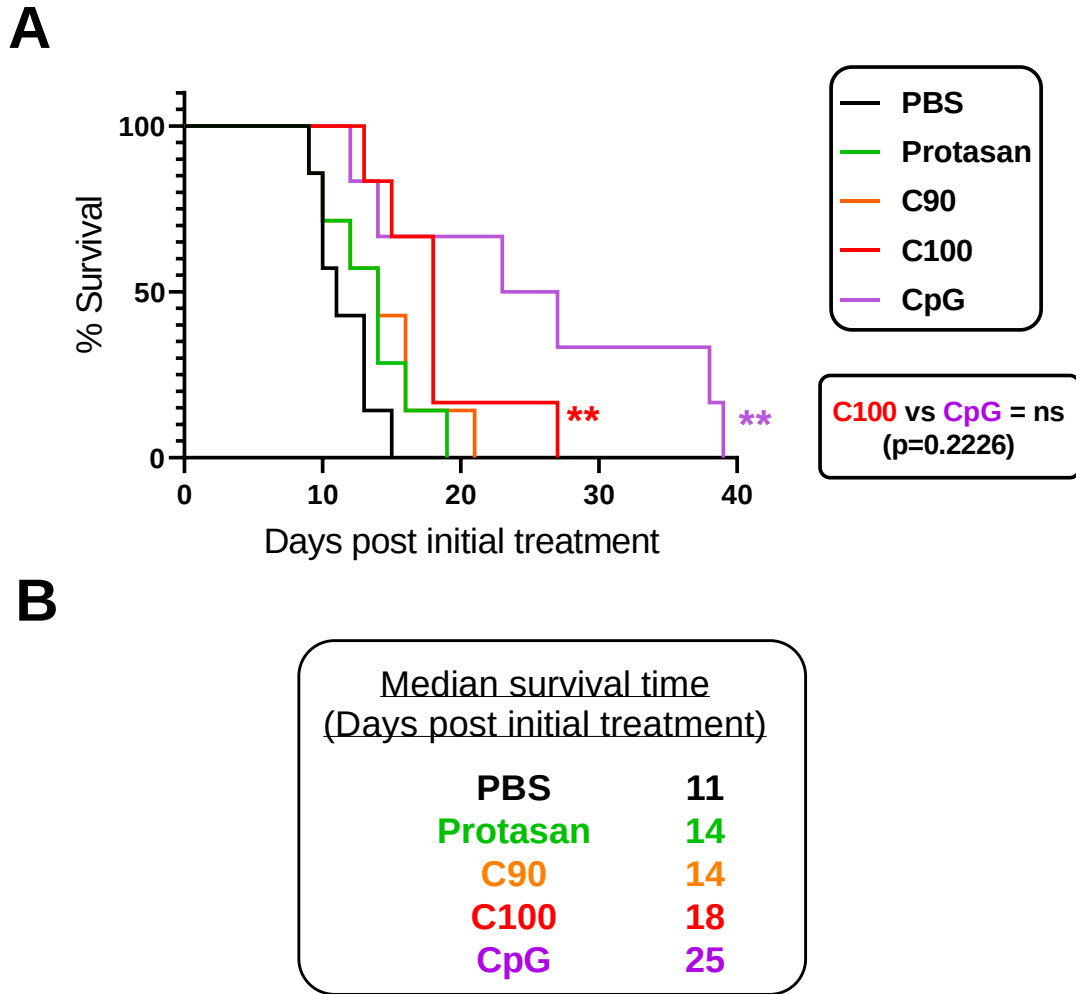
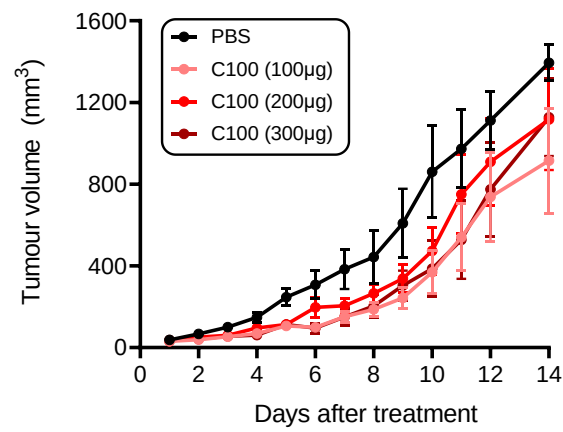


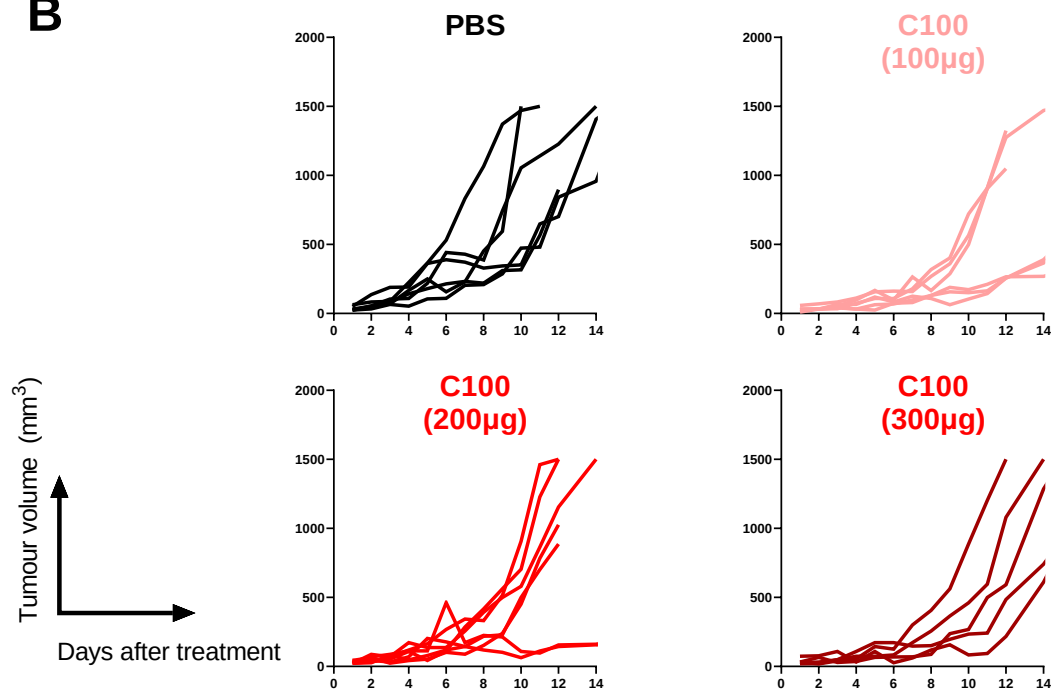
Fig.5.5.3 Intratumoral treatment with C100 or CpG prolongs survival in a therapeutic B16F10 model

Survival analysis data for experimental mice detailed in Fig.5.5.2. (A) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Table displaying median survival times of treated mice post initial treatment.

A



B



C

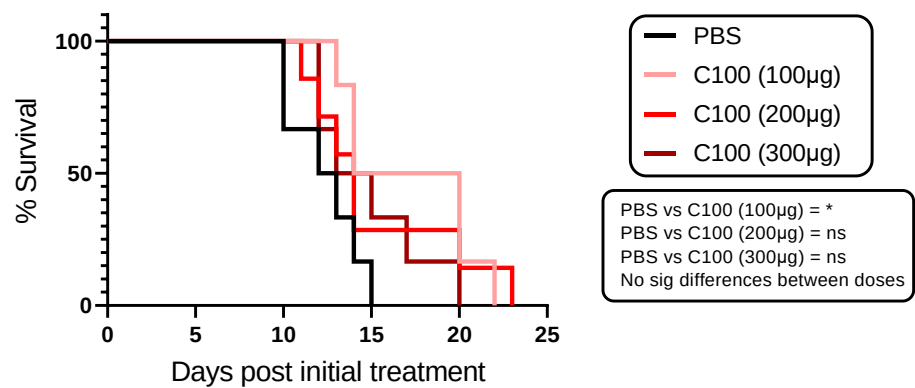


Fig.5.5.4 Doses higher than 100µg of C100 intratumorally do not improve therapeutic efficacy in a therapeutic B16F10 model

C57BL6/J mice were injected s.c with 3.5×10^5 B16 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS, C100 (100µg), C100 (200µg), or C100 (300µg) in a volume of 30µl with injections repeated twice more, 4 days apart. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (A) Initial tumour growth rate. (B) Spider plots displaying individual tumour growth rates. . (C) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < .05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Table displaying median survival times of treated mice post initial treatment.

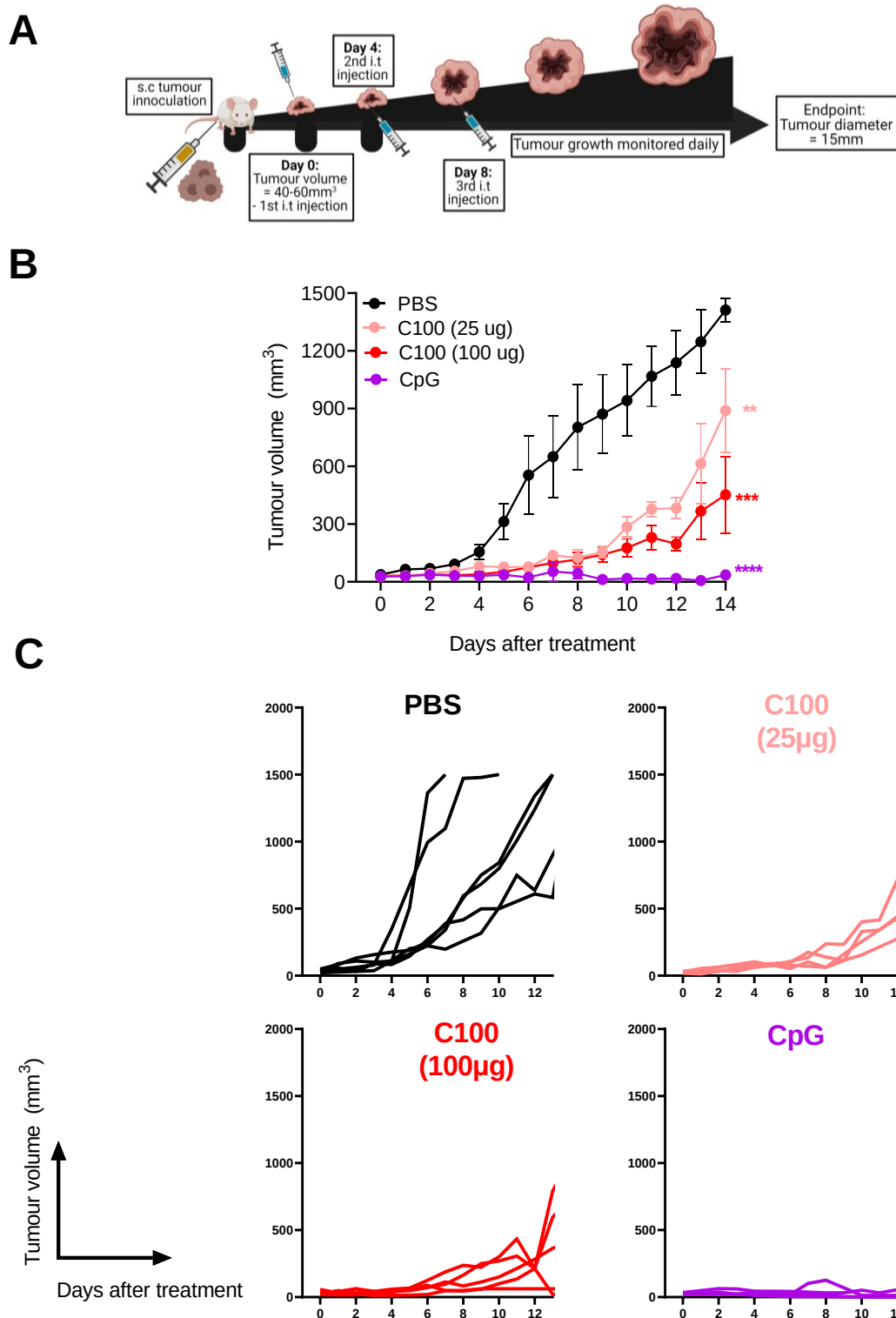
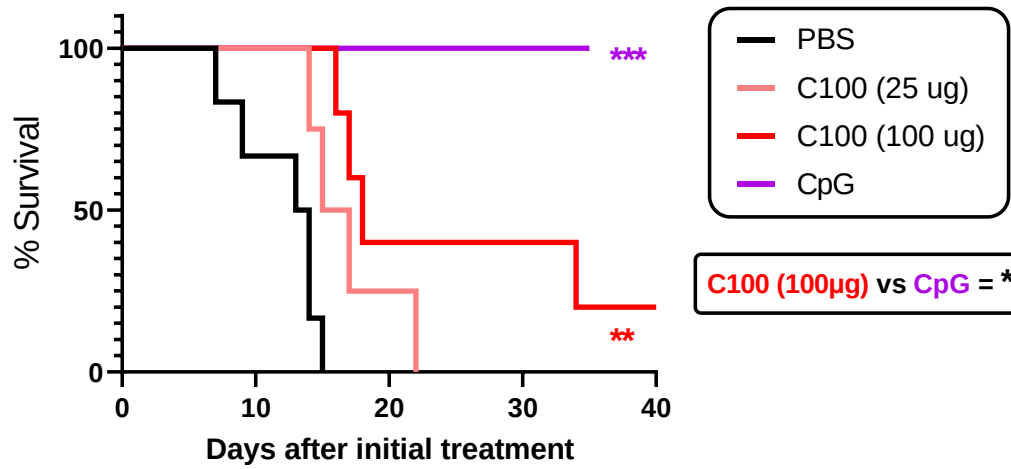


Fig.5.5.5 Intratumoral treatment with C100 and the CpG control significantly stunt initial tumour growth rate in a therapeutic MC38 model

(A) C57BL6/J mice were injected s.c. with 5×10^5 MC38 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS, C100 (100µg), C90 (100µg), Protasan CL213 (100µg) or CpG (50µg) in a volume of 30µl with injections repeated twice more 4 days apart. Tumour

growth was monitored daily until a clinical humane endpoint of $\geq 15\text{mm}$ in diameter was reached. (B) Mean initial tumour growth rate data for experimental mice detailed in. (C) Spider plots displaying individual tumour growth rates. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of differences between the tumour growth curves of treated mice and the PBS control group. Asterisks indicate significant differences. Not significant – ns (not displayed on graph), P value > 0.05 , *P < 0.05 , **P < 0.01 , ***P 0.001 and ****P < 0.0001 .

A**B**

Median survival time (Days post initial treatment)	
PBS	13.5
C100(25µg)	16
C100 (100µg)	18
CpG	Undefined

Fig.5.5.6 Intratumoral treatment with C100 or CpG prolongs survival in a therapeutic MC38 model

Survival analysis data for experimental mice detailed in Fig.5.5.5. (A) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < .05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Table displaying median survival times of treated mice post initial treatment.

5.6 Intratumoral C100 therapy outperforms the standard chemotherapeutic cisplatin in a therapeutic B16F10 model

With C100 identified as the most effective chitin-derived polymer for use intratumorally, an experiment was carried out to compare how this intratumoral therapy would perform against a clinically used chemotherapeutic agent. Cisplatin is a platinum based chemotherapeutic, which exerts cytotoxic effects via its ability to bind DNA and form crosslinks between and within strands³⁷⁰. It is mostly used clinically as a first line therapeutic for a wide range of malignancies and is administered intravenously to patients. It has been previously used in B16 models, delaying tumour growth and extending survival times when given systemically via the intraperitoneal route^{371–373}.

The intraperitoneal route of cisplatin administration was chosen to appropriately mimic its systemic use clinically. C57BL6/J mice received i.p injections of cisplatin or PBS, one day prior to intratumoral treatment with PBS or C100, this experimental design is illustrated in Fig.5.6.1.

Mice that received cisplatin displayed a reduction in initial tumour growth, however this was not statistically significant when compared to the PBS treated group (Fig.5.7.2). Like the previous experiment, C100 and CpG treated mice displayed a significant reduction in their initial tumour growth rate when compared to the PBS treated group (Fig.5.7.2).

However, all three treatment regimens – Cisplatin, C100 and CpG, proved effective in extending survival times. Cisplatin and C100 both significantly prolonged survival, while CpG was the most effective therapeutic for extending survival when compared to the PBS treated group (Fig.5.6.2). There was no statistically significant difference between the survival curves for C100 and cisplatin treated mice, but in this experiment CpG treated mice had significantly better protection than those given C100 (Fig.5.6.3).

In summary, intratumoral C100 therapy was shown to be consistently effective at inhibiting tumour growth and extending survival. This protection was comparable, if not slightly more effective than systemic treatment with cisplatin, a routinely used chemotherapeutic.

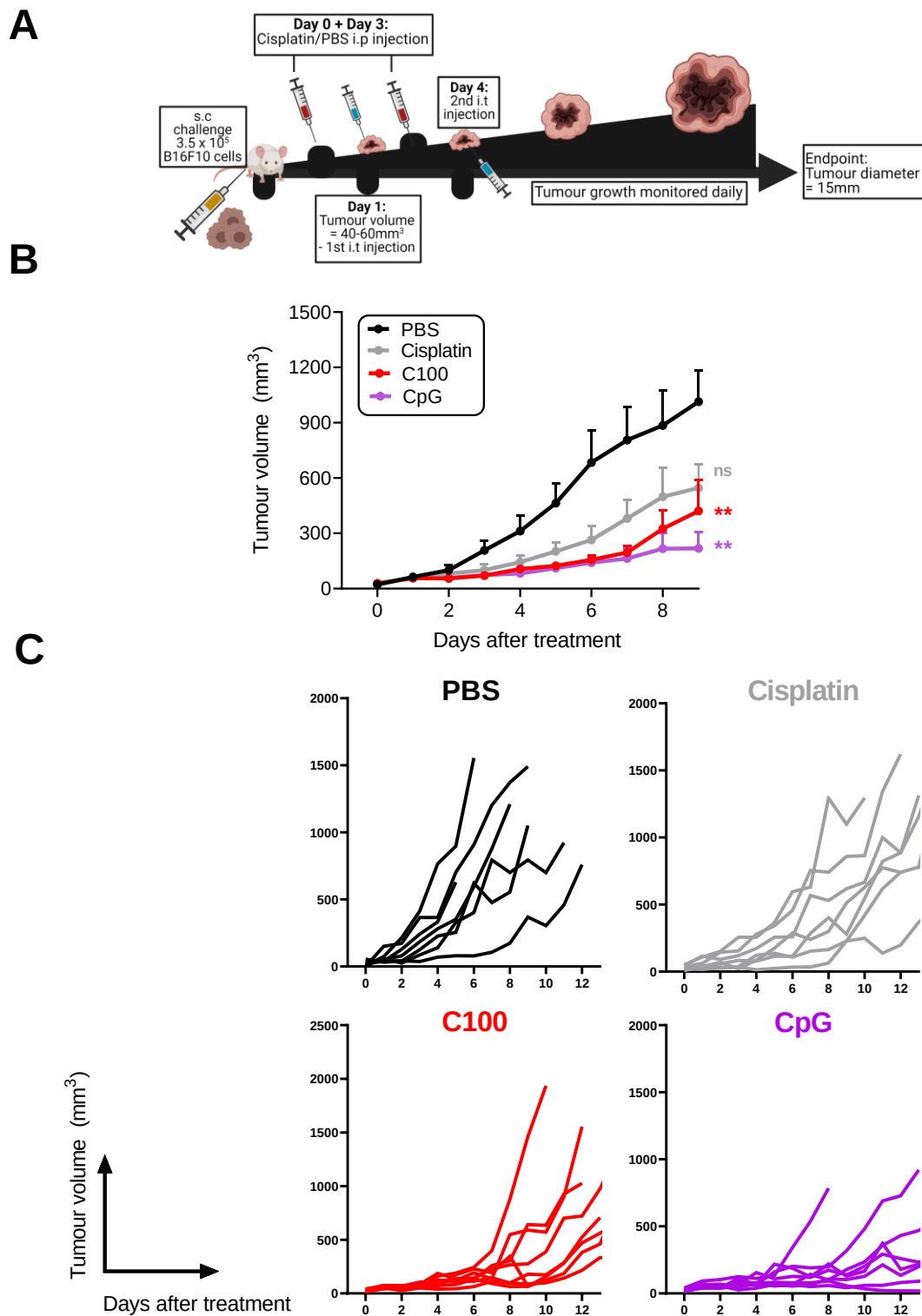
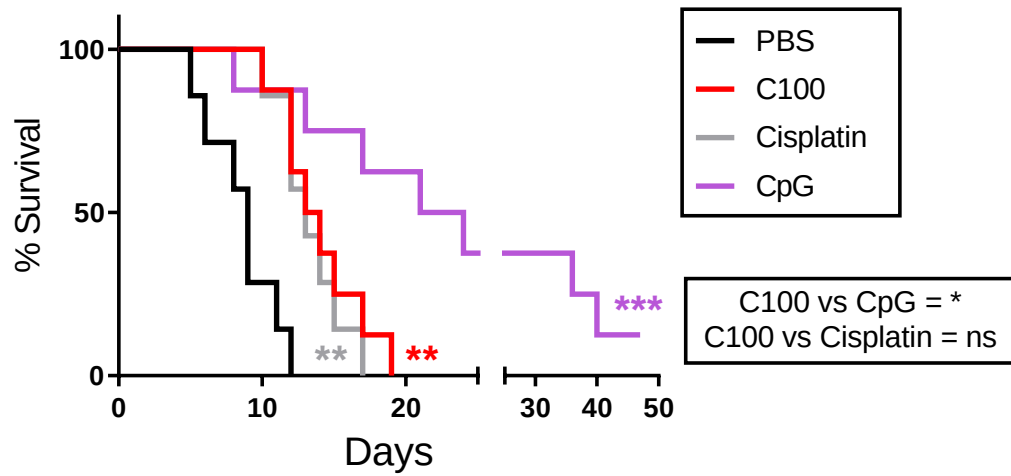


Fig.5.6.1 Intratumoral C100 is more effective than cisplatin at stunting initial tumour growth rate in a therapeutic B16F10 model.

C57BL6/J mice were injected s.c with 5×10^5 B16F10 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS, C100 (100µg) or CpG (50µg) with injections repeated once more 4 days later. 1 day prior to each injection mice were injected intraperitoneally with PBS or

Cisplatin (3mg/kg). Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (B) Mean initial tumour growth rate data for experimental mice detailed in. (C) Spider plots displaying individual tumour growth rates. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of differences between the tumour growth curves of treated mice and the PBS control group. Asterisks indicate significant differences. Not significant – ns, P value > 0.05 , *P < 0.05 , **P < 0.01 , ***P < 0.001 and ****P < 0.0001 .

A**B**

Median survival time (Days post initial treatment)	
PBS	9
C100	13.5
Cisplatin	13
CpG	22.5

Fig.5.6.2 Intratumoral treatment with C100 outperforms Cisplatin in prolonging survival in a therapeutic B16F10 model

Survival analysis data for experimental mice detailed in Fig.5.6.1. A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < .05$, ** $p < 0.01$, and *** $p < 0.001$. (B) Table displaying median survival times of treated mice post initial treatment.

5.7 The therapeutic efficacy of C100 is dependent on IFNAR signalling in host cells and STING expression in host or tumour cells

As previously discussed, research conducted in the Lavelle Lab has characterised C100 as a potent activator of the cGAS-STING pathway, the NLRP3 inflammasome and type I IFN signalling^{218–220}. It is therefore crucial to probe the importance of these pathways *in vivo* in the context of C100s therapeutic anti-tumour efficacy.

Firstly, to address whether C100 mediated activation of NLRP3 contributes to its's therapeutic efficacy, a therapeutic study involving the use of NLRP3 KO mice was utilised. No significant impact was observed on the therapeutic efficacy of C100 in NLRP3 KO mice in terms of inhibition of tumour growth kinetics (Fig.5.7.1A+B) or in the enhancement of survival (Fig.5.7.1C+D). This data indicates no significant role for NLRP3 signalling in therapeutic benefit.

To investigate the role of Type I IFN signalling in therapeutic efficacy, experiments were conducted utilising IFNAR KO mice. As before, intratumoural C100 successfully inhibited tumour growth (Fig.5.7.2A+B) and prolonged survival (Fig.5.7.2C+D) in WT mice. Crucially however, this therapeutic efficacy was completely abrogated in IFNAR KO mice, with IFNAR KO, C100-treated mice having no therapeutic benefit in terms of tumour growth kinetics (Fig.5.7.2A+B) or prolonging survival (Fig.5.7.2C+D). It is worth highlighting that the MC38 cells implanted into these mice were WT for IFNAR expression, and as such it can be concluded that IFNAR signalling in host immune cells is essential for therapeutic benefit.

As C100 has been identified as an activator of the cGAS-STING pathway and C100 mediated BMDC maturation is dependent on type I IFN and STING signalling²⁸⁶, therapeutic experiments involving the use of STING KO mice and sourced STING KO B16F10 cells were conducted. It was previously demonstrated that WT B16F10 cells retained functional STING expression (Fig5.5.1C). Here, therapeutic benefit from intratumoral treatment with C100 was still observed in WT mice challenged with STING KO tumours (Fig.5.7.3B+E). However, in STING KO mice challenged with STING KO B16F10 tumours, no therapeutic benefit was observed (Fig5.7.3D+E). Intriguingly, therapeutic efficacy could be rescued when STING KO mice were challenged with B16F10 cells with functional STING (Fig.5.7.3C+E). These data indicate that STING signalling is essential in the therapeutic efficacy of C100, suggesting that its expression in the tumour or in responding host immune cells is sufficient.

Altogether these data strongly align with previous work in the Lavelle Lab demonstrating roles for the cGAS-STING pathways and type I IFN signalling in C100 adjuvanticity *in vitro*. These observations confirm C100 as an intratumoral therapeutic that is reliant on functional STING expression in the host or tumour cell compartment and suggest that type I IFN signalling is essential for C100-mediated anti-tumour immune responses.

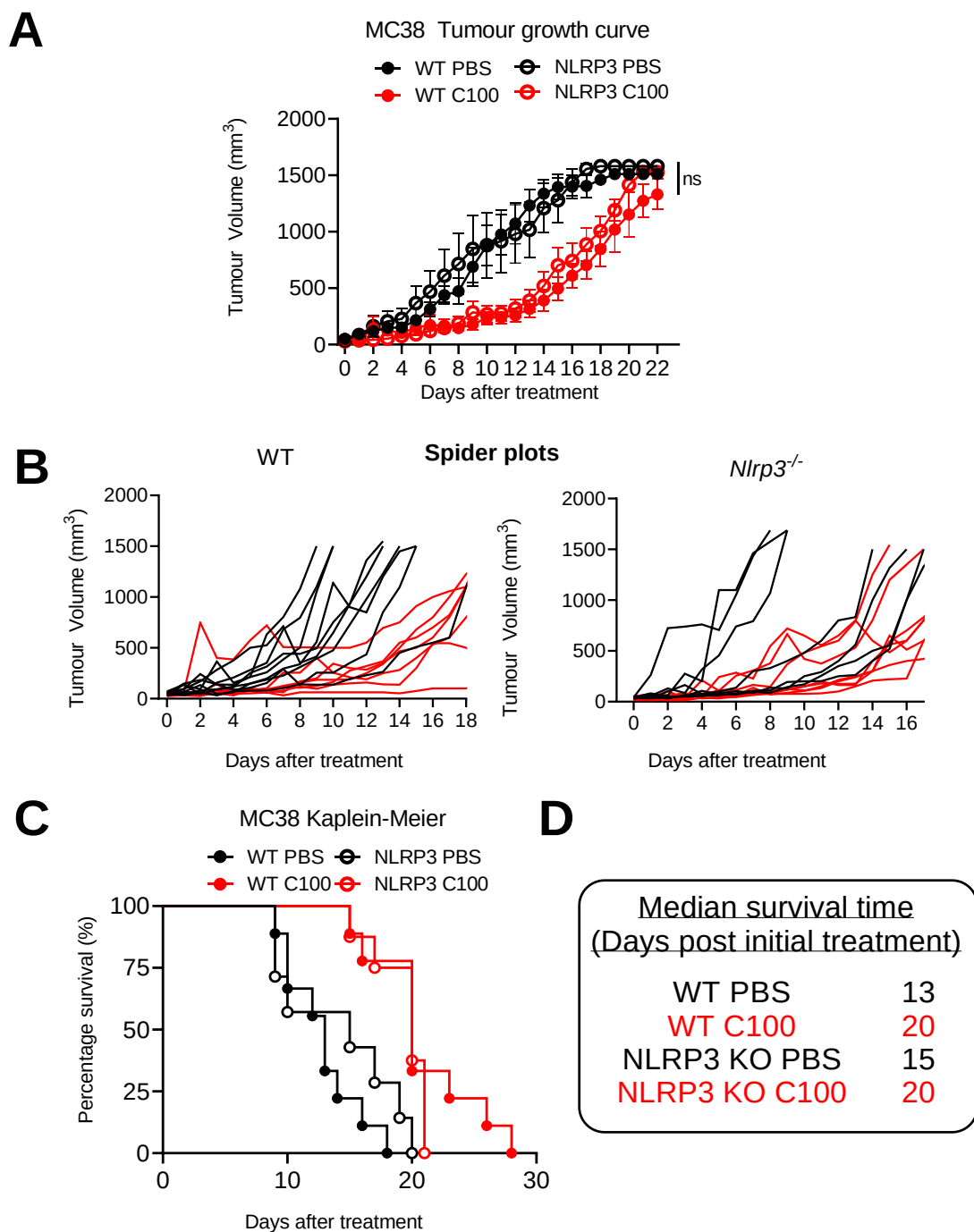


Fig.5.7.1 The therapeutic efficacy of C100 is not dependent on NLRP3 signalling *in vivo*

WT and NLRP3^{-/-} C57BL6/J mice were injected s.c with 5×10^5 MC38 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100µg) in a volume of 30µl with injections repeated twice more 4 days apart. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (A) Initial tumour growth rate. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of tumour growth curve differences between C100 treated mice and the PBS control group. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001. (B)

Spider plots displaying individual tumour growth rates. (C) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and C100 treated groups, where $*p<.05$, $**p<0.01$, and $***p<0.001$. (D) Table displaying median survival times of treated mice post initial treatment.

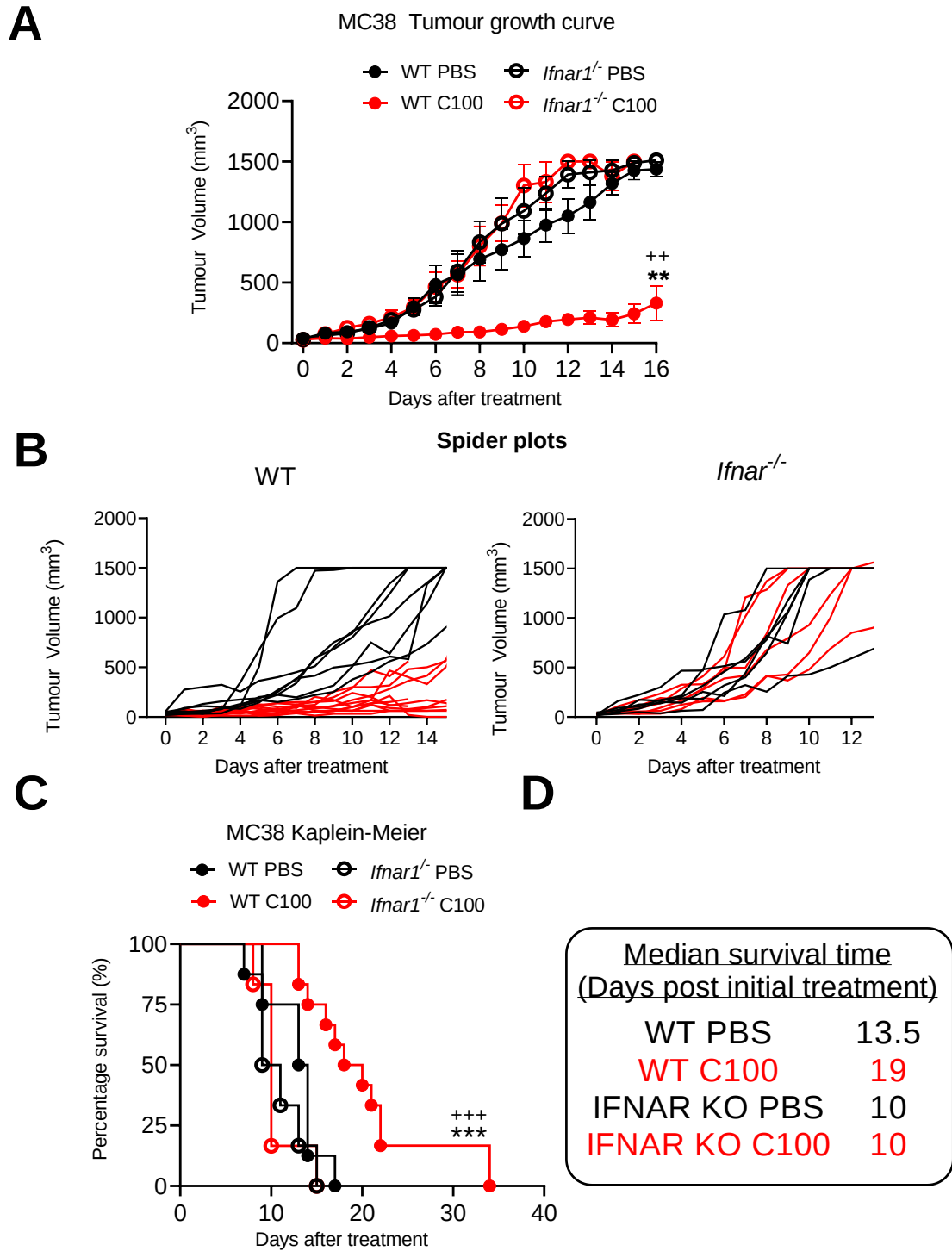


Fig.5.7.2 The therapeutic efficacy of C100 is dependent on host IFNAR signalling *in vivo*

WT and IFNAR^{-/-} C57BL/6J mice were injected s.c with 5 x 10⁵ MC38 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100µg) in a volume of 30µl with injections repeated twice more 4 days apart. Tumour growth was monitored daily until a clinical humane endpoint of ≥15mm in diameter was reached. (A) Initial tumour growth rate. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of tumour growth

curve differences C100 treated mice and the PBS control group. Asterisks indicate significant differences between WT PBS and C100 treated mice, + symbols indicate significant differences between WT and IFNAR KO C100 treated mice. Not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001. (B) Spider plots displaying individual tumour growth rates. (C) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and C100 treated groups, where *p<.05, **p<0.01, and ***p<0.001. Asterisks indicate significant differences between WT PBS and C100 treated mice, + symbols indicate significant differences between WT and IFNAR KO C100 treated mice. (D) Table displaying median survival times of treated mice post initial treatment.

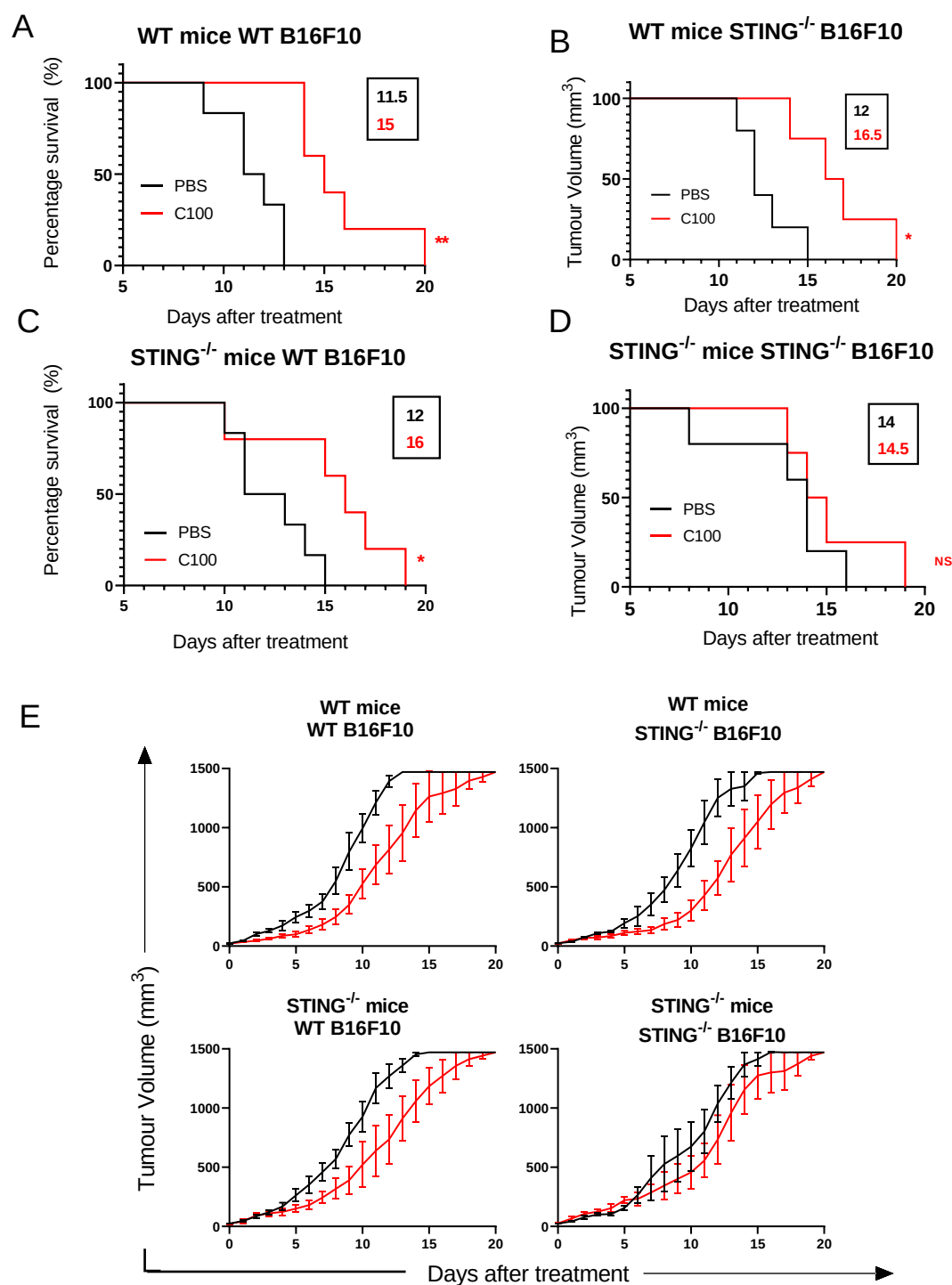


Fig.5.7.3 The therapeutic efficacy of C100 is dependent on STING expression in the host or tumour cells

WT or STING KO C57BL6/J mice were injected s.c with 3.5×10^5 B16 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100µg) in a volume of 30µl with injections repeated twice more 4 days apart. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. A Kaplan-Meier survival analysis of challenged

mice is displayed. (A) WT mice WT B16F10 (B) WT mice STING KO B16F10 (C) STING KO mice WT B16F10 (D) STING KO mice STING B16F10. A Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < .05$, ** $p < 0.01$, and *** $p < 0.001$. (E) Mean tumour growth rates are displayed as mean tumour volume $\pm$ SEM.

5.8 Intratumoral C100 therapy can synergise with systemic checkpoint blockade

A key application of cancer vaccines are combination approaches with existing immunotherapeutics⁶⁶. This is crucial as there remains significant room for improvement for response rates to checkpoint inhibitor therapies clinically¹⁸, with lower responses typically in tumours where there is a low immune infiltrate. As a result there has been a movement towards combination strategies utilising ISV to promote immune infiltrate and transition tumours from immunologically 'cold' to 'hot'⁶⁶. STING activating agonists have been identified as promising candidates for intratumoral therapeutics and have been trialled as monotherapies or in conjunction with checkpoint blockade¹⁵⁵.

To determine if intratumoral C100 therapy could synergise with α -PD1 therapy, a therapeutic MC38 tumour challenge model was designed involving administering intratumoral C100 therapy and α -PD1 therapy via the intraperitoneal route on the same treatment day as detailed in Fig.5.8.1A.

As demonstrated previously, C100 treatment alone again prolonged survival (Fig.5.8.2) and inhibited tumour growth (Fig.5.8.1B+C). Importantly a potent synergistic effect was observed in the group receiving intratumoral C100 and systemic anti-PD-1 in combination, significantly inhibiting tumour growth rate when compared to PBS, C100 and PD-1 alone groups (Fig.5.8.1B+C). Crucially, 50% of mice in the combination group had cleared their tumours/were alive at study endpoint (Fig. 5.8.2). Additional therapeutic experiments utilising a single dose of α -PD1 therapy further illustrate the synergistic effect of this combination therapy with 20% of mice alive at study endpoint in the PD-1 alone group compared to 60% survival in the combination groups (Fig 5.8.3).

This data is line with the literature demonstrating MC38 tumour responsiveness to immunotherapeutic interventions³⁷⁴, owing to intrinsic immunogenicity, in contrast α -PD1 therapy has not been observed to exert therapeutic benefit in less immunogenic, immunologically cold B16F10 tumours. Therefore, potential synergy in therapeutic efficacy was evaluated between C100 and α -PD1 in B16F10 tumours in a pilot study. These mice were ultimately culled 6 days following initial treatment, with tumours, spleens and draining lymph nodes harvested for FACs analysis, and these results will be discussed in the next section. Tumour growth analysis from this pilot study indicates that intratumoral C100 therapy and systemic α -PD1 could synergise in a B16F10 model to inhibit tumour growth (Fig.5.8.4). This is a promising result, warranting further studies investigating impact on survival to confirm synergy.

Altogether, these results strongly support the use of C100 as part of a combinatorial treatment approach with checkpoint therapy generating proof of concept in the immunologically hot MC38 and immunologically cold B16F10 tumour models. Further studies should investigate combinations with different checkpoint inhibitors to identify the optimal combination with C100.

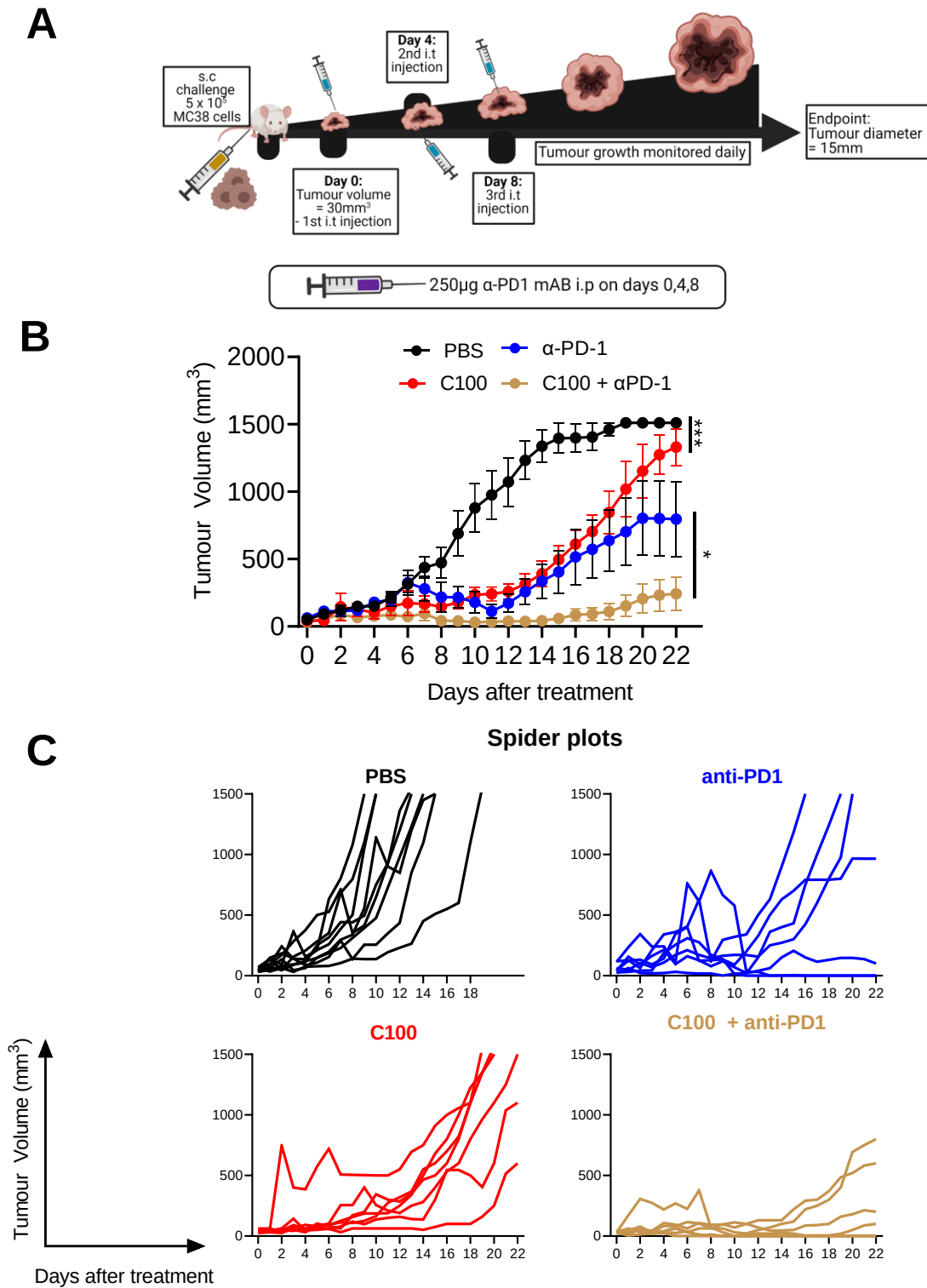


Fig.5.8.1 Intratumoral C100 therapy can synergise with systemic α-PD1 blockade in an MC38 model tumour model

C57BL6/J mice were injected s.c. with 5×10^5 MC38 melanoma cells. When tumours reached 40-60 mm^3 in size, they were injected intratumorally with PBS or C100 (100μg) with injections repeated twice more

4 days apart. On each of these treatment days mice received 250µg of a monoclonal anti-PD1 antibody or PBS via the intraperitoneal route. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (B) Mean initial tumour growth rate data. (C) Spider plots displaying individual tumour growth rates. A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of tumour growth curve differences between treatment groups. Asterisks indicate significant differences. Not significant – ns, P value > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

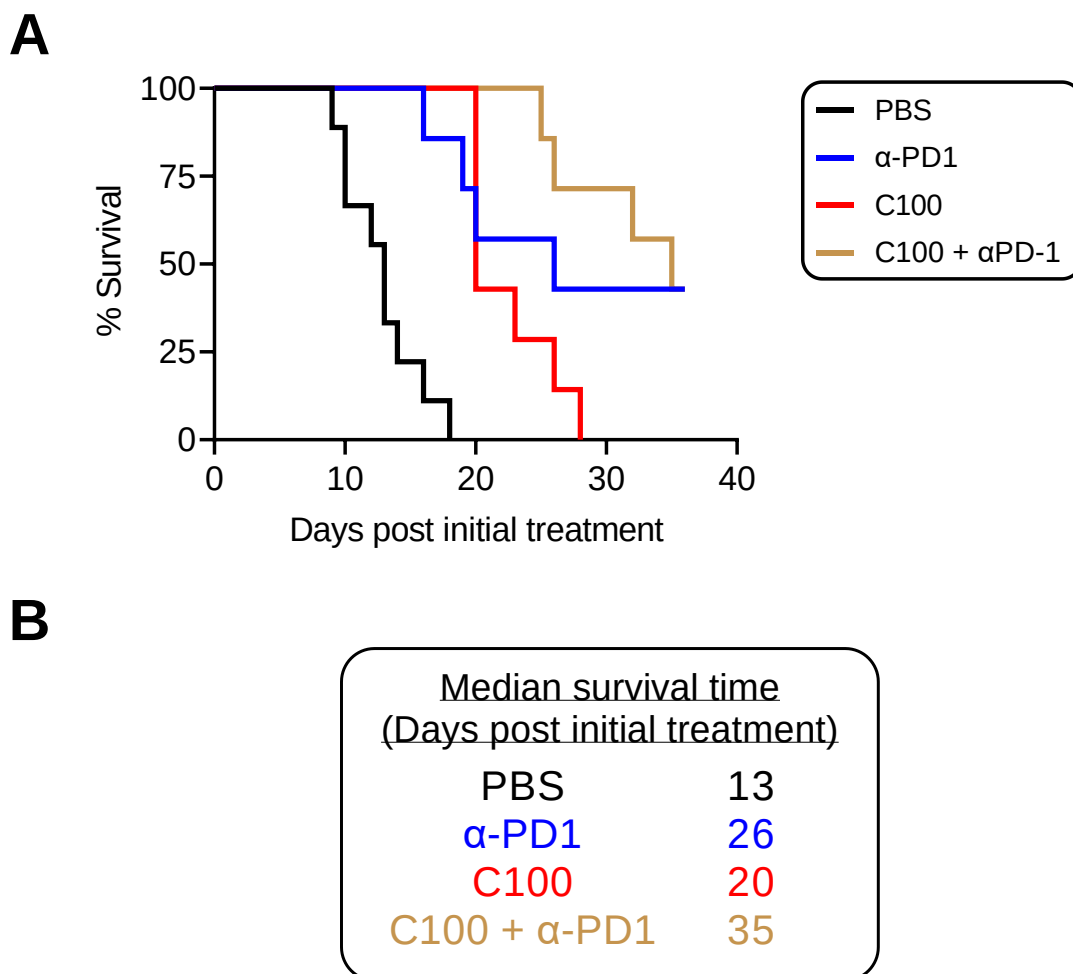


Fig.5.8.2 Intratumoral C100 therapy can synergise with systemic α -PD1 blockade to promote survival in a MC38 tumour model

Survival analysis data for experimental mice detailed in Fig.5.8.1. (A) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and C100 treated groups, where not significant – ns (not shown on graph) P value > 0.05, *p<.05, **p<0.01, and ***p<0.001. (B) Table displaying median survival times of treated mice post initial treatment.

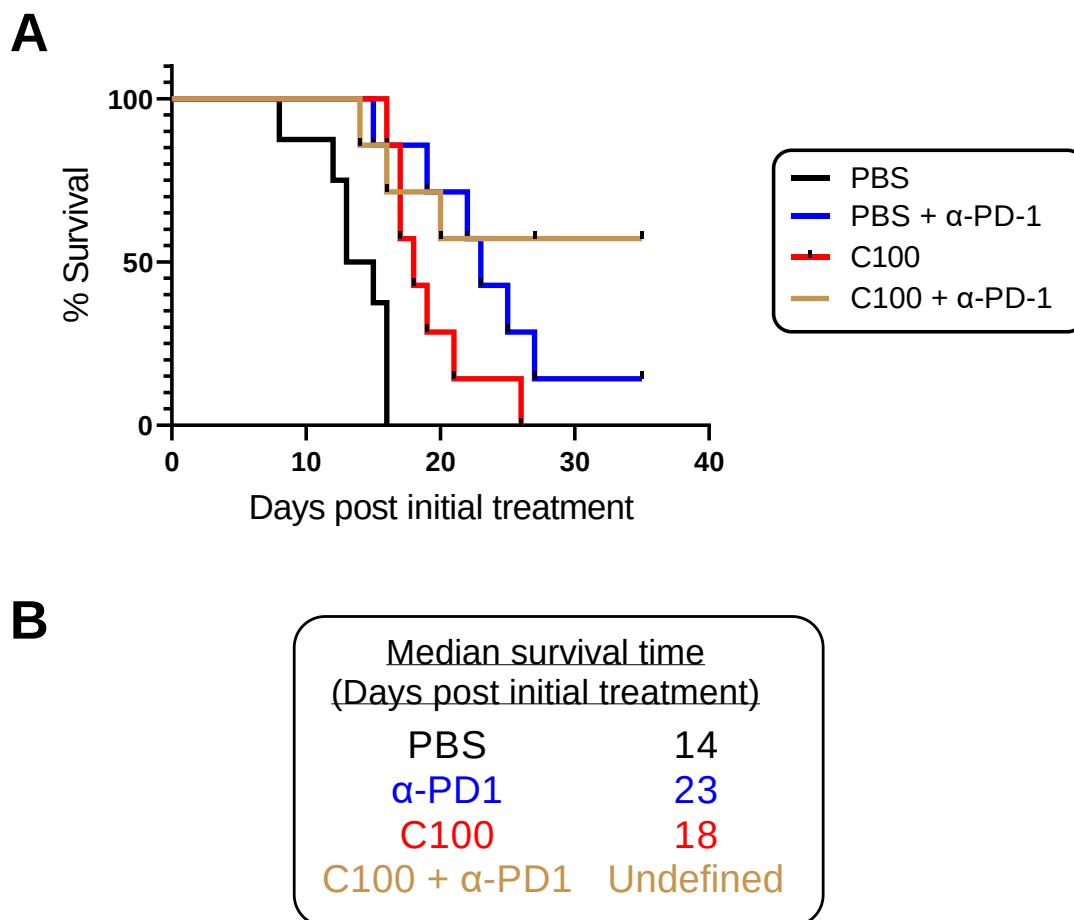


Fig.5.8.3 The synergistic effect of C100 therapy and α -PD1 is more evident when a single dose of anti-PD1 is administered

C57BL6/J mice were injected s.c with 5×10^5 MC38 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100 μ g) with injections repeated once more 4 days later. On day 0, in addition to the intratumoral injection, mice received 250 μ g of a monoclonal anti-PD1 antibody or PBS via the intraperitoneal route. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (A) A Kaplan-Meier survival analysis of challenged mice is displayed a Mantel-Cox test was used to determine statistical significance between PBS and C100 treated groups where, not significant – ns (not shown on graph) P value > 0.05, *p<.05, **p<0.01, and ***p<0.001. (B) Table displaying median survival times of treated mice post initial treatment.

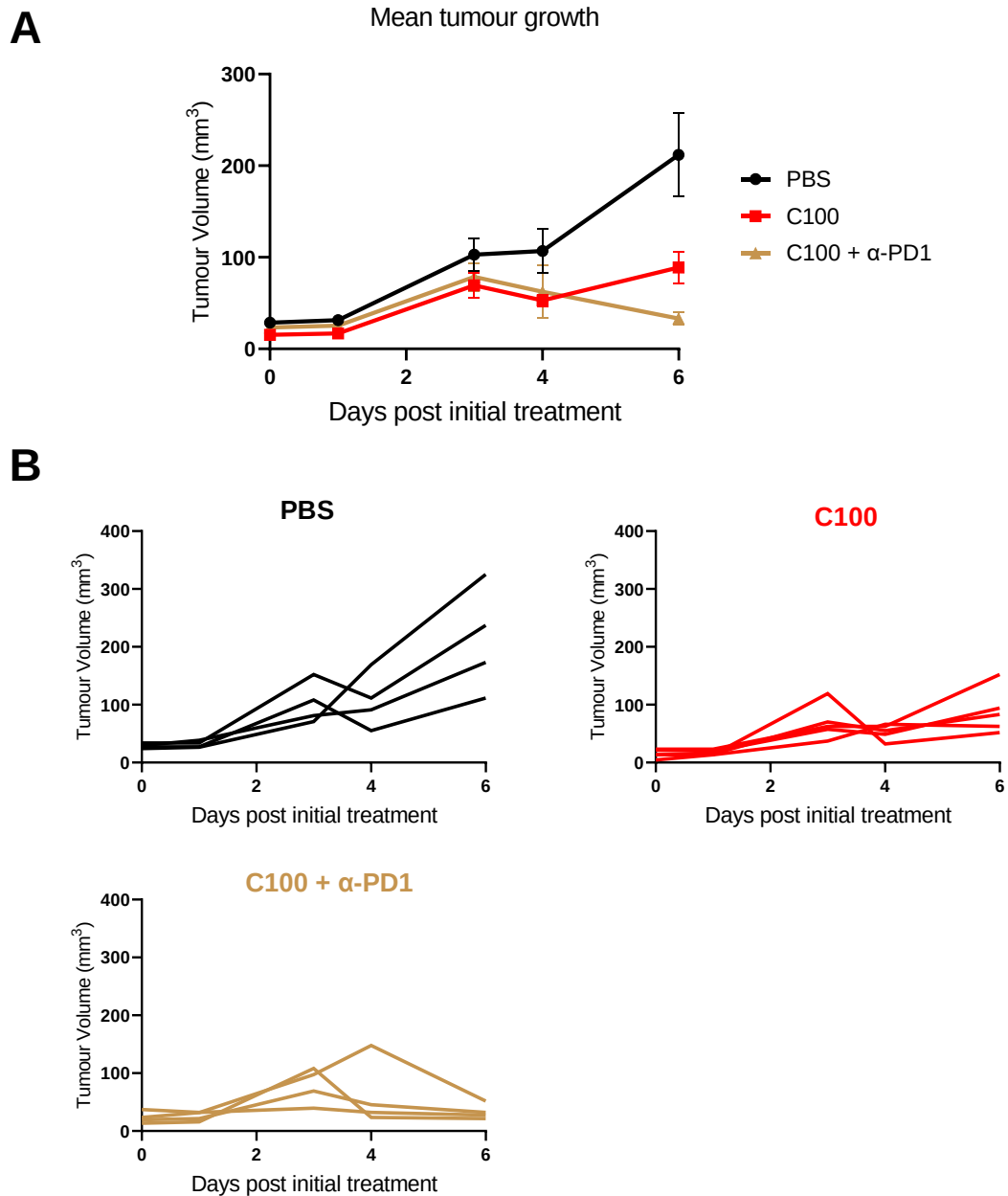


Fig.5.8.4 A pilot study indicates that intratumoral C100 therapy can synergise with systemic α -PD1 blockade in an B16 tumour model

C57BL6/J mice were injected s.c with 3.5×10^5 B16F10 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100μg) with injections repeated once more 4 days later. On each of these treatment days, mice received 250μg of a monoclonal anti-PD1 antibody or PBS via the intraperitoneal route. Tumour growth was monitored daily until day 6 when tissues were harvested from these mice. (A) Mean initial tumour growth rate. (B) Spider plots displaying individual tumour growth rates

5.9 The therapeutic efficacy of C100 is dependent on CD8⁺ T cells in an MC38 model

To investigate how C100 mediates its anti-tumour efficacy in the MC38 tumour model, either alone or in therapeutic combination strategies, cell depletion assays were performed.

Intratumorally administered STING agonists have been shown to elicit anti-tumour CD8⁺ T cell responses^{274,350} by facilitating DC mediated cross-priming through type I IFN induced APC maturation and production of cytokines. CD8⁺ T cells themselves can also be further expanded and activated through type I IFN signalling³⁷⁵. To assess the contribution of CD8⁺ T cells to the therapeutic efficacy of C100 in an MC38 model, a study involving intratumoural C100 therapy, systemic anti-PD1 therapy, and CD8⁺ T cell depletion (utilising a CD8 α targeted monoclonal antibody (Clone 2.43)) was utilised as detailed in Figure 5.9.1A. Successful depletion of CD8⁺ T cells was confirmed in experimental mice using peripheral blood sampling and FACS staining (Figure 5.9.1B+C). Crucially CD8⁺ T cell depletion was shown to completely abrogate the therapeutic benefit of intratumoural C100 therapy alone or in conjunction with systemic anti-PD-1 therapy resulting in a lack of impact on tumour growth kinetics (Fig.5.9.2A,B) or on survival (Fig.5.9.3).

In addition to CD8⁺ T cells, NK cells have potent anti-tumour functionality, and their activity has also been shown to be mediated by type I IFN signalling^{376,377}. Indeed, therapy with STING activating CDNs has been shown to promote NK mediated tumour clearance in some pre-clinical models downstream of DC activation^{24,350}. As a result, a therapeutic study involving the depletion of NK cells was conducted using a monoclonal antibody targeting NK1.1 (Clone PK136), with the experimental schematic displayed in Figure 5.9.4A. Peripheral blood was collected from a sample of experimental mice via the tail vein and stained for FACS analysis to evaluate cell depletion via the gating strategy outlined in Fig.5.9.4B. NK cells were successfully depleted in experimental mice as displayed in Fig.5.9.4C. No significant impact on therapeutic benefit was observed in mice depleted of NK cells when comparing those given intratumoural PBS vs C100, in terms of growth kinetics (Fig.5.9.5A,B) or survival (Fig.5.9.5C). However, it is worth highlighting that the MC38 tumour model is relatively insensitive to NK mediated killing due to high expression of MHC Class I molecules, and depletion of NK cells has even been shown to slow tumour growth in this model³⁷⁴. This is in line with our data showing a slight delay in tumour growth in NK cell depleted vs non-depleted mice when comparing those treated with intratumoral PBS.

In contrast to the MC38 model, the B16F10 melanoma model is more sensitive to NK cell mediated killing through downregulation of MHC Class I molecule expression. Therefore, further

work should characterise the relative contributions of NK cells and CD8⁺ T cells to C100-mediated protection in the B16 model.

Analysis of the immune infiltrate in B16F10 tumours of C100 treated mice may provide insights into its mechanism of action in this model. Tumours, spleens, and inguinal lymph nodes were harvested from mice 2 days after receiving their second C100/PBS intratumoral injection. The tumours and ILNs of mice that received C100 + α -PD1 had a significant increase in the frequency of NK cells/Live cells when compared to PBS treated (Fig.5.9.6A). A slight increase in frequency was observed in those given C100 alone.

Altogether, these results identify CD8⁺ T cells as the crucial mediators of C100 induced anti-tumour immunity when used alone or in conjunction with checkpoint blockade therapy in an MC38 model. Further work should characterise the cellular mediators in the phenotypically different B16F10 tumour model, with a focus on the contribution of NK cells.

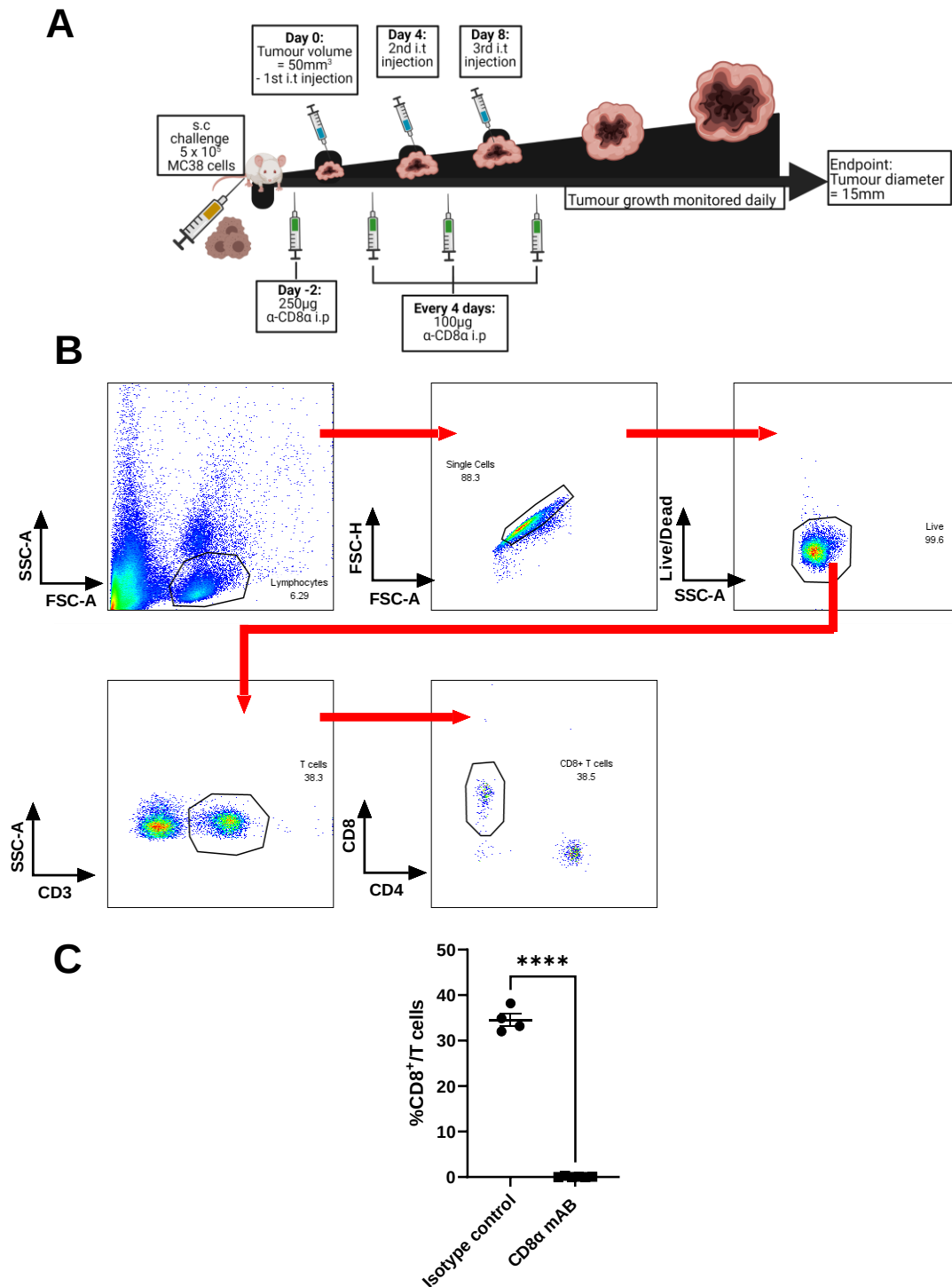


Fig.5.9.1 CD8⁺ T cells were successfully depleted by antibody treatment in experimental mice

C57BL6/J mice were injected s.c with 5×10^5 MC38 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100µg) with injections repeated twice more 4 days apart. On each of these treatment days, mice received 250µg of a monoclonal anti-PD1 antibody or PBS via the intraperitoneal route. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. CD8α⁺ T cells were depleted in experimental mice through

intraperitoneal injection of a CD8 α targeted monoclonal antibody (mAB) (Clone 2.43). Mice received 250 μ g of α -CD8 α mAB or PBS two days before tumour challenge, with subsequent 100 μ g of mAB or PBS every 4 days. B) Peripheral blood was sampled via the tail vein on day 0 and cell depletion was evaluated according to the displayed gating strategy. C) Frequency of CD8 α ⁺ T cells in peripheral blood displayed as mean $\pm$ SEM. A paired t test was used to determine statistical significance between depleted and non-depleted mice. Asterisks represent statistical significance where *p<.05, **p<0.01, ***p<0.001, and ****p<0.0001.

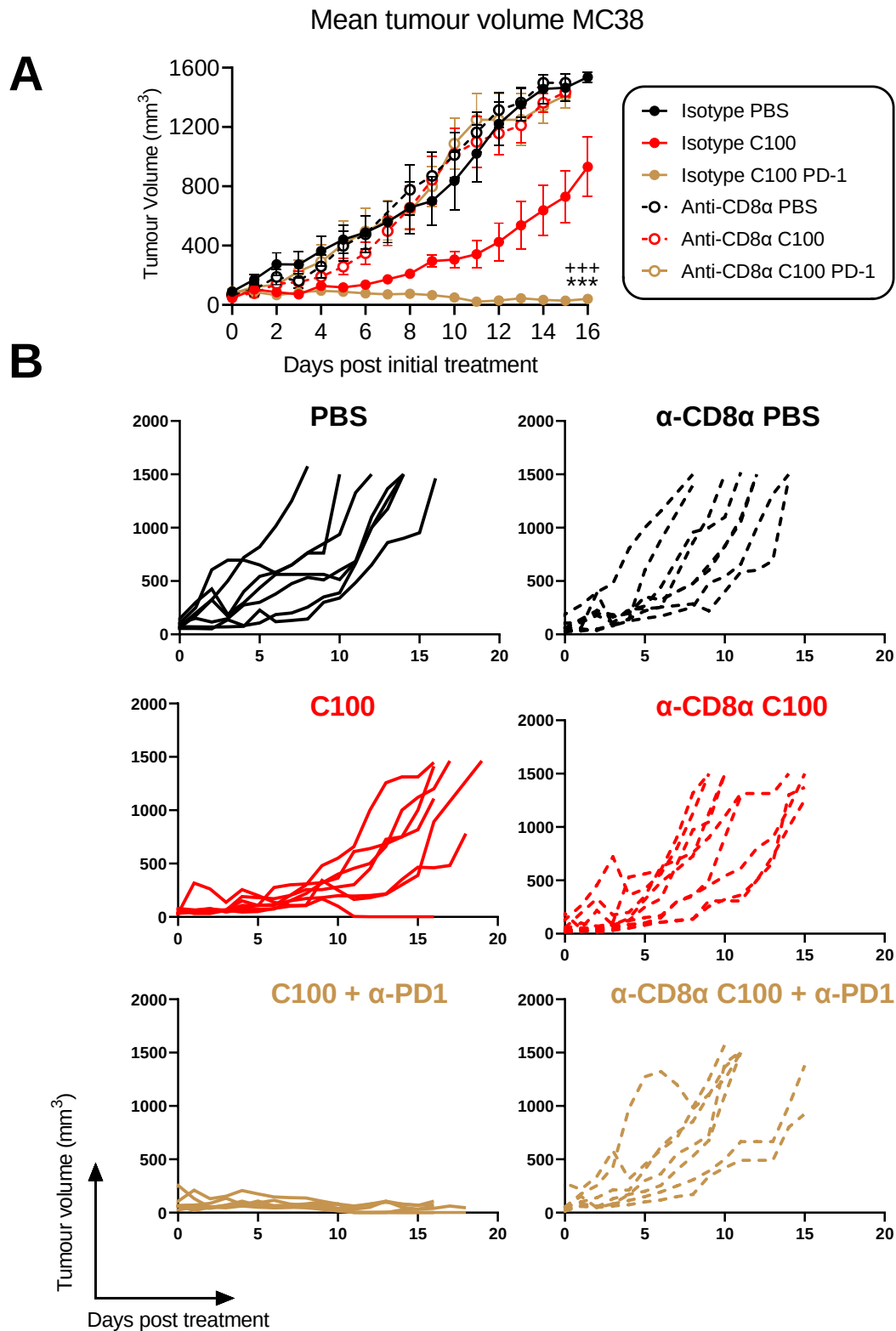


Fig.5.9.2 Therapeutic efficacy of C100 given as an intratumoral monotherapy or in combination with systemic checkpoint blockade is dependent on CD8⁺ T cells in an MC38 model

Tumour growth data for the mice described in Fig.5.9.1. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (A) Mean initial tumour growth rate data.

(B) Spider plots displaying individual tumour growth rates. . A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance of tumour growth curve differences between treatment groups. Asterisks indicate significant differences between PBS and C100 + anti-PD1 treated mice, + symbols indicate significance between depleted and non-depleted C100 + anti-PD1 treated mice. Not significant – ns (not shown on graph) P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

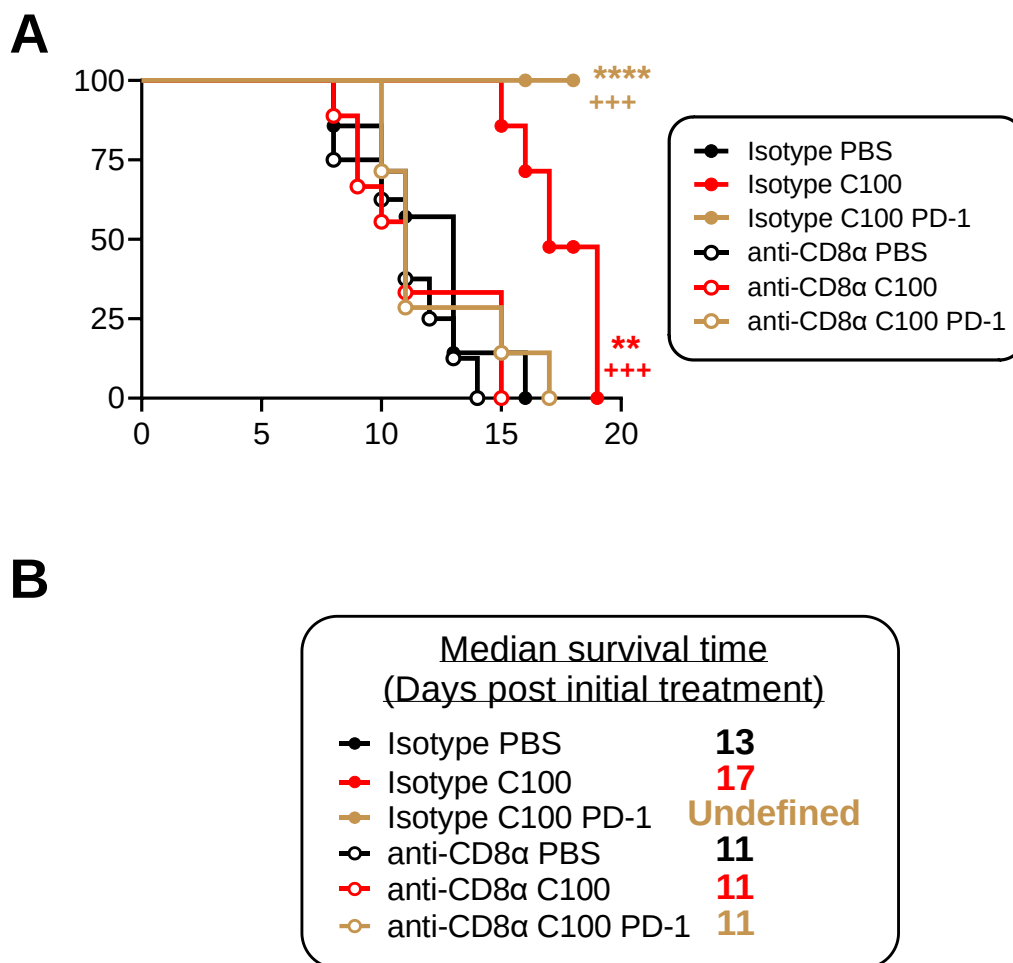


Fig.5.9.3 The enhancement of survival when C100 was given as an intratumoral monotherapy or in combination with systemic checkpoint blockade is dependent on CD8+ T cells in an MC38 model

Survival data for mice described in Fig.5.9.1. (A) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between PBS and experimental immunised groups, where * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Asterisks indicate significant differences between PBS and experimentally treated mice, + symbols indicate significance between depleted and non-depleted treated mice. Not significant – ns (not shown on graph) P value > 0.05 , * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. (B) Table displaying median survival times of treated mice post initial treatment.

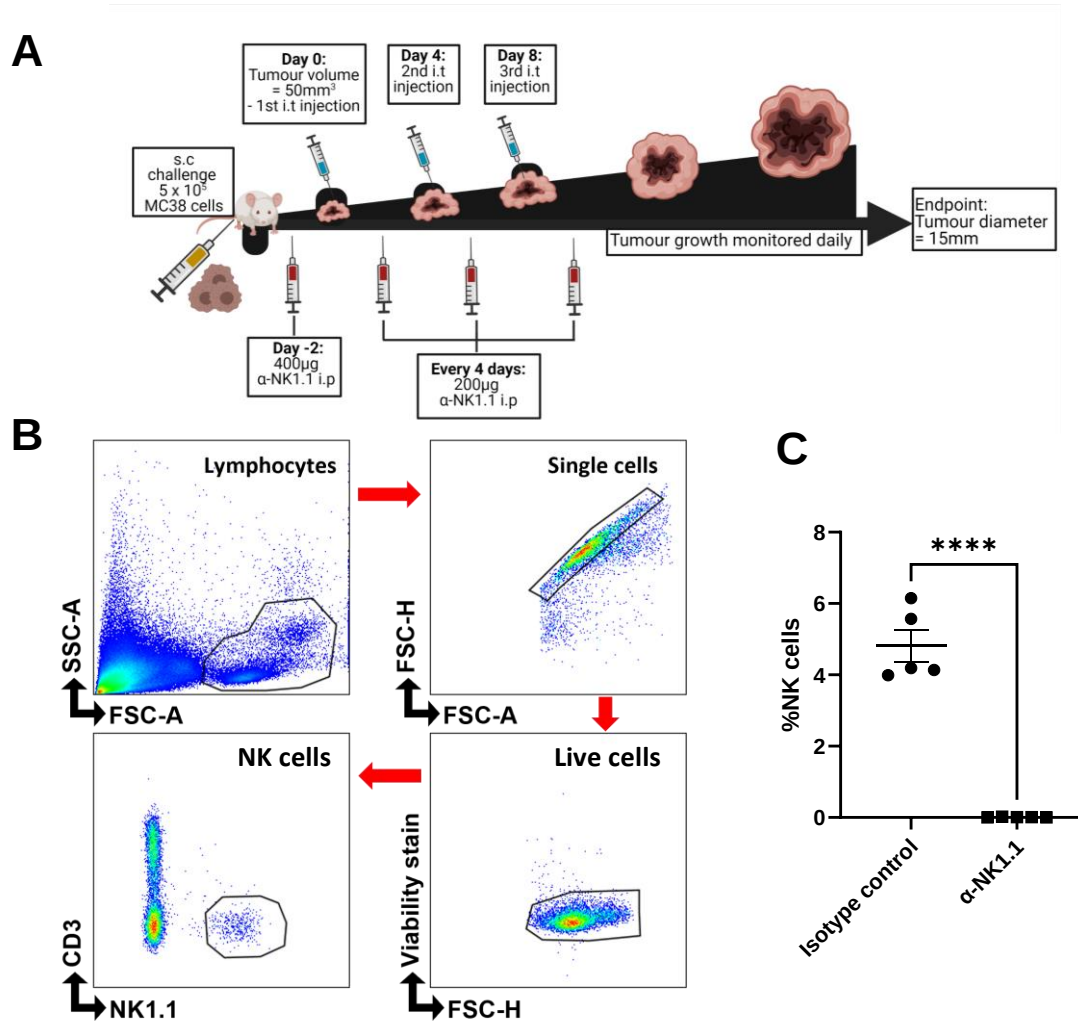


Fig.5.9.4 NK cells were successfully depleted by antibody treatment in experimental mice

C57BL6/J mice were injected s.c with 5×10^5 MC38 melanoma cells. When tumours reached 40-60 mm³ in size, they were injected intratumorally with PBS or C100 (100µg) with injections repeated twice more 4 days apart. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. NK cells were depleted in experimental mice through intraperitoneal injection of a NK1.1 targeted monoclonal antibody (mAB). Mice received 400µg of α-NK1.1 mAB or PBS two days before tumour challenge, with a subsequent injection of 200µg mAB or PBS every 4 days. B) Peripheral blood was sampled via the tail vein on day 0 and cell depletion was evaluated according to the displayed gating strategy. C) Frequency of CD8α⁺ T cells in peripheral blood displayed as mean $\pm$ SEM. A paired t test was used to determine statistical significance between depleted and non-depleted mice. Asterisks represent statistical significance where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

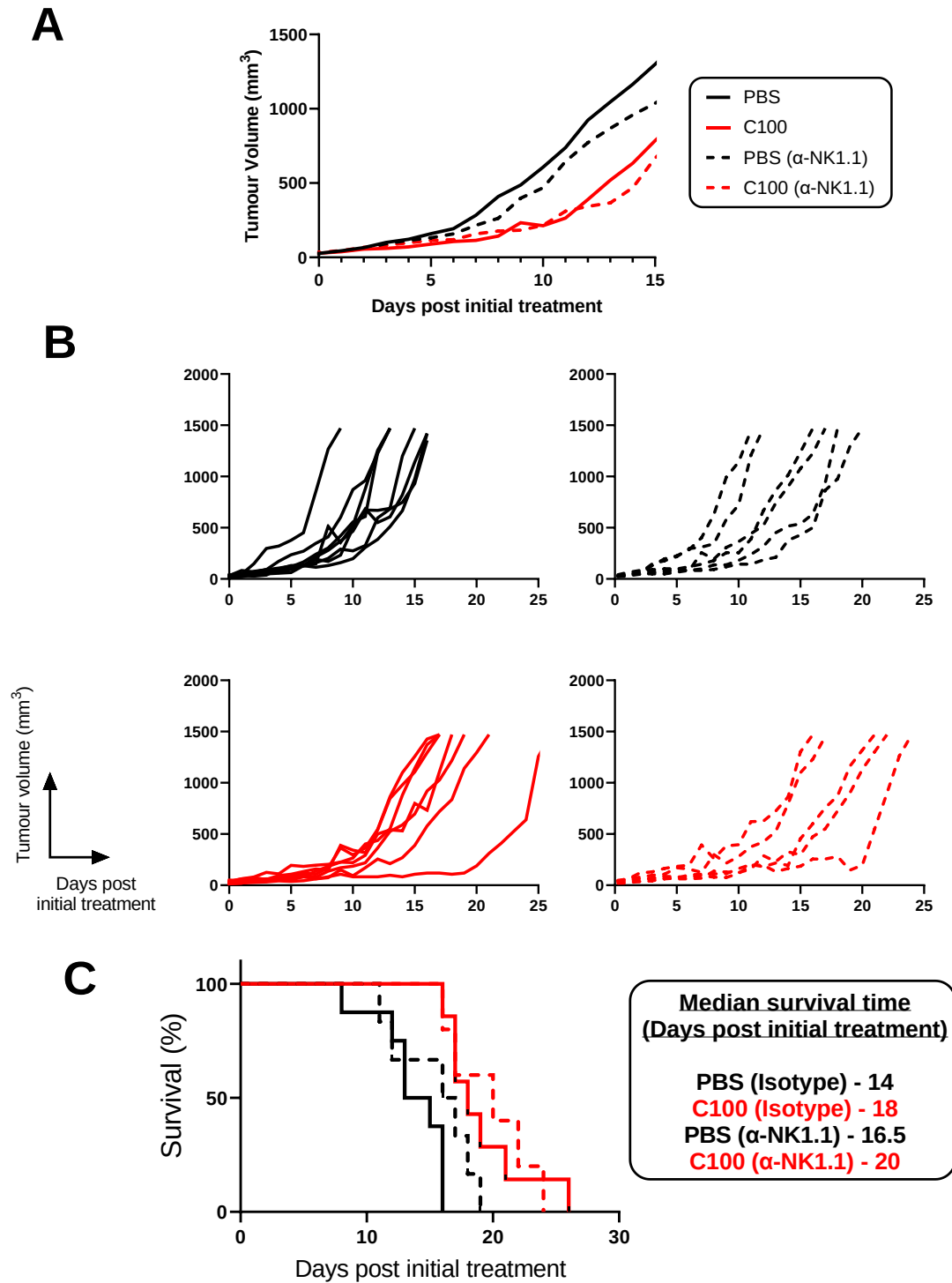


Fig.5.9.5 The therapeutic efficacy of C100 is not reliant on the presence on NK cells in an MC38 tumour model

Tumour growth and survival analysis for mice described in Figure 5.9.4. Tumour growth was monitored daily until a clinical humane endpoint of ≥ 15 mm in diameter was reached. (A) Mean initial tumour growth rate (B) Spider plots displaying individual tumour growth rates. (C) A Kaplan-Meier survival analysis of challenged mice is displayed, a Mantel-Cox test was used to determine statistical significance between

experimental groups, where Not significant – ns (not shown on graph) P value > 0.05, *p<.05, **p<0.01, and ***p<0.001. Table displaying median survival times of treated mice post initial treatment.

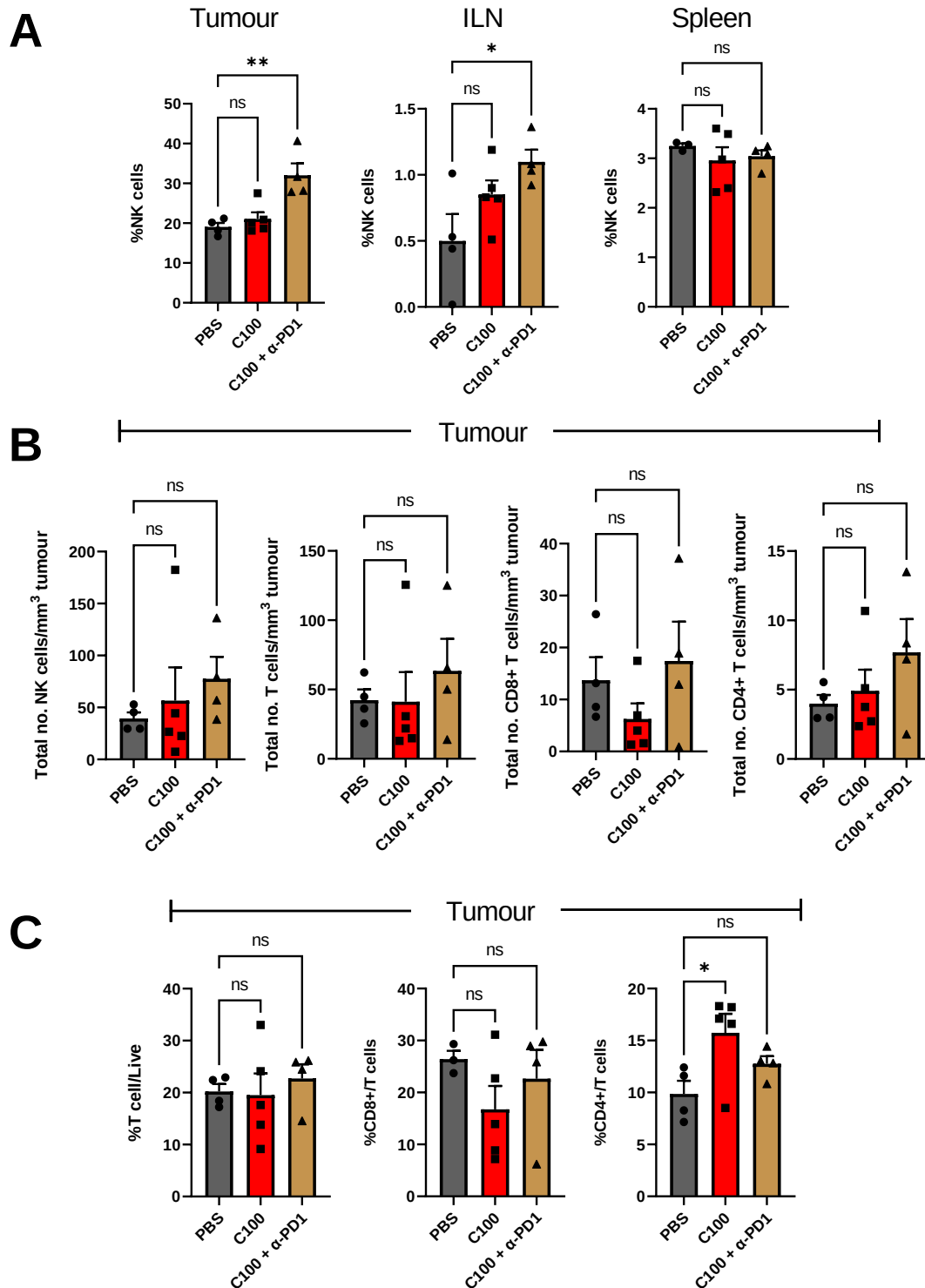


Fig.5.9.6 Intratumoral injection of C100 alone or in combination with systemic α-PD1 can promote NK cell infiltration into the tumour and draining lymph nodes in a B16 model

Tissue analysis of mice displayed in Figure 5.9.2. Tumours, spleens, and inguinal lymph nodes were harvested from tumour challenged mice on day 6. The spleens, ILNs and tumours were processed to obtain a single cell suspension and were subsequently stained for FACS analysis. (A) Frequency of NK cells in the tumour, ILNs or spleen. B) Total numbers of NKs, T cells, CD8+ T cells, and CD4+ T cells in the tumour.

(C) Frequency of T cells, CD8+ T cells and CD4+ T cells in the tumour. One way ANOVA with Dunnett's multiple comparisons test was used to compare statistical significance between experimental groups. Asterisks indicate significance between PBS and treated groups, not significant – ns P value > 0.05, *P < 0.05, **P < 0.01, ***P 0.001 and ****P < 0.0001.

5.10 Discussion

These results have crucially identified C100 as a dual-purpose adjuvant, suitable for prophylactic parenteral and therapeutic intratumoral anti-tumour vaccination strategies – paving the way for further development and a wide range of further preclinical and clinical applications.

Prophylactically, C90 and C100 had similar efficacy and further studies with more animals per group may be required to functionally differentiate these polymers. However, Protasan CL213 is far less effective than C90 and C100 in eliciting protective anti-tumour immunity when delivered intramuscularly. Deciphering the correlates of anti-tumour immunity following vaccination with C100 and C90 adjuvanted antigens is an important next step, and this could be achieved through cell depletion and KO mice studies like those described with 50nm PS NPs described previously. CD4⁺ CTLs may prove to be an important consideration in this elicited anti-tumour immunity, they have been documented in numerous *in vitro* and *in vivo* studies utilising B16 models^{80,82,378,379}, and killing has been shown to be via cognate MHC-II recognition, mediated by granzyme B and perforin. It may be possible that prophylactic vaccination with C100 and other highly deacetylated chitin-derived polymers leads to the induction of antigen-specific CD4⁺ CTLs, or that these cells undergo phenotypic alterations towards a CTL phenotype upon tumour encounter.

While there is a lack of detectable antigen-specific CD8⁺ T cell induction following intramuscular vaccination with OVA and C100, it cannot yet be definitively stated that CD8⁺ T cells are not playing a role in this protective immunity. It is possible that the robust OVA-specific CD4⁺ T cell response could play a role in enhancing the priming of OVA-specific CD8⁺ T cell responses following B16-OVA tumour challenge, through CD4⁺ T cell help mediated through DC licensing via cytokine production (IL-2 and IL-21⁷⁸), TCR-MHC-II and CD40-CD40L interactions⁵⁷. The expression of both MHC-I and MHC-II restricted neoantigens has been shown to indicate the most responsive tumours to immunotherapy approaches targeting T cell activation³⁸⁰. While 50nm PS NPs were more effective at preventing tumour establishment, it is possible that with further refinement of dosing and the prime boost regimen, that C100 or C90 may prove to elicit comparable levels of protection. In addition, formulation of these highly deacetylated chitosan polymers into nanoparticles, or into combination with other adjuvants e.g CpG, may improve their efficacy as cancer vaccine adjuvants.

C100 was also demonstrated to be the most effective highly deacetylated chitin-derived polymer for use as an intratumoral therapeutic adjuvant. Dose optimisation experiments were carried out for the intratumoral use of C100, of particular importance as the magnitude of STING

activation by targeted therapeutics has been shown to modulate resultant anti-tumour immune responses³⁶⁷. 100µg was determined to be the optimum dose for intratumoral use, with doses above and below resulting in lower therapeutic efficacy. Additionally, the therapeutic efficacy of C100 was confirmed in two distinct tumour models: MC38 and B16F10. These models differ in terms of mutational burden, immunogenicity and in response rates to immunotherapeutic interventions^{333–335,374} with B16F10 representing a more immunologically cold tumour than MC38. This is reflected in the results presented here, indicating an improved therapeutic response to C100 injection in the MC38 model. Building on previous work conducted in our lab²¹⁹ the utility of measuring mitochondrial stress induction and BMDC maturation as an *in vitro* readout for chitin-derived polymer adjuvanticity was confirmed. C100 was previously identified as the most potent chitin-derived polymer in driving mitochondrial stress and BMDC maturation²¹⁹ and the current work demonstrates that C100 possesses the most potent anti-tumour functionality, clearly demonstrating that the degree of deacetylation is an instructive parameter in a chitin-derived polymers potential as a cancer immunotherapeutic.

Mechanistically dissecting this C100 induced anti-tumour response, it was found that STING expression and IFNAR signalling were essential for the adjuvanticity of C100 *in vivo*, correlating with previous *in vitro* results using BMDCs^{224,286}. Intact type I IFN signalling in the host was identified as essential for C100s mechanism of action, this is not surprising as IFNAR KO mice have been demonstrated to fail to reject immunogenic tumours, correlating with impaired tumour infiltration of DCs and T cells^{252,381}. Type I IFN signalling in DCs has been shown to promote maturation^{382,383}, migration^{384,385}, antigen cross presentation and successful priming of anti-tumour T cell responses³⁸⁶. In addition, type I IFNs have been shown to potentiate the activation and cytotoxic potential of NK and T cells in the context of anti-tumour immunity. The success of C100 therapy in WT mice challenged with STING KO tumours is not surprising as DCs can successfully take up DNA or cGAMP from damaged or dying tumour cells or even through the transfer of cGAMP through tight junctions³⁸⁷. This indicates that C100 could activate cGAS in either the tumour cells or responding immune cells to elicit anti-tumour immune responses. Furthermore, it has been demonstrated that STING expression in host endothelial cells positively correlates with CD8⁺ TILs and enhanced survival³⁸⁸, and STING activation in these cells can promote effective anti-tumour immune responses²⁷⁴, identifying another cell type which C100 could be targeting in the TME. Similar therapeutic observations have been observed using different STING agonists in similar experimental designs³⁶⁷.

The success of C100 therapy in STING KO mice challenged with WT tumours was a surprising observation, as previous studies have demonstrated that STING KO mice lack the capacity to

mount spontaneous immune responses²⁷⁸, with diminished antigen specific CD8⁺ T cell responses observed³⁸⁹. However, it has been documented that functional STING expression and activation in tumour cells themselves not only correlates with increased CD8⁺ T cells in the TME but can promote susceptibility to NK and CD8⁺ T cell mediated killing³⁹⁰. This likely relies on the promotion of type I IFN signalling and chemokine production by the tumour cells themselves³⁹¹ and associated immune cell recruitment to the TME. In line with this, STING deficient tumours have been shown to express less CCL5 and CXCL10 which are essential for the infiltration of both CD4⁺ and CD8⁺ T and NK cells^{392,393}. However, other groups have observed that despite production of type I IFN by tumour cells, activation of STING by host immune cells was required to mount anti-tumour T cell responses²⁷³.

C100's observed therapeutic non-reliance on STING expression restricted to the host immune cell compartment, may be beneficial in a clinical setting as it is documented that most tumours retain cGAS and STING expression, although a caveat being that tumours can inactivate these proteins or reduce their activity post translation.

CD8⁺ T cells were identified as crucial mediators of C100-induced anti-tumour immunity in the MC38 tumour model, this is in line with the literature surrounding the use of STING agonists therapeutically in a cancer setting including Cgamp²⁷⁴, other synthetic CDNs³⁶⁷ and non-CDN agonists³⁹⁴.

The capacity of C100 to enhance the therapeutic efficacy of PD-1 blockade is particularly promising. Despite increases in the cohorts of patients eligible for checkpoint blockade therapy, there remains significant room for improvement in terms of response rates, with many patients developing resistance to therapy and subsequently relapse^{18,66}. Response to ICB has been shown to correlate with a number of factors including the immune contexture of tumours, providing prognostic value^{66,395}. This largely hinges on the presence of an existing, intact tumour intrinsic or circulating anti-tumour adaptive immune response involving CD8⁺ T cells or Th1 cells⁶⁶. Accordingly, patient/tumour stratification has been proposed, utilising immune parameters, and this has had success clinically in predicting response rates to ICB. According to this scoring method, immunologically 'cold' tumours, with low levels of tumour infiltrating lymphocytes (TILs) typically have lower response rates to ICB, while immunologically 'hot' tumours, with significant population of TILs have optimal response rates to ICB. Therefore, therapeutic regimens that culminate in the immunological heating up of tumours are attractive and may synergise with ICB⁶⁶. The current data indicate that intratumoral C100 therapy could be used both as a monotherapy but also in combination strategies aiming to sensitise tumours to respond to ICB through priming anti-tumour CD8⁺ T cell responses. This could be a broadly

applicable treatment option, as expression of STING in the tumour cells themselves, or in host cells is sufficient for therapeutic efficacy once IFN signalling pathways in the host remain intact.

Although there was no significant role for NK cells identified in the therapeutic efficacy of C100 in the MC38 model, there remains a possibility that NK cells could mediate therapeutic efficacy in the B16 tumour model. Digestion of tumours from mice treated with C100 or C100 combined with α -PD1 therapy indicated an increase in NK cell infiltration, which may be indicative of the role of NK cells here. This is in line with data showing that NK cells can mediate anti-tumour effects associated with α -PD1 blockade in poorly immunogenic tumours^{23,396}. B16 tumours have relatively low immunogenicity in terms of MHC class I molecule expression^{334,335}, rendering the tumour cells insensitive to CD8⁺ T cell mediated killing and increasing sensitivity to that mediated by NK cells. C100 has been shown to induce DNA damage in cells²⁸⁶, which could result in the upregulation of activatory NK cell ligands on the cell surface such as RAET1, MULT1 and H60a which serve as activatory ligands for NKG2D in mice²¹. This, coupled to the documented downregulation of MHC-I molecule expression in B16 cells, may contribute to increased susceptibility to NK cell mediated cell killing following C100 exposure^{21,397}. In addition, activation of cGAS-STING signalling in the TME has been shown to promote NK cell activation via type I IFN signalling²⁵. This has been shown to be dependent on cGAS but not STING expression in tumour cells, with cGAMP production occurring in response to DNA leakage into the cytosol of tumour cells. Intact STING was required by non-tumour cells, as cGAMP can be passed from tumour cells to other cells in the TME via tight junctions and exosomes²⁷². This bodes well for C100 mediated activation of NK cells in the TME and circumvents issues that may arise from downregulation of STING in tumour cells themselves. NK cells also contribute to the recruitment of Cdc1s intratumorally, via the production of FLT3L⁴¹. These Cdc1s play an integral role in mounting anti-tumour CTL responses and are proficient in trafficking tumour antigen to the draining lymph nodes³⁹, their abundance intratumorally is correlated with an increased responsiveness to immunotherapeutics targeting the PD-1/PD-L1 axis^{39,41}. A NK cell depletion experiment in the B16 model is currently being planned, unfortunately this work could not be completed prior to thesis submission due to expiration of the laboratory's HPRA project licence.

Altogether our data suggests that C100-mediated activation of the cGAS-STING pathway in tumour cells or host immune cells is sufficient to promote anti-tumour immune responses via type I IFN production. Fundamentally this requires IFNAR expression on host cells to ultimately prime anti-tumour CD8⁺ T cells to mediate anti-tumour effects, with a caveat being that the cellular mediators of protection may be tumour phenotype dependant.

Chapter 6 – General Discussion

Successful vaccination strategies have the capacity to reduce the mortality and morbidity associated with their targeted disease. Over the last few years, we have been reminded not only of the importance of vaccine research as a whole but of the importance of having a broad vaccination toolset, comprising multiple vaccination technologies and approaches. Effective therapeutic and additional prophylactic cancer vaccines would be a welcome addition to our vaccine arsenal, with potential to both prevent and treat cancers and complement existing immunotherapeutic approaches. The identification of adjuvants suitable for cancer vaccination approaches, through rational design principles, is an essential step in their development and this project aims to characterise the potential of 50nm NPs and highly deacetylated chitosan polymer adjuvants in a cancer vaccination setting. However, it should be noted that applications of these adjuvants are not just limited to cancer vaccines, as cancer and many pathogens share similar protective immune responses e.g., the importance of antigen-specific CD8⁺ CTLs and IFN γ in anti-viral and anti-tumour immunity.

For example, in this project 50nm NPs have been identified as potent vaccine adjuvants for driving functional cellular immune responses and while the functionality of responses has been validated here in an anti-tumour context, these adjuvants may have a broad range of vaccine applications, including those targeting viral and other intracellular pathogens. These include diseases with unmet vaccination needs, with the prominent examples of respiratory syncytial virus (RSV), M.tb and malaria. RSV causes lower respiratory tract infections worldwide, especially in young and elderly cohorts³⁹⁸. There is no approved vaccine for use in humans, although there are multiple candidates in clinical trials including GSKs AS01 adjuvanted RSV-OA and Pfizer's unadjuvanted RSVpreF protein based vaccines which have received 'Breakthrough therapy designation' in the US and have nearly completed phase III clinical trials³⁹⁹. In terms of correlates of vaccine induced protection, neutralising antibodies have been shown to be protective in infants, with IFN γ ⁺ Th1 and CD8⁺ T cell responses additionally implicated in protection in older individuals¹¹⁶. M.tb is endemic in low income regions and it is estimated that 1/4 of the world's population is latently infected⁴⁰⁰. In 2019 alone, 1.4 million people died from TB⁴⁰¹. Poor vaccine efficacy in adults is a major obstacle to global efforts aimed at reducing M.tb related deaths by 95% by 2035⁴⁰². However, an AS01_E adjuvanted protein subunit TB vaccine from GSK (now bought by IAVI), GSK692342, was recently reported to be 54% effective, warranting further phase III trials⁴⁰³ ((NCT01755598) and the other vaccine candidates, MTBVAC⁴⁰⁴ and VPM1002⁴⁰⁵, have been suggested as possible replacements for BCG vaccination with phase III trials underway. Polyfunctional IFN γ ⁺ Th1 cells, IFN γ ⁺ CD8⁺ T cells and neutralising IgG antibodies have been suggested as correlates of vaccine induced protection, although these

correlates have traditionally been a challenge to establish for M.tb⁴⁰³. Malaria represents another pathogen that has proved difficult to combat through vaccination, in 2019 there were an estimated 230 million cases and roughly 400,000 deaths⁴⁰⁶, and similar to RSV, children under 5 are at the highest risk of disease. The AS01 adjuvanted RTS,S vaccine from GSK represents one the most successful vaccine candidate to date with 30% efficacy, eliciting predominantly antigen-specific antibodies and to a lesser extent CD4⁺ T cell responses⁴⁰⁷. Based on these results, the WHO recommended a broad rollout in sub-Saharan Africa. However recently another protein subunit vaccine candidate, the Matrix M adjuvanted R21 vaccine developed at the University of Oxford, demonstrated 77% efficacy in a 12 month vaccination follow up study, associated with impressive antibody titres¹⁷⁵ with follow up trials suggesting seasonal booster vaccinations to maintain immunity¹⁷⁵. On the back of these exciting results, this R21/MM vaccine candidate is undergoing further phase II/III clinical trials. Correlates of vaccine induced protection have been difficult to fully decipher for malaria, but because of its complex life cycle in the human host, a varied immune response is likely needed for optimum protection⁴⁰⁷ and this would likely be composed of antigen specific antibodies, IFN γ ⁺ Th1 cells and CD8⁺ CTLs. Our lab group has previously demonstrated that 50nm NPs elicit robust neutralising antibody titres²⁸⁹ alongside durable antigen-specific IFN γ ⁺ Th1 and CD8⁺ T cell responses, with functionality confirmed via tumour models. As a result, 50nm NPs have been identified as not only suitable adjuvants for cancer vaccine approaches but also potentially suitable candidate adjuvants for vaccination approaches targeting RSV, M.tb and malaria amongst other pathogens. It will be crucial to determine if adjuvanticity is retained in aged and neonatal mice, to infer whether vaccination in these cohorts will be successful – as these age groups often constitute the most at-risk populations.

Crucially 50nm NP adjuvanticity has been successfully validated when using a biodegradable and biocompatible polymer – PLGA. This is a crucial step towards clinical translatability as PS NPs are unlikely to be approved for clinical use due to a lack of biodegradability and subsequent concerns over safety and long-term health effects. Styrene, the monomeric component of polystyrene has been classed as a potential carcinogen by the IARC⁴⁰⁸ but this has not been established for polystyrene. PS NP are frequently found in humans and other organisms, obtained from environmental sources, predominantly in the food chain⁴⁰⁹. However effects of their accumulation are controversial with some reports showing ill effects on health, predominantly of the digestive, respiratory and nervous system and others showing no impact on health whatsoever⁴⁰⁹. However, to avoid such potential issues, PLGA is an attractive alternative as it is readily broken down into lactic and glycolic acid through hydrolysis *in vitro*

and *in vivo* and has an excellent safety track record with FDA approval for use in humans in a wide range of clinical applications including drug delivery. Additionally, the PLGA NPs produced originally by Prof Yvonne Perries lab, and now the Vaccine Formulations Institute (VFI) in the University of Geneva, can be freeze dried without impacting particle size and adjuvanticity, this is an important practical and logistical feature as PLGA particles have poor stability in suspension. It is worth noting that these 50nm NPs are also compatible with filter sterilisation techniques frequently utilised in industrial production of vaccines. In addition, another key advantage is that our formulations do not require complex antigen addition, with simple admixing techniques used. This can be done shortly before vaccination and does not require extensive training of personnel.

Moving forward it will be essential to repeat all tumour challenge experiments conducted using 50nm NPs made of PLGA instead of PS, this was unfortunately not feasible in this project due to issues with consistent supply of these PLGA adjuvants. This supply issue has recently been addressed with a new collaboration with the VFI.

Another crucial step in rational vaccine adjuvant design is resolving an adjuvants MOA, and the current thesis has contributed to demonstrating the importance of components of the canonical and non-canonical pyroptotic pathway in the adjuvanticity of 50nm NP, tying in both *in vitro* and *in vivo* observations. Particles larger than 50nm in size fail to induce significant levels of cellular ROS, GSDMD cleavage and cellular death *in vitro* and this correlates with a lack of induction of cellular immune responses *in vivo*. Based on these findings, it can be hypothesised that an endogenous ligand for caspase 11 is responsible for mediating cell death, and this ligand remains to be identified. Oxidised phospholipids (oxPAPCs) which are generated and accumulate in damaged/dying tissues have been shown to activate caspase 11²⁴², and this is in line with our results demonstrating the importance of local ROS generation at the injection site in 50nm NP adjuvanticity. Additionally, cellular ROS can sensitise GSDMD for subsequent activation²³⁷. To determine whether oxPAPCs are responsible for caspase 11 activation in the current setting, mass spectrophotometry techniques could be utilised to characterise caspase 11-ligand complexes, or pull-down assays could be utilised with immobilised caspase 11 to create an affinity column⁴¹⁰ with subsequent ligand purification. Although oxPAPCs have been identified as caspase 11 ligands, primarily in BMDCs, they have been documented to elicit hyperactivity and not pyroptotic cell death²⁴². This is characterised by GSDMD pore formation and the sustained release of IL-1 family cytokines, and these hyperactive DCs have been shown to be able to mount potent anti-tumour adaptive immune responses^{242,411}. Therefore, it remains a possibility that the 50nm NP mediated induction of cellular hyperactivity and not cell death

contributes to adjuvanticity *in vivo*. Experiments designed to track 50nm NPs *in vivo* and detect cell death locally at the injection site and in draining lymph nodes via methodologies such as TUNEL staining will be important going forward. These experiments will aim to determine the precise role of cell death elicitation, the kinetics of particle uptake and induction of cytotoxicity, in addition to identifying the key cell types involved in 50nm NP adjuvanticity.

Nevertheless, the insights into 50nm NP MOA provided by this thesis present potential strategies to enhance adjuvanticity and this is a key concept of rational vaccine adjuvant design. For instance, the IL-1 family DAMP, IL-33 was previously shown to negatively regulate 50nm PS NP mediated immune responses²⁶³. Therefore, it is possible that co-administration of 50nm NPs with an IL-33 blocking mAb may therefore provide a feasible means to improve adjuvanticity. Similarly, this thesis shows that blockade of local pyroptosis through chemical inhibition abrogates adjuvanticity, therefore it is plausible that strategies to potentiate pyroptotic signalling could improve adjuvanticity. This could be achieved through coadministration of 50nm NPs with LPS or its non-toxic derivative MPL-A. Caspase 11 binds to LPS via CARD domain interactions with the LPS Lipid A moiety⁴¹², however, although MPL-A is composed of a detoxified lipid A LPS region, it remains to be determined if it can activate caspase 11. Similarly IFN γ has been shown to predispose cells to die via pyroptosis, with guanylate binding proteins (GBP)⁴¹³ and other unknown factors⁴¹⁴ implicated in this effect, so co-delivery of IFN γ could constitute another strategy to potentiate adjuvanticity. If instead, nanoparticle induced cellular hyperactivation is revealed, then co-administration with oxPAPCs or individual oxPAPCs components^{242,411} could be explored to potentiate 50nm NP mediated immune responses. Although the immune responses observed following intramuscular vaccination with OVA and 50nm NPs are robust and provide almost complete protection from tumour establishment, further increasing the durability and magnitude of immune responses may have important applications when utilising therapeutic cancer vaccination approaches.

The data presented in the thesis identify 50nm NP nanoparticles as potent adjuvants for use in prophylactic cancer vaccination approaches, with this class of vaccines including the licenced and highly effective HPV and HBV vaccines. There remains an unmet need for vaccines targeting other oncogenic viruses such as Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). While the identification of suitable antigenic targets has complicated their development, bioinformatic and reverse vaccinology approaches can provide solutions here⁴¹⁵. However, there remains a need for suitable adjuvants to drive antigen specific IgG, IFN γ ⁺ Th1 and CD8⁺ CTL responses required to prevent/control these infections, with CD8⁺ CTLs particularly

important for the control of EBV⁴¹⁶, supporting the rationale for use of 50nm NPs as vaccine adjuvants in these prophylactic settings.

Cancer vaccines however have a much broader application in the treatment of existing disease either alone or in combination approaches with other therapies⁸⁴. The results presented here demonstrate the suitability of 50nm NP adjuvants for use in these therapeutic approaches, successfully mounting effective anti-tumour adaptive immune responses when mice with established tumours were vaccinated via the intramuscular route. As highlighted in Section 1.2 there is a need for more adjuvants that can drive CMI, in particular antigen specific CD8⁺ T cells. In addition, it may be beneficial to include adjuvants that can promote the generation of antigen specific T cells with a high degree of 'stemness' in cancer vaccines. These 'stem-like' T cells have been shown to correlate with improved anti-tumour immunity and response to immunotherapeutic interventions^{74,76,84,418}. This is line with data presented in this thesis showing synergistic effects between 50nm NP adjuvanted cancer vaccination and systemic α -PD1 therapy. As such 50nm NP are proposed as suitable alternative adjuvants for these cancer vaccine approaches. Further work should be conducted to expand therapeutic experiments with 50nm NPs in different tumour models and other immunotherapeutic strategies. Additionally, the phenotype of CD8⁺ TILs induced following therapeutic 50nm + OVA vaccination should be determined through FACs and sequencing approaches which may further inform applicable therapeutic combination strategies.

This project has also characterised the highly deacetylated chitin-derived polymer, C100, as a potent, dual purpose cancer vaccine adjuvant capable of eliciting protective immune responses when used prophylactically and therapeutically. Although prophylactically, the polymers C90 and C100 had similar efficacy, *in vitro* studies suggest that C100 is a more effective adjuvant^{219,224}. Protasan CL213 on the other hand was outperformed by C100 in both prophylactic and therapeutic settings. Prophylactically, C100 adjuvanted vaccine-induced protection likely depends on the robust antigen specific IFN γ (Th1) responses that are elicited; however, this would have to be confirmed through cell depletion strategies and the use of IFN γ KO mice for experimentation. As previously mentioned, it is possible that CD4⁺ CTLs, documented in numerous *in vitro* and *in vivo* studies utilising B16 models^{80,82,378,379} and in human cancer biopsies⁸³ may prove to be an important consideration in this C100 elicited anti-tumour immunity.

However, prophylactically C100 is less effective in eliciting anti-tumour protective immune responses when compared to 50nm NP adjuvants, while the adjuvant yielded exciting results in

an intratumoral therapeutic setting. As such, further investigation into the therapeutic capacity of C100 was prioritised.

Therapeutic *in situ*/intratumoral vaccinations rely on the release of antigen locally following injection³⁶¹, which can be effectively achieved through induction of cell death^{227,234,362} and promotion of immune cell recruitment and activity. Additionally, cell death mediated by CD8⁺ T cells has been shown to be a form of ICD in murine models, resulting in the induction of further anti-tumour immune responses, with caspase 3 being identified as a crucial mediator of this effect⁴²⁰. The capacity of CD4⁺ CTLs and NK cells to promote such ICD is less well defined, but likely results in similar effects due to shared cell death effector pathways.

The use of chitin-derived polymers as intratumoral therapeutics is a novel concept, Protasan G 213 has been included as a delivery platform before in the context of intratumoral cytokine (IL-12) delivery in a mouse tumour model⁴²¹, however there was no observed therapeutic effect of Protasan G 213 alone on tumour growth. Here it is shown in a therapeutic tumour model, that intratumoral delivery of C100 outperformed the systemic administration of the routinely used chemotherapeutic cisplatin, and C100 was demonstrated to have greater efficacy when compared to C90 and Protasan CL213. Crucially, C100 was also observed to exert a somewhat comparable therapeutic effect to CpG. This is an impressive result as SD-101, a CpG compound, has recently been trialled clinically as a standalone intratumoral therapeutic, and in combination with checkpoint therapies. C100 may have an advantage over CpG/SD-101, in terms of mouse to human translatability, in that the expression of TLR9 is far more restricted in humans when compared to mice⁴²² as human TLR9 expression is generally restricted to the myeloid cell compartment, and as such anti-tumour effects relying on the expression of TLR9 in tumour cells or other cells of the immune system may be blunted. In contrast the expression of cGAS, STING and IFNAR is far less restrictive, and additionally both STING signalling and type I IFN production have been shown to be crucial for the spontaneous priming of cellular anti-tumour immunity mediated by the innate immune system^{275,276,278} and endothelial cells²⁷⁴.

As highlighted in section 1.4, STING agonists are attractive therapeutics for use intratumorally but yet have yielded underwhelming results clinically. C100 may be a suitable cGAS-STING alternative to these suboptimal agonists, which as previously highlighted does not rely on direct binding to cGAS and STING, but instead relies on the release and accumulation of endogenous DNA (Both mitochondrial and nuclear in origin)²⁸⁶ which activate cGAS, leading to the subsequent production of 2'3'-cGAMP and STING activation. This may circumvent issues that have plagued STING agonists clinically, whereby mouse-human translation in terms of STING binding is suboptimal²⁸², and differences in STING haplotypes in human populations can

complicate the affinity of STING binding^{283,423}. Importantly, C100 polymers are readily taken up into cells. In addition the chitosan polymer with high degrees of deacetylation are less susceptible to enzymatic degradation⁴²⁵, which may indicate a favourable stability profile. Furthermore, C100 mediated activation of STING does not result in concomitant activation of NFκB signalling²⁸⁶, and this is unique when compared to other STING agonists. STING mediated activation of NFκB is thought to predominantly occur at the ER, as blockade of STING translocation to the ERGIC/perinuclear compartment successfully blocks optimum type I IFN production, but not NFκB activation^{426–428}. This may be of significant value in an intratumoral therapeutic setting, where activation of NFκB signalling in tumour cells may exert pro tumourigenic effects through inhibition of cell death pathways, upregulation of checkpoint molecules⁴²⁹, promotion of tumour angiogenesis and epithelial-mesenchymal transition (EMT)⁴³⁰.

Dose optimisation experiments have indicated that 100µg of C100 is the optimum dose for use intratumorally. This may prove to be due to achieving the correct balance between the C100 mediated induction of cell death and the immune modulation achieved presumably via production of type I IFNs, CXCL10, IL-1β^{218–220} in the TME. While intratumoral injection of C100 successfully delayed tumour growth and prolonged mouse survival, there were only a low occurrence of complete tumour regression. In part this may be due to the aggressiveness of B16 and MC38 tumour growth, but it may also be highlighting the possible benefit of extended treatment where mice could receive more than 3 intratumoral doses of C100. This sustained treatment may provide enhanced therapeutic efficacy if death of responding immune cells in the TME is avoided. To further refine dosing schedules, the kinetics of immune cell recruitment to the TME should be characterised.

Furthermore, it may be of interest to determine if C100 can effectively induce tumour cell ICD via the gold standard ICD vaccination assay^{225,227,234}, where tumour cells killed via C100 stimulation *in vitro* will be used to vaccinate mice. If ICD is apparent, mice will be protected from subsequent tumour challenge^{225,227,234}. Additionally, it will be important to determine if tumour cells have an increased susceptibility to C100 mediated death rather than immune cell populations *in vivo*. It is hypothesised that due to high basal levels of DNA damage, that tumour cells may have increased susceptibility. Additionally, the use of C100 as an intravenous anti-tumour therapeutic could be examined.

Crucially the MOA of C100 has now been well defined through *in vitro* and *in vivo* experimentation and validation^{224,286}. Results indicate that activation of the cGAS-STING

signalling pathway in host or tumour cells and subsequent signalling via IFNAR signalling in immune cells is crucial for therapeutic efficacy. The cellular mediators of this protection may be tumour phenotype context dependent, with CD8⁺ T cells identified as the cellular mediators of protection in MC38. Unfortunately, due to a lapse in time in the labs animal licence approval it was not possible to carry out an NK cell depletion experiment in B16 challenged mice, but this will be prioritised upon licence approval. In the case of B16 tumours, NK cells may be playing a more important role in C100 mediated anti-tumour immune responses. Moving forward, components of the NK-cDC1-CD8⁺ axis of tumour infiltration will be examined

Through experiments combining intratumoral C100 therapy with systemic PD-1 blockade, it was demonstrated that C100 may be a suitable therapeutic for immunologically 'heating' up tumours. A significant proportion of mice receiving both C100 and α -PD1 displayed full tumour regression, and sustained treatment could further improve the outcome. It will be important to characterise the effect of C100 on the expression of checkpoint molecules on host immune cells, as therapeutic efficacy is reliant on the expression of these coinhibitory molecules on TILs^{431,432}. These results may infer future/applicable combinatorial treatment regimens. An excellent example of this proposed strategy is demonstrated by Sagiv-Barfi et al¹⁵⁰, where OX-40 upregulation was noted following CpG treatment, and combination strategies with intratumoral CpG and systemic OX-40 agonist mAbs were demonstrated to drive potent systemic anti-tumour immunity. The ability of C100 to sensitise tumours for response to subsequent checkpoint inhibitor therapies will be conducted in the less responsive B16 tumour model upon animal licence approval. building on promising results in a preliminary pilot experiment. This would be an important validation as the key tumours where these combination approaches may improve response rates are those that are immunologically 'cold'⁶⁶.

There is also potentially an application for combination strategies utilising 50nm NPs and C100 therapeutically. Intratumoral C100 therapy could be combined with subsequent delivery of 50nm NPs intratumorally, or targeted 50nm delivery to tdLNs with an aim to mount potent adaptive immune responses to the C100-released tumour antigens.

In summary, this project has characterised the potent anti-tumour functionality of two vaccine adjuvants – 50nm PS NP and C100. The potential applications of 50nm NP adjuvants in prophylactic and personalised therapeutic cancer vaccines, and of C100 in 'off-the-shelf' *in situ* cancer vaccines were successfully demonstrated. Components of the pyroptotic pathway/non-canonical inflammasome have been identified as essential for 50nm NP adjuvanticity. Additionally, the mediators of 50nm NP induced anti-tumour protection have been identified and the unique capacity of 50nm NPs to drive antigen specific 'stem-like' effector CD8⁺ T cells

has been characterised. C100 has been identified as a dual-purpose cancer vaccine adjuvant, and as the most effective highly deacetylated chitosan polymer candidate for these approaches. IFNAR signalling in the immune compartment and STING activation were identified as crucial for the therapeutic efficacy of C100 *in vivo* following intratumoral administration. Importantly, both intratumoral C100 therapy and intramuscular vaccination with 50nm NPs and tumour antigen were shown to synergise with checkpoint blockade and these results highlight the potential combination strategies between vaccination with these adjuvants and other immunotherapeutic interventions.

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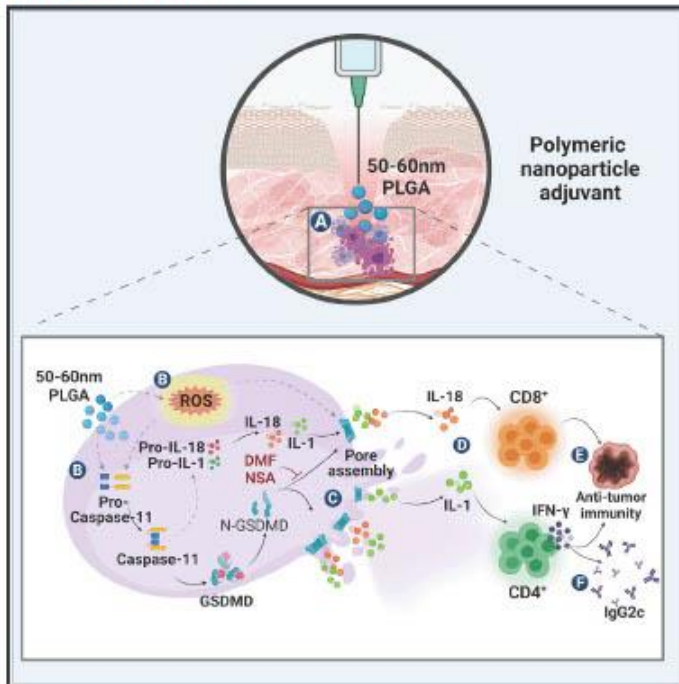
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Appendix

Non-canonical inflammasome activation mediates the adjuvanticity of nanoparticles

Graphical abstract



Authors

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In brief

Muñoz-Wolf et al. show that particle size dictates the induction of cell-mediated immunity by polymeric nanoparticulate adjuvants, identifying 50–60 nm as the optimal size for induction of Th1/CD8⁺ responses. Authors provide mechanistic insights uncovering the roles of caspase-11, gasdermin D, and radical oxygen species in nanoparticle adjuvant-induced **Q11** cell-mediated immunity.

Highlights

- Adjuvant particle size dictates induction of Th1/CD8⁺ responses but not antibodies
- 50- to 60-nm polymeric particles optimally induce cell-mediated (CMI) responses
- ROS, caspase-11, and gasdermin D are key mediators of nanoparticle-induced CMI
- Biodegradable 50- to 60-nm PLGA particles are promising CMI-inducing adjuvants

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Article

Non-canonical inflammasome activation mediates the adjuvanticity of nanoparticles

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SUMMARY

The non-canonical inflammasome sensor caspase-11 and gasdermin D (GSDMD) drive inflammation and pyroptosis, a type of immunogenic cell death that favors cell-mediated immunity (CMI) in cancer, infection, and autoimmunity. Here we show that caspase-11 and GSDMD are required for CD8⁺ and Th1 responses induced by nanoparticulate vaccine adjuvants. We demonstrate that nanoparticle-induced reactive oxygen species (ROS) are size dependent and essential for CMI, and we identify 50- to 60-nm nanoparticles as optimal inducers of ROS, GSDMD activation, and Th1 and CD8 responses. We reveal a division of labor for IL-1 and IL-18, where IL-1 supports Th1 and IL-18 promotes CD8⁺ responses. Exploiting size as a key attribute, we demonstrate that biodegradable poly-lactic co-glycolic acid nanoparticles are potent CMI-inducing adjuvants. Our work implicates ROS and the non-canonical inflammasome in the mode of action of polymeric nanoparticulate adjuvants and establishes adjuvant size as a key design principle for vaccines against cancer and intracellular pathogens.

Q3 Q4 Q7 INTRODUCTION Q8 Q2

Vaccination prevents an estimated 6 million deaths per year globally and allows enormous savings in medical care costs and infection-related disability.¹ Vaccine-induced protection has been mainly linked to potent and durable humoral immunity that can effectively control pathogens with a relatively stable antigen repertoire and associated toxins.² However, emerging viruses like SARS-CoV-2,³ infections caused by other intracellular pathogens, and cancer⁴ are more effectively controlled by CD8⁺ cytotoxic T lymphocytes (CTL) and IFN- γ -producing T helper 1 (Th1) lymphocytes alone, or together with protective antibodies. Therefore, induction of CMI remains a critical frontier for modern vaccinology.

Subunit vaccines based on purified antigens require adjuvants to boost their immunogenicity and efficacy. However, while the particulate adjuvants in licensed subunit vaccines effectively promote antibody responses, they do not induce optimal cell-mediated immunity (CMI).⁵ Several inorganic and polymeric nano- and microparticles have shown promise as CMI-inducing adjuvants in preclinical studies^{6–11}; however, the use of formulations containing particles with dissimilar physicochemical attributes, modification of their surface, and the use of co-adjuvants have yielded contradictory results.¹² Our understanding of how physicochemical characteristics of particles (i.e., size, surface charge, topography, shape, etc.) influence the magnitude and type of adjuvant-induced immune responses and their mode of action (MOA) is therefore limited.¹³ Identifying the design

principles that link particulate adjuvant characteristics to immune activation, particularly strong CMI, could transform particulate adjuvant design and accelerate vaccine development for intracellular pathogens and cancer.

Unlike pathogens, inorganic particulate vaccine adjuvants including alum and synthetic polymeric particles lack inherent ligands to engage pathogen recognition receptors such as Toll-like receptors (TLRs) that can activate antigen-presenting cells (APCs) to prime cognate naive T cells. Instead, their adjuvanticity is thought to rely on damage/danger-associated molecular patterns (DAMPs) and inflammatory cytokines released in response to cell stress or death. Cellular stress and immunogenic cell death lead to the release of IL-1 family cytokines that act as inducible DAMPs,¹⁴ enhancing APC maturation, naive T cell priming, and T cell effector functions.^{9,15–18} However, their roles in adjuvant-induced T cell responses are highly context dependent and not fully understood.¹⁹

Regulated immunogenic cell death (ICD) pathways, including pyroptosis and necroptosis, contribute to the release of the IL-1 family cytokines, IL-1 α , IL-1 β , and IL-18. Unlike necrosis, which typically leads to sudden DAMP release and transient inflammation, regulated ICD promotes the coordinated synthesis and activation of inducible DAMPs including pro-IL-1 β and pro-IL-18, prior to cell death. This is crucial to provide the appropriate immunogenic environment for T cell activation.²⁰ While secretion of IL-1 and IL-18 has been traditionally linked to the activation of canonical caspase-1 inflammasomes, a non-canonical inflammasome using caspase-11 as a sensor has been also described.²⁰ Both canonical and non-canonical inflammasomes converge on the activation of pro-IL-1, pro-IL-18, and gasdermin D. GSDMD, the executor of pyroptosis, forms membrane pores facilitating release of IL-1, IL-18, and other leaderless proteins.²¹ In addition, cellular stress leading to production of reactive oxygen species (ROS) has been linked to formation of the GSDMD pores and resultant pyroptosis.²² Different types of cell death including necrosis,²³ apoptosis-like death,²⁴ and caspase-independent cytotoxicity²⁵ have been linked to adjuvant MOA. However, the role of regulated ICD downstream of non-canonical inflammasome activation has not been addressed for particulate adjuvants.

Using biocompatible polystyrene and biodegradable polylactic co-glycolic acid (PLGA) particles (50 nm–30 μ m), we demonstrate that size critically determines the ability of particles to induce antigen-specific T CD8⁺ and Th1 responses, without affecting their ability to induce antibodies. We unequivocally identify 50–60 nm as the optimal particle size range for eliciting specific Th1 and CD8⁺ T cells to subunit protein antigens and show that vaccination with 50-nm particles induced a long-lasting and functional response that protected mice from B16-OVA melanoma. We show that the non-canonical inflammasome sensor caspase-11 and the pyroptosis effector GSDMD are essential components of nanoparticle-induced Th1 and CD8⁺ T cell responses. We uncover a clear division of labor for the IL-1 family cytokines with IL-1 supporting Th1 and IL-18 promoting CD8⁺ T cells responses. Finally, we show that polymeric nanoparticle-induced GSDMD activation and CMI after intramuscular vaccination are linked to ROS induction, which is a

size-dependent phenomenon promoted by 50- to 60-nm particles.

RESULTS

Particle size critically determines adjuvant-induced CD8⁺ T cell responses

Polymeric particulate adjuvants have exciting potential to enhance the effectiveness of subunit vaccines. While the ability of such adjuvants to enhance antibody responses is well established, their ability to promote CMI is ambiguous,¹² potentially as a result of a lack of consistency in the physicochemical properties of the particles tested. Size is the most basic particulate attribute that influences immunostimulatory properties,^{12,26} but precisely how particle size influences the type and quality of adjuvant-induced immune responses remains unclear. For example, some reported micron-sized polymeric PLGA particles (~1 μ m) as more effective enhancers of antibody responses than nanoparticles,²⁷ whereas others found similar antibody responses for PLGA particles between 110 and 900 nm.²⁸ Reports on induction of CTLs and Th1 responses have also been contradictory, depending on the system used.^{12,29–31}

Taking advantage of their monodisperse and biocompatible nature, we used endotoxin-free, negatively charged polystyrene (PS) particles ranging from 50 nm to 30 μ m to establish the role of size in CMI induction in an intramuscular (i.m.) prime-boost regime with ovalbumin (OVA). Antigen (ag)-specific CD8⁺ T cell responses were quantified by flow cytometry using H-2 Kb OVA SIINFEKL epitope tetramers (Figure S1A). Remarkably, only 50-nm particles significantly increased ag-specific CD8⁺ T cells in spleens of vaccinated animals (Figures 1A–1C) and enhanced ag-specific secretion of IFN- γ in splenocytes (Figure 1D), which we previously demonstrated was CD4⁺ T cell mediated.⁹ In addition, 50-nm NPs outperformed both CpG 1826 and 2'3'cGAMP in eliciting antigen CD8⁺ T cells (Figures S1B and S1C), and elevated class I tetramer⁺ frequencies were also observed in magnetically sorted CD8⁺ T cells (Figure S1E), suggesting that tetramer staining efficacy is dependent on sample composition. In contrast, all particles enhanced ag-specific IgG (Figure 1E), and this was comparable to responses following vaccination with antigen and CpG 1826 or 2'3'cGAMP (Figure S1E). Interestingly, ag-specific secretion of IL-10 was noted in splenocytes of animals vaccinated with 50-nm particles and restimulated with OVA (Figure S2A). This cytokine, although regulatory, has been found to enhance CD8⁺ responses, anti-tumor immunity, and vaccine efficacy when present just after booster vaccination.³² In contrast, IL-17 was not detected for any particle size (Figure S2B).

To further confirm that size was the main determinant of particle-induced CMI, we used the same formulations in an intraperitoneal (i.p.) vaccination protocol since vaccination route can impact factors regulating CD8⁺ T cell cross-priming, including the kinetics of antigen and adjuvant transport and subsets of APC being targeted.^{33,34} Only 50-nm particles elicited a robust ag-specific CD8⁺ response via the i.p. route (Figures S3A and S3B), confirming the importance of size for CMI induction independent of vaccination route, whereas all particle sizes induced strong IgG responses (Figure S3C).

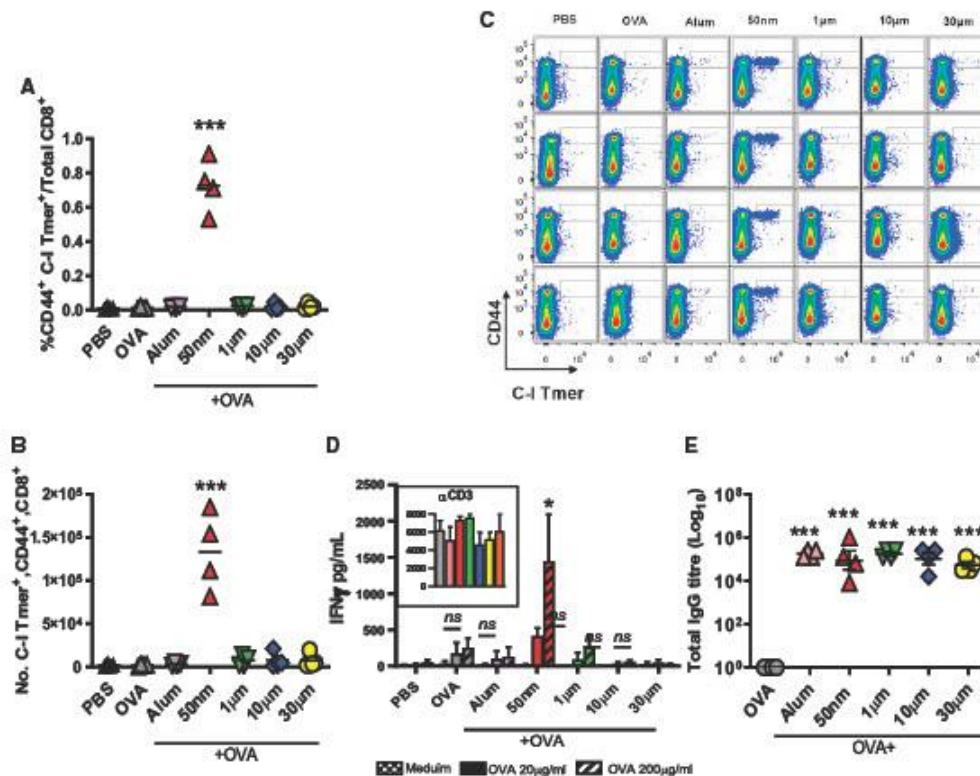


Figure 1. Particle size dictates the induction of T CD8⁺ and IFN-γ responses

Effector CD8⁺ T cells specific to the H-2 Kb OVA epitope SIINFEKL (C-I Tmer) were quantified tetramers 7 days after booster.

(A and B) Percentage of CD44⁺ Tmer⁺ cells over total CD8⁺, CD8⁺, and total numbers in spleens.

(C) Representative dot plots for SIINFEKL-specific CD8⁺ and CD44⁺.

(D) IFN-γ in splenocyte supernatants after ex vivo stimulation with OVA for 72 h.

(E) Total OVA-specific IgG titers in serum 7 days after booster. Statistical differences were calculated per multiple comparison ANOVA and Tukey-Kramer post-hoc test to determine significant differences between all groups on Log₁₀ data. Representative of two experiments, n = 4/group.

Small nanoparticles elicit CD4⁺ T cell responses and long-term memory after vaccination

CD4⁺ T cell help during CD8⁺ lymphocyte priming enhances CD8⁺ memory and long-term protection.^{35,36} Due to the poor performance of OVA I-A(b) tetramers, we took advantage of the influenza A nucleoprotein peptide QVYSLIRPNENPAHK (NP₃₁₁₋₃₂₅), a known class II restricted epitope in C57BL/6 mice, to evaluate the influence of particle size on ag-specific CD4⁺ responses. Clinical grade alhydrogel (alum), an effective inducer of CD4⁺ T cell responses,³⁷ was used as positive control. Particles in the 50-nm range were the only adjuvant to induce a potent ag-specific CD4⁺ T cell response along with alum (Figure S4A).

To simultaneously evaluate T CD8⁺ and CD4⁺ effector and memory responses driven by 50-nm PS particles, we generated a fusion antigen by covalent linkage of NP₃₁₁₋₃₂₅ to the OVA protein (OVA-NP₃₁₁₋₃₂₅). The response was measured 7 days after i.m. booster. Co-administration of the fusion antigen with 50-nm particles significantly enhanced CD4⁺

(Figure S4B) and CD8⁺ T cell responses (Figure S4C) compared with the antigen alone. Importantly, ag-specific CD8⁺ T cells remained significantly upregulated 100 days after immunization with admixed antigen and 50-nm particles, compared with mice vaccinated with OVA-NP₃₁₁₋₃₂₅ alone, and produced IFN-γ (Figure S4D). These long-lived CD8⁺ T cells expressed the canonical memory marker CD69 and but no CD62L or CD103 (Figure S4D, histograms). Altogether these results reveal that size critically determines the ability of polymeric particles to promote ag-specific Th1 and CD8⁺ T cell responses and identify 50 nm as the optimal size for induction of long-lasting CMI.

Polymeric 50-nm particles induce durable CD8⁺ T cell-dependent responses against melanoma

To further assess the functionality of nanoparticle-induced CMI, we evaluated if vaccination with OVA and 50-nm particles conferred protection against B16-F10 melanoma cells expressing OVA (B16-OVA), where protection requires IFN-γ,

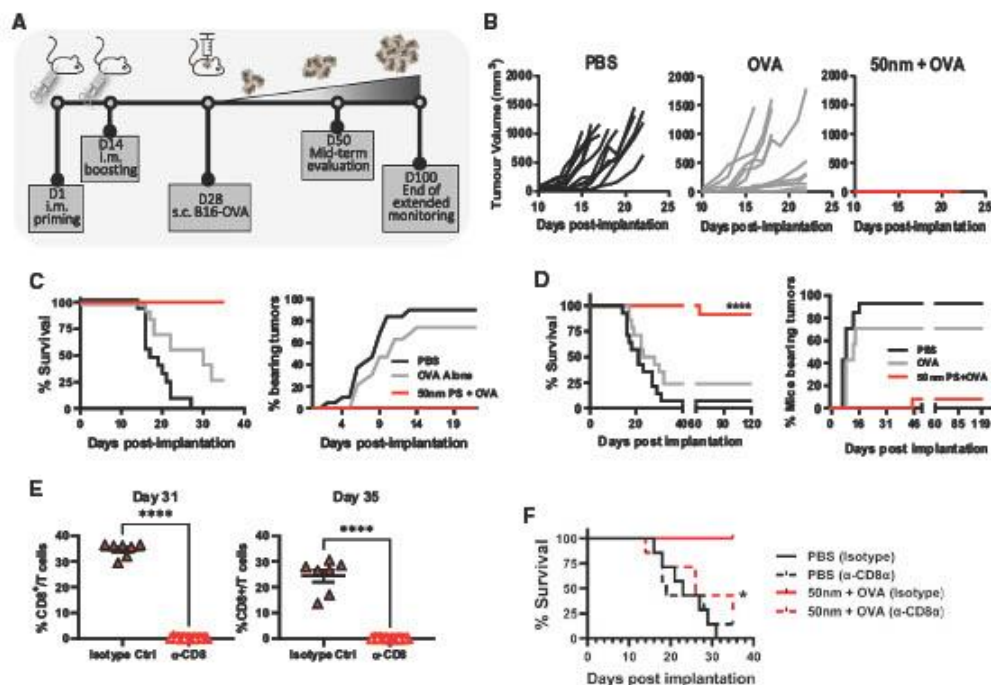


Figure 2. 50-nm PS nanoparticles drive potent antigen-specific anti-tumor immunity

(A) Immunization scheme and s.c. challenge with 3.5×10^5 B16-OVA cells. Tumors were measured daily.

(B) Spider plots show early development of tumors. Lines represent individual mice.

(C) Kaplan-Meier survival graphs up to 25 days after challenge.

(D) Extended survival analysis up to day 100. (A–C) $n = 19$ in PBS and OVA groups, $n = 17$ in 50-nm particle + OVA group. (D) $n = 14$ for PBS and OVA, $n = 12$ for 50-nm particle + OVA. Statistical significance for survival analyses determined by Mantel-Cox test.

(E) CD8 α T cell depletion efficacy in peripheral blood by fluorescence-activated cell sorting over total CD3 $^{+}$ T cells on day 31 and 35 post priming displayed as mean frequency $\pm$ SEM. An unpaired T test was used to determine statistical significance where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

(F) Kaplan-Meier survival analysis of challenged mice; a Mantel-Cox test was used to determine statistical significance between 50-nm NP OVA immunized mice with CD8 $^{+}$ T cell depletion and non-depleted 50-nm NP OVA immunized mice given isotype control where * $p < 0.05$. $n = 7$ mice per group.

ag-specific CD8 $^{+}$, and CD4 $^{+}$ T cell responses. Mice were vaccinated i.m. with OVA alone or in combination with 50-nm particles. 14 days after booster, B16-OVA cells were implanted subcutaneously, and tumor growth was measured (Figure 2A). Mice vaccinated with 50-nm PS particles plus OVA were fully protected against melanoma, whereas all animals in the mock group (PBS) developed tumors and reached the endpoint by day 25 post-implantation. Vaccination with OVA alone delayed the development of tumors compared with PBS, but 80% of the animals still developed tumors within 2 weeks of implantation, and 75% succumbed to challenge by day 35 (Figures 2B and 2C). Extended monitoring for 100 days showed long-lasting protective immunity against melanoma in animals vaccinated with 50 nm + OVA (Figure 2D). To address whether the vaccine-induced CD8 responses were required for protection, CD8 $^{+}$ T cells were depleted in vaccinated mice using an anti-CD8 α targeted monoclonal antibody³⁸ (Figure 2E) leading to a reduction in vaccine-mediated protection from 100% to 30% (Figure 2F).

Size dictates the induction of CMI by biodegradable PLGA particles

To establish if size impacts the ability of particles made of a clinically relevant polymer to induce CMI, we used endotoxin-free PLGA (Tables S1 and S2). Owing to its safety, biodegradability, and biocompatibility, PLGA products including particles have been approved by the US Food and Drug Administration and the European Medicines Agency for parenteral, mucosal, subcutaneous, intradermal, and intramuscular drug delivery in humans.³⁹ While PLGA particle adjuvant properties were first described by O'Hagan et al. in the 1990s,⁴⁰ PLGA-based adjuvants have failed to show a clear advantage of PLGA particles (i.e., as a CMI-inducing adjuvant) compared with alum and other licensed adjuvants.³⁹

Standardization of PLGA nanoparticle manufacturing by traditional methods such as double emulsion or solvent evaporation has been challenging, especially below 100-nm-diameter range, leading to formulations with high polydispersity indexes (PDI) that contain a mix of nano- and microparticles.⁴¹ To overcome

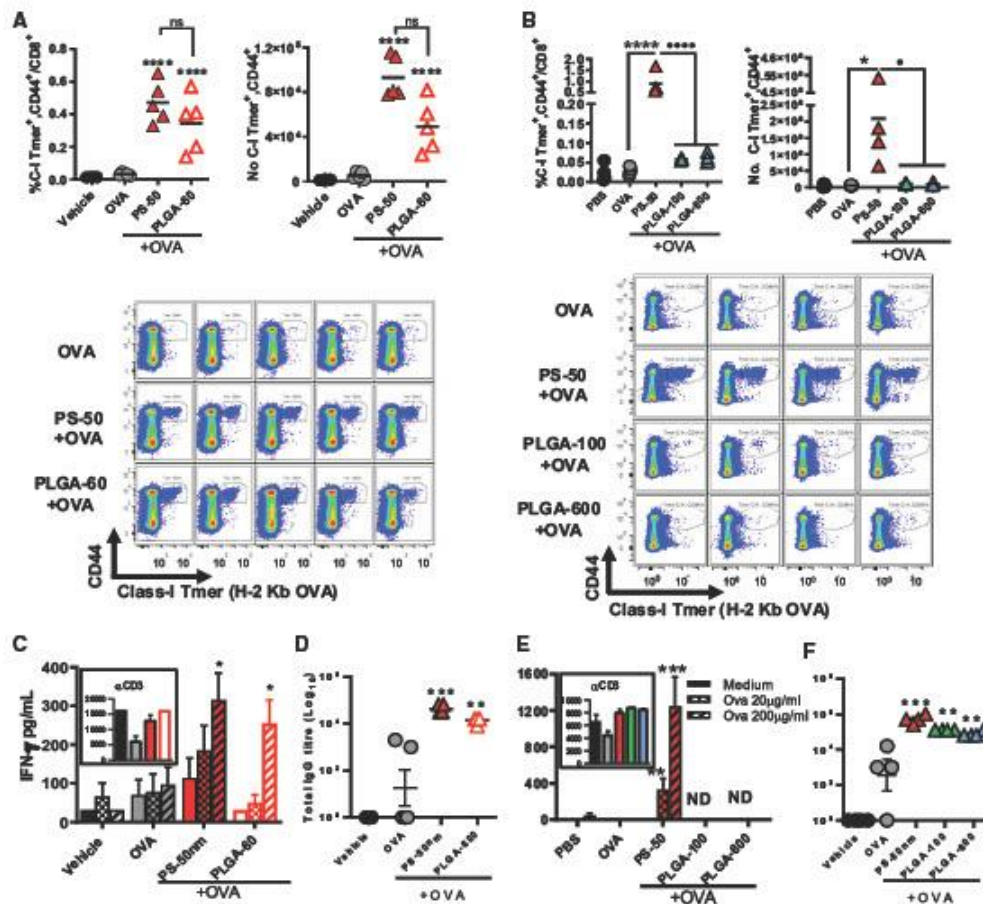


Figure 3. Induction of cell-mediated immunity by PLGA adjuvants depends on particle size

(A) An increase in percentage and total numbers of H-2Kb SIINFEKL-specific CD8⁺ T cells is observed on day 21 after vaccination with 50-nm PS and 60-nm PLGA particles (graphs and dot plots).

(B) 100- and 500-nm PLGA particles fail to induce CD8⁺ responses in the spleen after vaccination.

(C–E) IFN- γ in splenocytes supernatants after 72 h of antigen restimulation. Data are displayed as mean of triplicate samples $\pm$ SEM.

(F) OVA-specific IgG titers in serum 7 days after booster.

Statistical differences per multiple comparison ANOVA and Dunnett's or Fisher LSD post-hoc test. Representative of two experiments for (A), (C), and (D) and one for (B), (E), and (F). (A, C, D) $n = 5$ mice per group. (B, E, F) $n = 4$ mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; asterisks (*) represent differences to antigen alone, dots (•) represent comparisons between groups with adjuvant; ns: not significant.

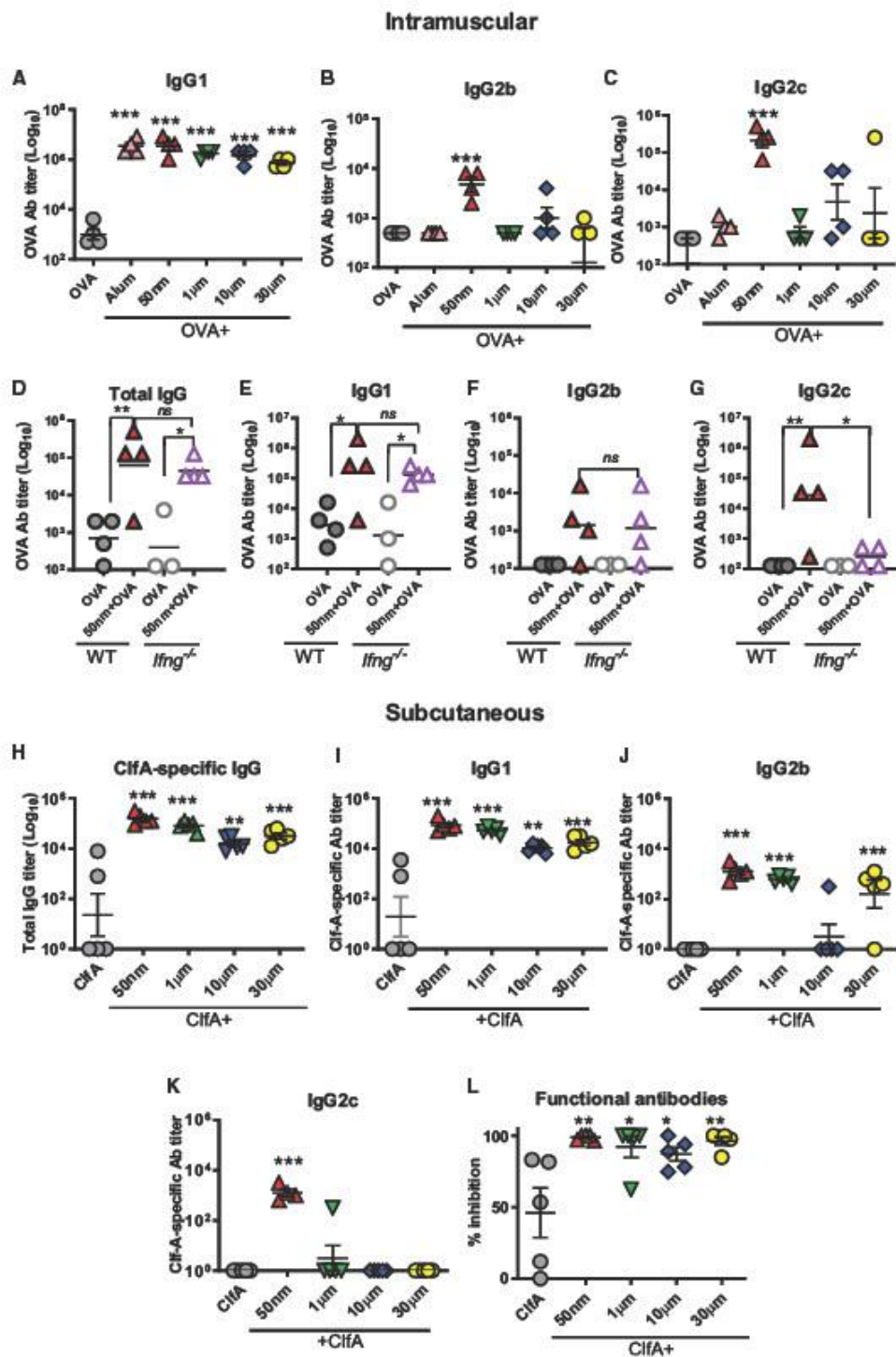
this issue, we used the Nanoassembly technology, a continuous-flow microfluidic platform designed for scale-up manufacturing of nanoparticles compliant with good manufacturing practice, and we produced PLGA nanoparticles of 61.6 ± 1.2 nm (PLGA-60) comparable to 53.7 ± 2.0 nm of the PS particles (PS-50), both with similar low PDIs and net negative charge (Table S1).

PLGA-60 and PS-50 were mixed with OVA and injected i.m. as before, both significantly enhancing ag-specific CD8⁺ T cells compared with OVA alone (Figure 3A). PLGA-60 also induced IFN- γ secretion in ag-stimulated splenocytes (Figure 3C). In contrast, PLGA particles of 100 or 600 nm failed to induce CD8⁺ T cell or Th1 responses when combined with OVA (Figures 3B

and 3E). All particles induced significant ag-specific IgG independent of their size or polymer composition (Figures 3D and 3F). These results demonstrate that PLGA nanoparticulate adjuvants are potent inducers of CMI when formulated in the 50- to 60-nm size range.

Size-dependent induction of antigen-specific IFN- γ drives IgG2c class switching

T cell-derived cytokines influence antibody class switching. In rodents IFN- γ stimulates IgG2c/IgG2a in C57BL/6 or BALB/c mice respectively. Therefore, we investigated if the IFN- γ response induced by 50- to 60-nm particles influenced antibody isotypes induced by vaccination. As seen for total IgG titers



(legend on next page)

(Figures 1E, 3D, 3F, and S3C), particles across all sizes tested significantly enhanced OVA-specific IgG1 titers compared with OVA alone, similar to alum (Figure 4A). In contrast, only 50-nm particles significantly increased IgG2b and IgG2c titers (Figures 4B and 4C). To confirm whether class switching was IFN- γ dependent, we assessed antibodies *Ifng*^{-/-} mice vaccinated with 50-nm PS + OVA. OVA-specific IgG, IgG1, and IgG2b were similar in *Ifng*^{-/-} and WT mice (Figures 4D–4F), whereas IgG2c was negligible in *Ifng*^{-/-} (Figure 4G). Interestingly, IFN- γ deficiency was associated with an increase in the number of ag-specific CD8⁺ T cells (Figure S5).

To test if particle size influenced the functionality of antibodies, we measured humoral responses using a clinically relevant antigen: the staphylococcal clumping factor A (CfA). This surface protein of *Staphylococcus aureus* binds to fibrinogen and fibrin, promoting bacterial adhesion to blood clots and is an important virulence factor during blood infection, septic arthritis, endocarditis, and skin infection.^{42–45}

Mice were vaccinated subcutaneously (s.c.) with recombinant CfA alone or in combination with particles, and serum antibodies were measured 7 days post-booster. All particles enhanced total CfA-specific IgG and IgG1 titers (Figures 4H and 4I). However, only 50-nm particles were able to induce class switching to IgG2c (Figure 4K). Increased IgG2b titers were observed in animals vaccinated with 50-nm, 1- μ m, and 30- μ m particles (Figure 4J). This contrasts with the response seen with OVA for which only 50-nm particles were able to induce IgG2b and suggests that 50-nm particles are more appropriate adjuvants for poorly immunogenic antigens as they can induce a more diverse antibody response. Finally, we determined the capacity of the serum of vaccinated mice to inhibit *S. aureus* adhesion to immobilized fibrinogen *in vitro*. Importantly, despite the different isotype profiles observed, all particle sizes were able to induce antibodies with comparable neutralizing activity (Figure 4L).

IL-1 and IL-18 differentially regulate the IFN- γ and CD8⁺ T cell responses induced by nanoparticles

Cytokines of the IL-1 family play central roles in priming, expansion, and survival of T cells, either by promoting APC maturation or acting directly influencing lymphocytes.¹⁹ IL-1 and IL-18 have been implicated in the MOA of GLA-SE and saponin-containing adjuvants,^{46,47} but other than the role of IL-33 as a negative regulator of polymeric particle-induced CMI previously described by our lab,⁹ IL-1 family cytokines have not been shown to support nanoparticle adjuvant-induced CMI *in vivo*. Given the importance of IL-1 or IL-18 as CMI modulators, we investigated their role in Th1/CD8⁺ responses induced by

50-nm particles and compared the response in WT, *Il1r1*^{-/-}, and *Il18*^{-/-} mice after vaccination. Neither IL-1 nor IL-18 were required for IgG or IgG1 responses (Figures 5A and 5B). Surprisingly, IL-18 deficiency boosted IgG2b and IgG2c production, which remained unaffected by the lack of IL-1 signaling (Figures 5C and 5D).

On the other hand, IL-18 was required for ag-specific CD8⁺ T cell responses induced by 50-nm particles: ag-specific CD8⁺ T cell numbers were reduced in the spleens of *Il18*^{-/-} mice compared with wild-type mice (Figures 5E and 5F). Although IL-1 β can directly support the expansion and effector function of CD8⁺ T cells,^{15,16} there was no statistically significant decrease of OVA-specific CD8⁺ T cells in *Il1r1*^{-/-} mice compared with WT (Figures 5E and 5F). In contrast, nanoparticle-induced Th1 responses were impaired as revealed by the lower secretion of IFN- γ seen in splenocytes of vaccinated *Il1r1*^{-/-} mice after *ex vivo* restimulation (Figure 5G). Intriguingly, vaccination of *Il18*^{-/-} with nanoparticles consistently induced increased ag-specific IFN- γ secretion upon restimulation, which is in line with the increased IgG2c responses observed in *Il18*^{-/-} mice (Figure 5G). Antigen-specific IL-10 secretion was reduced in *Il1r1*^{-/-} mice but unaffected in *Il18*^{-/-} mice (Figure 5H). In sum, IL-1 and IL-18 support different aspects of CMI induced by 50-nm particles, with IL-18 playing a prominent role in the induction of ag-specific CD8⁺ T cell responses, and IL-1 stimulating Th1/IFN- γ and IL-10 secretion after vaccination with nanoparticles.

The non-canonical inflammasome sensor caspase-11 and the pyroptosis effector GSDMD are essential for nanoparticle-induced cell-mediated immunity

Infectious and non-infectious stimuli including PAMPs, particulate matter, and other cellular stressors can trigger the assembly of inflammasomes and secretion of IL-1 α , IL-1 β , and IL-18. Both canonical caspase-1 and non-canonical caspase-11 inflammasomes promote secretion of these cytokines via direct or indirect activation mechanisms.²¹ Therefore, we sought to determine if either of these inflammatory caspases could contribute to Ag-specific CMI induced by 50-nm polymeric nanoparticles.

Given that pyroptotic ICD is also a feature of inflammasome activation in certain contexts, we first tested whether 50-nm particles induced cell death *in vitro*. Quantification of cell death by lactate dehydrogenase (LDH) release assay in murine myotubes (the most abundant cell type exposed to the vaccine components during i.m. vaccination) after exposure to PS 50-nm particles showed a dose-dependent increase in cytotoxicity. In contrast, 1- μ m particles did not induce LDH release. Importantly, the cytotoxic effect of 50-nm particles was moderate in

Figure 4. Particle size influences antibody class switching without affecting antibody neutralizing activity

Wild-type (WT) or *Ifng*^{-/-} mice received i.m. OVA (A–G) or s.c. CfA (H–L) alone or in combination with PS particles. PBS was used as vehicle control and alum as positive control. Serum antibodies were measured by ELISA on day 21.

(A–C) OVA-specific IgG1, IgG2b, and IgG2c after i.m. vaccination.

(D–G) Total antibodies and isotypes in WT and *Ifng*^{-/-}.

(H) Total CfA-specific antibodies after s.c. vaccination.

(I–K) CfA-specific titers for IgG1, IgG2b, and IgG2c after s.c. vaccination.

(L) Neutralizing activity as percent inhibition of *S. aureus* binding to fibrinogen, with 100% being bacterial adherence in the absence of serum.

Results are shown as mean $\pm$ SEM; symbols represent individual animals. Statistical differences were calculated per multiple comparison ANOVA and Dunnett's test compared with antigen alone or selected Fisher LSD for selected groups. Representative of two experiments for WT mice immunized with OVA (n = 4/group) or CfA (n = 5/group) and one for *Ifng*^{-/-} (n = 4/group).

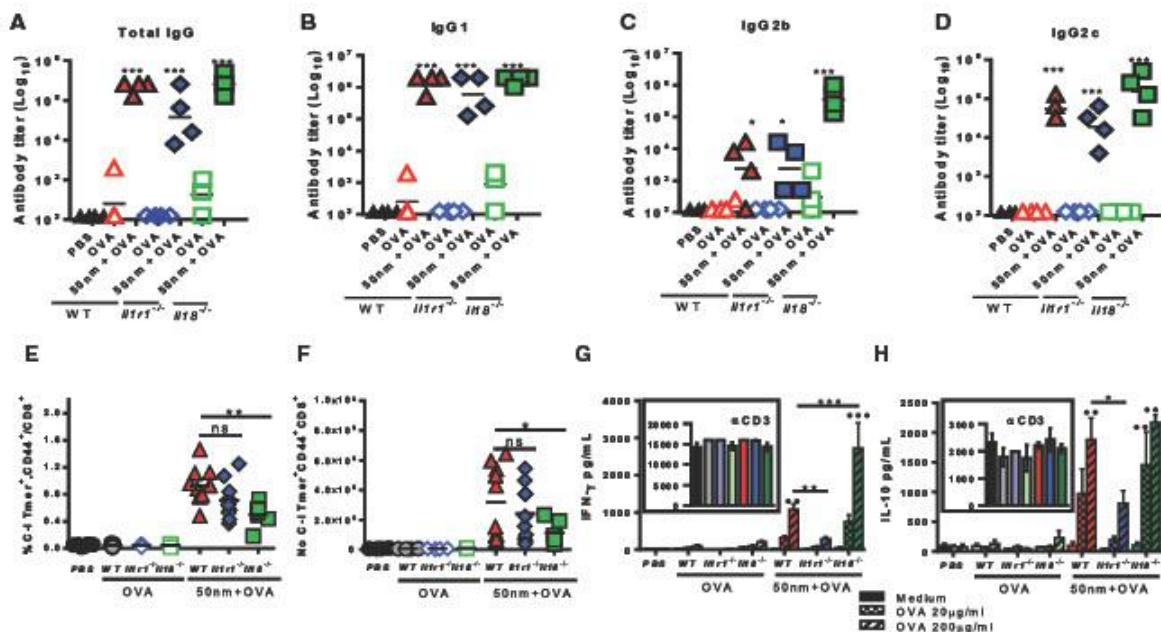


Figure 5. IL-1 and IL-18 differentially regulate nanoparticle-induced IFN-γ and CD8⁺ T cell responses

(A–D) Ag-specific isotypes in serum 7 days after Im. booster.

(E and F) Percentage and number of OVA H-2Kb CD8⁺ T cells in spleens 7 days after booster.

(G and H) IFN-γ and IL-18 secretion in splenocytes on day 21, after 72h *ex vivo* ag-stimulation. Results are presented as mean ± SEM. Statistical differences are per one-way ANOVA and Fisher LSD (for Tmem⁺ cell quantification) or Dunnett's (for cytokine quantification) post-hoc tests. IFN-γ and IL-18 data are shown as mean ± SEM; * denotes differences between selected groups, + denotes difference compared with control (OVA alone), ns: not significant. Representative of three experiments for *Il1r1*^{-/-} mice and two for *Il18*^{-/-} mice (n = 4/group for antibodies and 8/group for Tmem and cytokines).

comparison to other stimuli including alum, which typically induces necrotic cell death (Figure S6A), suggesting that 5-nm particles promote regulated ICD.

To assess the involvement of caspase-1 in 50-nm particle adjuvanticity, mice were vaccinated intramuscularly with 50-nm particles and OVA with or without the caspase-1 inhibitor Ac-YVAD-fmk (YVAD) during prime and boost. Pharmacological inhibition of caspase-1 did not significantly compromise induction of ag-specific CD8⁺ T cells (Figures 6A and 6B), although a decrease in IFN-γ was noted (Figure 6C). YVAD did not affect antibody production induced by OVA + 50-nm particles (Figure S7A). In contrast, vaccination of caspase-11 knockout (*casp11*^{-/-}) mice with 50-nm PS + OVA failed to promote ag-specific CD8⁺ T cell (Figures 6D and 6E) and Th1 responses (4-fold decrease in IFN-γ) compared with wild-type mice (Figure 6F), while antibody responses remained intact in *casp11*^{-/-} mice (Figure S7B).

Activation of caspase-11 leads to downstream cleavage of GSDMD oligomerization and transmembrane pores formation facilitating the release of IL-1β and IL-18 and pyroptotic cell death.^{49–50} Therefore, we assessed whether polymeric particles could promote GSDMD cleavage in a size-dependent manner. Bone marrow-derived dendritic cells (BM-DCs) were primed with LPS and stimulated with 50-nm or 1-μm particles. Cell lysates were probed for cleaved GSDMD by western blot. As

shown in Figure 6G, only 50-nm particles were able to induce GSDMD cleavage in BM-DCs.

To further define the contribution of GSDMD in particle-induced cell death, necrostatinamide (NSA) and disulfiram (DMF) were used. NSA blocks pore formation by binding to the GSDMD Cys191 residue (murine Cys192) and blocking p30-GSDMD oligomerization. As a result, NSA selectively blocks pyroptosis and IL-1β release in human and murine cells without affecting TLR signaling, inflammasome assembly, or gasdermin E-mediated cell death.⁵¹ It is important to note that while NSA has been reported to inhibit the necroptosis effector mixed lineage kinase domain-like protein, this effect only affects human cells.⁵² DMF also blocks pyroptosis and cytokine release in murine and human cells by covalently modifying the Cys191/Cys192 residues of GSDMD and blocking pore formation.⁵³ Similar to our previous observation in myotubes (Figure S6A), 50-nm particles induced death in BM-DCs, which was prevented by the addition of the GSDMD inhibitors NSA and DMF (Figure S6B). These results suggest that 50-nm particles can promote pyroptotic cell death in immune and non-immune cells, favoring the release of active IL-1 and IL-18 to promote CD8⁺ T cell activation and IFN-γ secretion.

Based on the *in vitro* data, we hypothesized that local cytotoxicity and release of IL-1 and IL-18 at the injection site through GSDMD pores were key to CMI. Therefore the GSDMD inhibitor

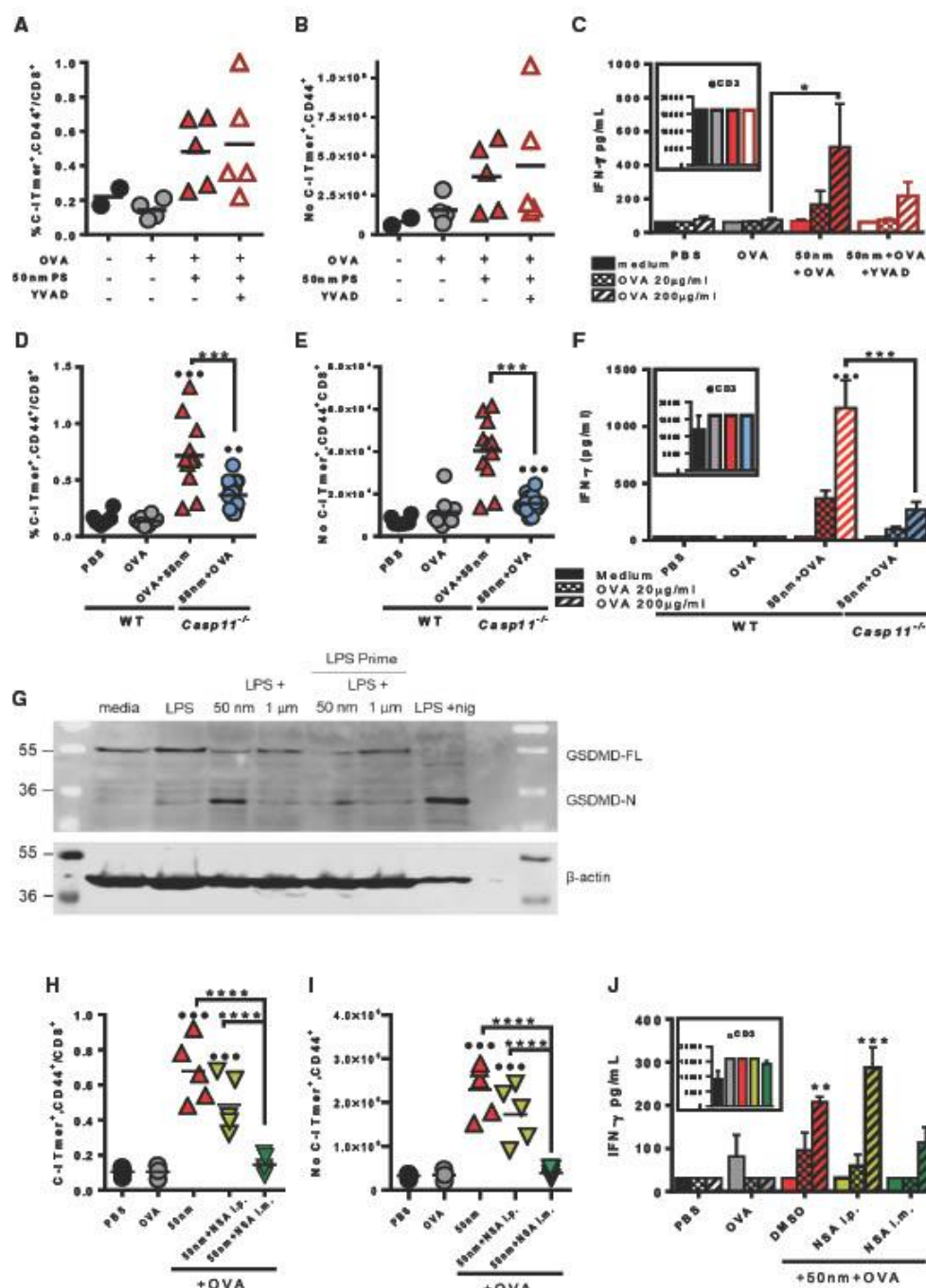


Figure 6. Caspase-11 and size-dependent induction of GSDMD are required for antigen-specific CD8⁺ responses

(A) WT mice were vaccinated i.m. with 50-nm particles and OVA, with or without the caspase-1 inhibitor Ac-YVAD-fmk (YVAD) on days 7 and 14. Ag-specific responses were quantified in splenocytes on day 21 using H-2Kb/OVA (SIINFEKL) MHC Tetramers. PBS or OVA alone were used as vehicle or antigen alone controls. Percentage of OVA-specific CD8⁺ over total CD8⁺ and CD44⁺ T cells.

(legend continued on next page)

NSA was administered *in vivo* either intraperitoneally 1 h prior to vaccination or locally in the muscle at the time of immunization. Systemic inhibition of GSDMD by i.p. injection of NSA induced a partial decrease in ag-specific CD8⁺ T cells. In contrast, intramuscular NSA abolished ag-specific CD8⁺ responses (Figures 6H and 6I), suggesting that local activation of GSDMD is necessary for CTL priming by 50-nm particles. Intramuscular administration of NSA at the time of vaccination also downregulated antigen-specific IFN- γ secretion, which was not affected in animals that received systemic NSA (Figure 6J). Inhibition of GSDMD had no impact on antibody responses regardless of the administration route (Figure S7C).

Polymeric nanoparticles promote size-dependent production of ROS-required ag-specific CD8⁺ responses Q13 after vaccination

ROS have been shown to influence the activity of both caspase-11 and GSDMD.^{22,54} We therefore investigated if particles could induce cellular ROS in a size-dependent manner and if ROS production could contribute to particle adjuvanticity *in vivo*. Firstly, BM-DCs were stimulated with polymeric particles of 50 nm, 200 nm, or 1 μ m diameter, and ROS production was measured by flow cytometry using Cell-ROX staining. Only 50-nm particles induced a notable increase in ROS production in viable cells (Figures 7A–7D), confirming that induction of cellular ROS by polymeric particles is a size-dependent phenomenon.

To assess whether ROS production was required for CD8⁺ responses *in vivo*, mice were vaccinated i.m. with OVA and 50-nm particles and the ROS scavenger TEMPOL (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), a membrane-permeable, metal-independent SOD mimetic specific for superoxide anions. Co-injection of OVA, 50-nm particles, and TEMPOL i.m. led to a significant reduction in the total number of ag-specific CD8⁺ T cells in the spleen. In contrast, systemic i.p. administration of TEMPOL prior to and just after i.m. vaccination did not lead to decreased CD8⁺ responses (Figure 7E).

These results indicate that local production of ROS elicited by 50-nm particles is essential for the induction of CD8⁺ T cell responses.

DISCUSSION

Rational subunit vaccine development requires knowledge of how adjuvants activate and direct innate and adaptive immunity at a cellular and molecular level. Polymeric nano- and microparticles can enhance the immunogenicity of microbial and cancer

antigens in preclinical vaccination models.^{30,31,40,55} However, the design principles dictating their efficacy and the molecular mechanisms involved in their adjuvanticity are largely unknown, which has hindered their clinical development. Previously we showed that intramuscular vaccination with polymeric non-degradable biocompatible nanoparticles induced potent Th1 and CD8⁺ T cell responses when combined with protein antigens.⁹ Here we defined the key attributes of polymeric particle design linked to the induction of CMI, and we unveiled the molecular pathways required for their adjuvanticity.

Given the known role of programmed ICD in Th1 and CD8⁺ T cell induction, we hypothesized that adjuvant-induced ICD could underlie activation of CMI by 50-nm particles. Supporting our hypothesis, 50-nm particles induced LDH release in skeletal muscle cells (Figure S6A), which was negligible for 1- μ m particles. Likewise, 50-nm particles induced cell death in BM-DCs, which was prevented by the addition of the GSDMD inhibitors NSA and DMF (Figure S6B). In addition, stimulation of BM-DCs with polymeric particles induced a size-dependent activation of GSDMD that was only observed for 50-nm-particle-treated cells (Figure 6G). These results are suggestive of induction of pyroptotic cell death by 50-nm particles. In line with this, our *in vivo* data revealed a critical role for the non-canonical inflammasome sensor caspase-11 (Figures 6D–6F), the pyroptosis effector GSDMD (Figures 6H–6J), and the pro-inflammatory cytokines IL-1 and IL-18 (Figures 5E–5G) in the induction of ag-specific CMI by 50-nm nanoparticles.

Enhanced ROS production has been shown to increase the expression and activation of caspase-11 downstream of c-Jun N-terminal kinase in a murine model of enteropathogenic bacterial infection,⁵⁴ and certain oxidized endogenous lipids have been identified as activator ligands for caspase-11.⁵⁶ Furthermore, ROS production influences the transition from GSDMD cleavage and membrane localization to oligomerization and pore formation.²² Therefore, we hypothesized that 50-nm-particle-induced CMI dependent on caspase-11 and GSDMD activation could be linked to a size-dependent induction of ROS. Evidence for this size-dependency was provided by treating BM-DCs with 50-nm-, 200-nm-, and 1- μ m-diameter particles and measuring ROS production by flow cytometry, which revealed that only 50-nm particles promoted significant ROS in these cells (Figures 7A–7D). Furthermore, intramuscular treatment of mice with the ROS scavenger TEMPOL at the time of vaccination abolished CMI, confirming the importance of locally generated ROS by 50-nm particles for induction of ag-specific CD8⁺ T cells (Figure 7E).

(B) Total number of OVA-specific CD8⁺ T cells in the spleen.

(C) Quantification of IFN- γ in culture supernatants 72 h after OVA stimulation.

(D and E) Analysis of H-2Kb/OVA (SIINFEKL)-specific CD8⁺ lymphocytes in vaccinated *Casp11*^{−/−} and WT mice.

(F) Quantification of IFN- γ in culture supernatants 72 h after OVA stimulation.

(G) Induction of GSDMD is size dependent. BM-DCs were primed with LPS or left unprimed and were then stimulated with 50-nm or 1- μ m particles in combination with LPS for 18 h. Cell lysates were probed for full-length and cleaved GSDMD protein, and β -actin was used as a loading control. LPS + Nigericin-treated BM-DCs were used as positive control for GSDMD cleavage. Data are representative of two independent experiments.

(H and I) WT mice were vaccinated as before. Some groups received the GSDMD inhibitor NSA i.p. 1 h before vaccination or i.m. at the time of vaccination. (J) Percentage and total number of OVA-specific CD8⁺ T cells in the spleen.

(K) IFN- γ in culture supernatants 72 h after OVA stimulation. Statistical differences are per one-way ANOVA and Tukey-Kramer post-hoc test. Symbols: * denotes differences between treatment groups and + to OVA alone.

Representative for one experiment for (A)–(C) and (H)–(J) and two for (D)–(F) and (K). Each symbol represents one mouse.

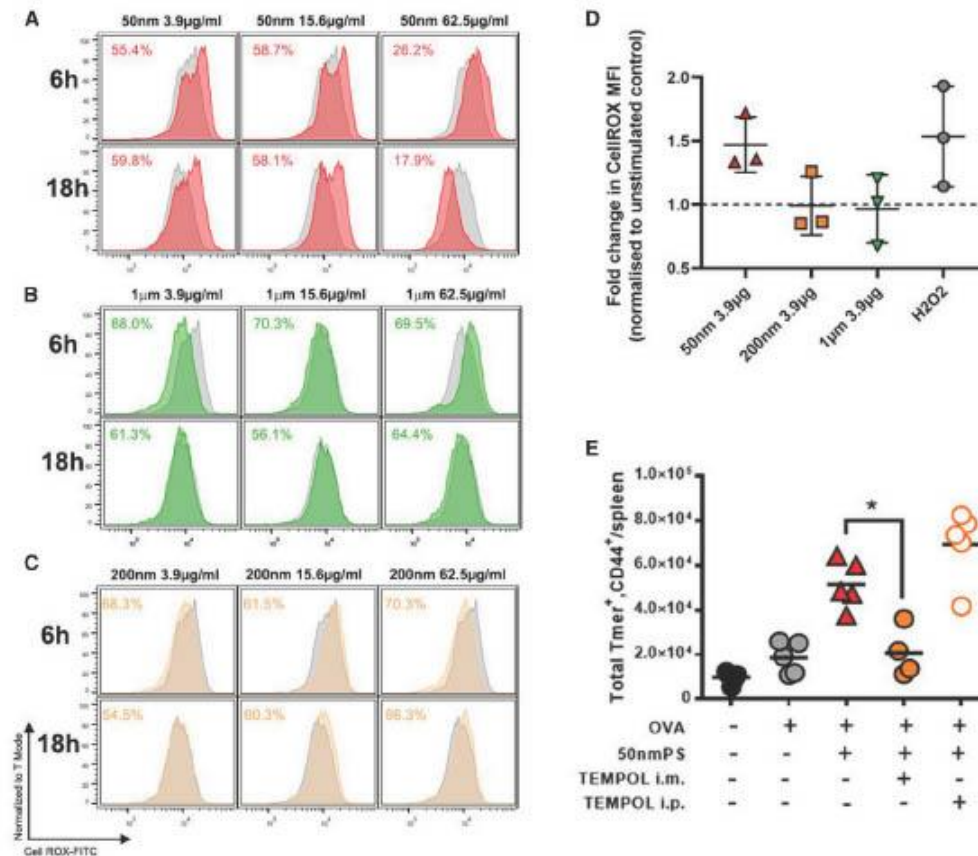


Figure 7. Size-dependent production of ROS controls the induction of CD8⁺ responses

(A–D) BM-DCs were stimulated with increasing concentrations of 50-nm (red), 200-nm (orange), or 1-µm (green) particles for 6 or 18 h or were left untreated (gray histograms). ROS production was measured by fluorescence-activated cell sorting on gated live cells using Cell-ROX gated on single live cells. Percentages in histograms indicate proportion of live cells after each treatment. (D) Mean fold change in Cell-ROX MFI compared with unstimulated bone marrow-derived dendritic cells controls is displayed $\pm$ SD. Data are representative of three biological/experimental repeats. (E) Numbers of CD44⁺, Tmem⁺, and CD8⁺ T cells in spleens of WT mice treated with the ROS scavenger TEMPOL administered i.m. or i.p. during prime/boost. Statistical differences are per one-way ANOVA and Dunn's post-hoc test. Data are representative of one experiment. Each symbol represents one mouse.

Downstream of caspase-11 and GSDMD activation is the release of the pro-inflammatory cytokines IL-1 and IL-18 that can modulate CD4⁺ and CD8⁺ T cell responses.^{9,15–19} IL-1 and IL-18 positively regulated nanoparticle-induced CMI, but we uncovered a clear division of labor for these inflammatory cytokines: while IL-1R1 signaling was required for optimal Th1 responses (Figure 5G), IL-18 supported ag-specific CD8⁺ T cells (Figures 5E and 5F). Total IgG and IgG1 responses were comparable in *Il1r1*^{−/−} and *Il18*^{−/−} mice (Figures 5A and 5B), whereas IFN-γ production supported by IL-1R1 signaling was key to IgG2c class switching as revealed by *Ifng*^{−/−} mice (Figures 5D and 5G). However, IFN-γ deficiency did not impair ag-specific CD8⁺ T cell responses after vaccination (Figures S4A and S4B). Indeed, greater numbers of ag-specific CD8⁺ T cells were seen in *Ifng*^{−/−} mice. IFN-γ deficiency may lead to increased expansion of ag-specific CD8⁺ T cells, possibly linked to altered

immunodominance and reduced activation-induced cell death of CD8⁺ lymphocytes.⁵⁷

While IL-1R1 signaling and IFN-γ were dispensable for nanoparticle-induced CD8⁺ T cell responses, IL-18 was required (Figures 5E and 5F). Intriguingly, we found an unexpected increase of IFN-γ production in *Il18*^{−/−} mice after vaccination with 50-nm particles (Figure 5G). This is an interesting observation as IL-18 is known to promote Th1 function and IFN-γ production. Yet, IL-18 also supports the function of a specialized subset of regulatory T cells (Tregs) involved in tissue repair by promoting the expression of the growth factor amphiregulin (Areg) in damaged skeletal muscle.^{58,59} Therefore, by impairing Areg expression on Tregs, IL-18 deficiency may lead to decreased Treg function and increased IFN-γ production.

Canonical (caspase-1) and non-canonical (caspase-11) inflammasomes lead to activation of IL-1α, IL-1β, and IL-18, and

secretion of these cytokines via formation of GSDMD pores, depending on the level of activation, may lead to pyroptotic cell death.²¹ Although both caspase-1 and caspase-11 can process GSDMD into the active pore-forming subunits, caspase-1 but not caspase-11 can process IL-1 β .⁶⁰ Both caspase-1 and caspase-11 (and its human ortholog caspase-4) can process IL-18.⁶¹ On the other hand, caspase-11 (and its human ortholog caspase-5) but not caspase-1 can process IL-1 α ,^{62,63} which can also be processed by calpain activated via Ca²⁺ influx through the GSDMD pores. In fact, GSDMD deficiency impairs IL-1 α maturation but does not affect IL-1 β processing during inflammasome activation.⁶¹ We found that local pharmacological inhibition of caspase-1 did not abrogate ag-specific cellular or humoral responses induced by 50-nm particles (Figures 6A–6C and S7A), whereas caspase-11 deficiency and GSDMD inhibition led to impaired Th1 and CD8 responses. This may point toward a role for IL-1 α in promoting IL-1R1-dependent Th1 responses induced by the nanoparticulate adjuvant.

Finally, our study demonstrates that particle size critically determines the ability of particulate adjuvants to promote CMI and identifies 50–60 nm as the optimal range for induction of protective and long-lasting Th1 and CD8⁺ T cell responses (Figures 1, 3, and S3). We also unveiled a dichotomy in terms of the particle qualities influencing the induction of humoral versus cellular responses: while only particles in the 50–60 nm range induced CMI, particles over a broad size range induced humoral responses of similar magnitude, irrespective of the polymer or immunization route (Figures 1E, 3F, 4H–4L, and S3C).

PLGA particles are attractive adjuvant candidates,⁶⁴ but to date, they have not demonstrated a unique selling point beyond widely used adjuvants including alum and oil in water emulsions. Our results suggest that the limited efficacy of PLGA particles as CMI-inducing adjuvants is linked to the use of inadequate particle sizes. We demonstrate that 50- to 60-nm PLGA nanoparticles effectively promote Th1 and CD8⁺ T cell responses, which are abolished as soon as particle size reaches 100 nm (Figures 3A–3C). Until recently, limitations in PLGA particle formulation processes restricted their production to sizes between 80 nm and 250 μ m. New nanoparticle manufacturing platforms such as the microfluidic technology used for preparation of our PLGA nanoparticles allow for fine control of particle size and generation of particles with low polydispersity index under GMP conditions that are appropriate for large-scale production.⁶⁵

In summary, our study identifies size as a key design principle linking adjuvant particle characteristics to induction of CD8⁺ and Th1 responses, demonstrates the efficacy of clinically relevant PLGA particles as CMI-inducing adjuvants, and implicates ROS production, the non-canonical inflammasome components caspase-11, GSDMD, and the effectors IL-1 and IL-18 as critical components of nanoparticulate adjuvant MOA. Our findings are a significant step forward toward the development of polymeric nanoparticles as next-generation adjuvants for anticancer and antiviral vaccines.

Limitations of the study

The current study is limited to a murine model of vaccination. Therefore, the magnitude of Th1 and CD8⁺ responses elicited

by polymeric nanoparticles in human subjects will require further investigation. Our results demonstrate the size-dependent nature of CMI and an optimal size range of 50–60 nm for the induction of CD8⁺ T cell responses using polymeric nanoparticles, which differ in their composition, biodegradability profile, and charge. However, it remains to be determined if these findings apply to other particulate adjuvants including emulsions and liposomes. Finally, the *in vitro* studies mainly focused on analyzing the response of dendritic cells and myotubes, but further work is required to resolve the cell targets of adjuvant-induced IDC following injection locally and in the draining lymph nodes.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, E.C.L. and N.M.W. Methodology, N.M.W., R.W.W., Y.P., K.A.S., C.G., and A.G.B. Investigation, N.M.W., R.W.W., C.P.M., K.O., C.H.H., F.A.S., C.B.C., G.A., J.G., K.A.S., C.G., and C.W. Writing – original draft, E.C.L. and N.M.W. Writing – review & editing, E.C.L., N.M.W., R.W.W., A.G.B., and E.C. Funding acquisition, E.C.L. Resources, E.C.L., Y.P., M.C., and E.C. Supervision, E.C.L., N.M.W., and A.G.B. Project management, E.C.L. Formal analysis and validation, N.M.W., C.H.H., and R.W.W. Visualization, N.M.W. and R.W.W.

DECLARATION OF INTERESTS

E.C.L. and N.M.W. are named inventors on patent application, WO2021123430, Polymeric nanoparticles as vaccine adjuvants. E.C.L. is a founder of AllseVax.

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Q5 Q6 STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD16/CD32 mAbs	BD Pharmingen	553142
Anti-mouse F4/80 AF100 (Cl:A3-1)	Bio-Rad	MCA497A700
Anti-mouse CD8 RPE-AF750(KT15)	Bio-Rad	MCA609P750
Anti-mouse CD4 APC-eFluor780 (GK1.5)	Invitrogen	47-0041-82
Anti-mouse CD3 BV650 (17A2)	Biolegend	100229
Anti-mouse CD11c BV605 (N418)	Biolegend	563057
Anti-mouse CD44 FITC (IM7)	BD Biosciences	553133
Anti-mouse B220 AF700 (RA3-6B2)	BD Biosciences	557957
InVivoMab anti-mouse CD8 α , Clone 2.43,	BE0061	BioXCell
InVivoMab rat IgG2b isotype control, Clone LTF-2,	BE0090	BioXCell
Biotinylated rat anti-mouse IgG1	BD Pharmingen	553441
HRP conjugated goat anti-mouse IgG2c	AbD Serotec	STAR135P
HRP conjugated Goat anti-mouse IgG	Southern Biotech	1030-05
Biotinylated rat anti-mouse IgG2b	BD Biosciences	553393
Mouse anti- β -actin (AC-74) antibody	Sigma	A5316
Rabbit anti-GSDMD (126-138) antibody	Sigma	G7422
IRDye 680RD Goat anti-mouse IgG Secondary AB	Licor	926-68070
IRDye 800CW Goat anti-Rabbit IgG Secondary AB	Licor	926-32211
Bacterial and virus strains		
<i>Staphylococcus aureus</i> PS80	Laboratory of Joan Geoghegan	N/A
Chemicals, peptides, and recombinant proteins		
PE-labelled H-2K(b) Chicken OVA257-264 SIINFEKL	NIH	N/A
APC-labelled H-2K(b) I-A(b) NP311-325 QVYSLRPNENPAHK	NIH	N/A
CellROX Green reagent	Invitrogen	C10444
Fixable Viability stain BV510	BD Biosciences	564406
Endograde Apyrogenic Ovalbumin	Lionex GmbH	LET0027
Necrosulfonide (NSA)	Millipore	480073
Dimethylfumarate (DMF)	Sigma	50744
Ac-YVAD-cmk	Cayman Chemical	10014
Influenza nucleoprotein peptide NP ₃₁₁₋₃₂₅ (QVYSLRPNENPAHK)	JPT Peptide Technologies	12489
Maleimide-activated OVA	Thermo-Fisher	77125
Recombinant <i>S. aureus</i> clumping factor A (ClfA)	Laboratory of Joan Geoghegan	N/A
Critical commercial assays		
IFN- γ DuoSet ELISA kit	R&D Systems	DY485
IL-17 DuoSet ELISA kit	R&D Systems	DY421
IL-10 ELISA MAX kit	Biolegend	431411
CyQUANT LDH Cytotoxicity Assay	Invitrogen	C20300

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pierce Chromogenic Endotoxin Quantification Kit	Thermo Scientific	A39552
Experimental models: Cell lines		
B16F10-OVA	Laboratory of Kingston Mills, TCD	N/A
C2C12	ATCC	CRL-1772
Experimental models: Organisms/strains		
C57BL/6J	Comparative Medicines Unit, TCD	N/A
<i>Il18</i> ^{-/-}	The Jackson Laboratory	Strain #:004130
<i>Il1r1</i> ^{-/-}	The Jackson Laboratory	Strain #:003245
<i>caspase11</i> ^{-/-}	Laboratory of Emma Creagh, TCD	
<i>Ifng</i> ^{-/-}	The Jackson Laboratory	Strain #:002287
Other		
Polystyrene particles (50nm–30µm)	Phosphorex Inc	50nm: 102; 1µm: 112; 10µm: 118; 30µm: 130
Alhydrogel (alum)	Brenntag Biosector/Croda	
Poly-lactic co-glycolic acid (PLGA) particles (>50nm size)	Phosphorex Inc	100nm: LG100; 500/600nm: LG500
50nm PLGA nanoparticles	Laboratory of Yvonne Perrie	N/A
Detoxi Gel	Thermo Fisher	20339

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ed Lavelle (lavellee@tcd.ie).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data: All data reported in this paper will be shared by the lead contact upon request.
- Code: This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Eight to 12 weeks old wild-type (wt) C57BL/6J, *Il18*^{-/-}, *Il1r1*^{-/-}, *Casp11*^{-/-}, *Ifng*^{-/-} all on a C57BL/6 background were bred in-house in the Comparative Medicine Unit, TCD as per College and The Health Products Regulatory Authority (HPRA) welfare guidelines. All animal studies were approved by the TCD Animal Research Ethics Committee (Ethical Approval Number 091210) and HPRA license AE191364/P079. Animals were kept in IVC cages, SPF conditions with a 12h light cycle, 21°C, 50% relative humidity and food and water *ad libitum* for the duration of study. In selected experiments *Il18*^{-/-}, *Il1r1*^{-/-} and wt were maintained in the same conditions but in a low-barrier facility. Pilot studies were conducted to assess the response in males and females. No significant differences were found between sexes, therefore age- and sex-matched animals were used in all subsequent experiments. Power analyses were used to determine the minimal number of animals needed to achieve statistical significance according to 3Rs principles.

Cell culture conditions

All cells were cultured at 37°C with 95% humidity and 5% CO₂, using a cell culture incubator. Base medium used for cell culture (RPMI 1640 (Gibco) or DMEM (SIGMA)) was supplemented with 100 U/mL penicillin and 100µg/mL streptomycin (Gibco); 10% heat inactivated fetal bovine serum (FBS) (Biosera) and 2mM glutamine (Gibco) to make complete media (cRPMI or cDMEM). T cell media was made by supplementing RPMI 1640 0.04mM β-mercaptoethanol (Gibco), 0.88mM sodium pyruvate (Gibco),

0.88mM L-Glutamine (Gibco), 4.4 U/mL penicillin, 4.4μg/mL streptomycin (Gibco), 10mM MEM Non-Essential Amino Acid (Gibco), 0.35% (v/v) 100X MEM Vitamins (Gibco), 10% (v/v) heat-inactivated FBS (Biosera).

B16F10-OVA

B16F10 and B16F10-OVA cell lines were cultured in cDMEM and upon reaching logarithmic growth between 50–70% confluency were used for experimentation *in vivo*.

Splenocytes

Spleens were isolated from euthanised mice, and single cell suspensions were obtained after homogenizing the organs with a sterile plunger and filtering cell suspension in complete RPMI through 70μm cell strainers. Cells were centrifuged at 300 × g for 5 minutes at 4°C, and red blood cells (RBC) were lysed by suspending the cells in 1mL of 0.88% ammonium chloride (NH₄Cl) for 2 minutes, with subsequent quenching of reaction with cRPMI and centrifugation. Cells were resuspended in cRPMI and live cells counted with trypan blue. For *ex vivo* antigen restimulation assays, cells were plated in sterile U-bottom 96 well plates in 200μL of T cell media at 2 × 10⁶ cells/mL. Cells were then restimulated with appropriate antigen concentrations and controls as detailed in the figure legends for each experiment.

Culture of bone marrow derived dendritic cells (BMDCs)

On day 1, the femurs and tibiae were removed from culled mice, and were cleaned of muscle tissue with a sterile scissors, tissue and 70% ethanol (EtOH). The separated bones were then briefly washed in petri dishes containing 70% EtOH and rinsed in cRPMI. A 20 mL syringe was filled with cRPMI and attached to a 27G needle. The tips of the femur and tibia bone were minimally cut using a sterile scissors and the bone marrow was flushed out into a petri dish utilising a 20mL syringe filled with cRPMI and a 27G needle. Cell clumps were disrupted by repeatedly passing the cell suspension through a 19G needle. The cells were transferred to 50 mL falcon tubes and centrifuged at 300 × g for 5 minutes at room temperature. The supernatant was removed, and the cell pellet was resuspended in 1 mL of 0.88% NH₄Cl de (NH₄Cl) for RBC for 2 minutes, with subsequent quenching of reaction with cRPMI and repeat of centrifugation. Supernatants were discarded and the cell pellet was resuspended in 10 mL cRPMI. Cells were counted as outline above and cell suspension volume was adjusted with cRPMI containing 20 ng/mL GM-CSF to give a final concentration of 4.25 × 10⁵ cells/mL. Cells were transferred to T175 flasks (12.75 × 10⁶ cells/flask) gently handling to avoid DC activation. On day 3 of culture protocol, 30 mL of cRPMI containing 20 ng/mL GM-CSF was added to each T175 flask. On day 6 flask supernatants were removed and replaced with 30 mL fresh cRPMI containing 20 ng/mL GM-CSF. On Day 7, 30 mL of cRPMI containing 20 ng/mL GM-CSF was added to each T175 flask. On day 10, loosely adherent cells were removed by gentle repeat pipetting. Cells were transferred to 50 mL falcon tube and centrifuged at 300 × g for 5 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 10 mL cRPMI. Cell suspensions were adjusted to a final concentration of 6.25 × 10⁵ cells/mL in cRPMI with 10 ng/mL GM-CSF. Cells were plated in round bottom 96 well plates at a volume of 200 μL/well. The plates were incubated for at least 2 hours at 37°C, 95% humidity and 5% CO₂ before stimulation. Cells were treated with indicated inhibitors 40 minutes prior to stimulation with particles/controls. Control cells ("vehicle control") were incubated with in the presence of corresponding amount of DMSO used in the inhibitor groups. Cells were then stimulated with particles/controls as indicated in the figures and were incubated overnight.

Culture and differentiation of mouse myoblast cell line C2C12 cells

Mouse myoblast C2C12 culturing protocol was based on the cell line product data sheet (ATCC). C2C12 cells were cultured at 37°C, 95% humidity and 5% CO₂ in T75/T175 flasks in cDMEM, and it is crucial to avoid cells becoming confluent over course of their sub-culture. To remove cells from flasks they were first washed with pre-warmed PBS, followed with trypsin-EDTA treatment for 5 minutes at 37°C. Cells were then harvested and centrifuged at 300 × g for 5 minutes at 37°C, and the cell pellet was resuspended in cDMEM for cell counting as described above. Cells were then plated in 24 well plates for cytotoxicity studies. C2C12 cells were plated at 5 × 10³ cells per cm² of cell culture surface area. These cells were allowed to become confluent, usually after 3–4 days of culture and following confluency media was changed from standard cDMEM to cDMEM supplemented with 2% horse serum instead of 10% FBS, to encourage differentiation, usually occurring within another 3–4 days of culture. C2C12 cells undergo differentiation from myoblasts into myotubules, with indicative changes in morphology towards tightly packed, striated cells. Fully differentiated cells were then used for *in vitro* experimentation. 24 hours before stimulation cells were returned to cDMEM supplemented with 20% FBS. Particles were then added at concentrations indicated in the figures and plates were centrifuged at 200 × g for 3 minutes before cells were returned to the incubator.

METHOD DETAILS

Particle washing

Ultrapure, endotoxin-free, negatively charged polystyrene particles were purchased from Phosphorex Inc (USA). Particles were vortexed for 2 minutes and washed using 2 cycles of centrifugation/suspension in 20 volumes of apyrogenic PBS to remove surfactants or stabilizers. Centrifugation velocity was determined as per Stokes' Law according to particle size. Particles were suspended

at the appropriate concentration calculated as mg polymer/mL, mixed with the antigen and kept at room temperature for 40min before injection.

PLGA particle synthesis

To make 50–60nm particles, PLGA 50:50 (Sigma-Aldrich) and brain phosphatidylserine (PhS) (Lipoid) were suspended at a final concentration of 0.5 mg/mL (1:1 w/w) in DMSO (Sigma-Aldrich). Nanoparticles were produced using the NanoAssemblr® (Precision NanoSystems, Inc) at a flow rate ratio (DMSO/Tris buffer) 1:1 and a total flow rate 10 mL/min. Excess solvent removal and concentration of the particles to 20 mg/mL was achieved using the Krosflo Research III tangential flow filtration system (Spectrum Labs) with a 500 kD mPES column and a 12 mL diafiltrate volume. Particle size, polydispersity index and zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, UK) equipped with a 633 nm laser and a detection angle of 173°. All measurements were undertaken in triplicate with the attenuation value between 6 and 9.

Antigen production

Apyrogenic ovalbumin (OVA) was from Hyglos GmbH (Germany). Influenza nucleoprotein peptide NP311–325 was from JPT Peptide Technologies (Germany). OVA-NP311–325 was generated by conjugation of Cys-NP311–325 to maleimide-activated OVA (ThermoFisher) as per manufacturer instructions. Recombinant *S. aureus* clumping factor A (ClfA) was produced and purified using Detoxi-Gel; endotoxin levels were <0.005 EU/mL by limulus amoebocyte lysate (LAL) chromogenic endotoxin quantitation kit.

Mice immunisations

Intramuscular vaccination was done on days 0 and 14 using 27G/0.5in tuberculin needle in both hind limbs (biceps femoris muscles) using 50 µL/leg. Animals received 10 µg OVA alone or in combination with 1 mg of particles (or alum/CpG/cGAMP) or PBS alone. For studies investigating the role of GSDMD, mice received the GSDMD inhibitor necro-sulfonamide (NSA) i.p. (1.25mg) 1h before vaccination or i.m. (0.25mg) at the time of vaccination. For studies investigating the role of ROS, mice were treated with the ROS scavenger TEMPOL administered i.m. (25 mg/kg) or i.p. (100 mg/kg). Subcutaneous immunization was done in the flanks on days 0 and 14, with a total volume of 100 µL/mouse and previously determined optimal amounts of particles (4mg) and ClfA (1 µg). For intraperitoneal vaccination the same immunization scheme was used. Mice received PBS alone, 1 µg OVA alone or with 4mg of particles or 1mg alum in 200 µL.

B16F-OVA melanoma challenge & CD8 α T cell depletion

Mice were vaccinated i.m. as before. Two weeks after booster vaccination, 3×10^5 B16F-OVA cells in exponential growth phase were implanted subcutaneously in a volume of 100 µL. Mice were monitored daily; once the tumours became palpable the diameter of the lesion was measured using a callipers and tumour volume in mm³ was recorded. Animals were humanely euthanised when the lesion reached 1500 mm³ or if necrosis was noted. For the CD8 α T cell depletion, mice were injected i.p. with 250 µg of either a monoclonal α -CD8 α antibody (Clone 2.43) or a monoclonal isotype control antibody (Clone LTF-2) on day 26. Mice subsequently received injections of 100 µg of the respective monoclonal antibodies on days 29, 33, 37, 41 and 48. Blood was sampled from mice via the tail vein on days 31 and 35 to detect the presence of peripheral CD8 α T cells. B16-OVA tumour cells were implanted on day 28.

Quantification and characterization of ag-specific T cells

Seven days after booster single-cell suspensions were prepared by mechanical dissociation of spleens using a 40 µm mesh. After lysis with ammonium chloride buffer, 2×10^7 viable cells were suspended in 200 µL of complete RPMI. Cells were stained with PE-labelled H-2K(b) Chicken OVA257–264 SIINFEKL or APC-labelled H-2K(b) I-A(b) NP311–325 QVYSILRPNENPAHK from MBL or provided by the National Institutes of Health Tetramer Core Facility (Emory University) at 15 µg/mL for 2 h at 37°C in a tissue culture incubator (95% humidity and 5% CO₂), with orbital shaking (200 rpm). Anti-CD16/CD32 mAbs (1 µL/sample) (BD) was added prior labelling with fluorescent antibodies for mouse F4/80 (Cl:A3-1) and CD8 (KT15) (Biorad), CD4 (GK1.5, eBiosciences), CD3 (17A2) and CD11c (N418) (Biolegend), CD44 (IM7) and B220 (RA3-6B2) (BD) and incubated for 20 min. After washing in PBS live-dead Aqua staining (BD) was added (1/500 dilution) for 30 min. After washing cells were suspended in 2% v/v buffered paraformaldehyde for 20 min, washed in PBS and suspended in 200 µL of PBS+2% v/v fetal bovine serum and kept at 4°C in the dark until acquisition in a 4 laser/16 fluorescent parameter LSR-Fortessa analyzer at a maximum flow rate of 16,000 events/sec until reaching 1×10^6 live cells or 1.5×10^3 Tmer⁺ cells. Analysis was carried out in FowJo (V10X, BD).

Quantification of cytokines

Seven days after booster, 4×10^5 splenocytes obtained as before were plated in triplicate in sterile 96 well U-bottom plates (200 µL/well) in the presence of absence of 20 µg or 200 µg of OVA, or plate-bound anti-CD3 (0.2 µg/mL). Cells were incubated at 37°C with 5% CO₂ for 72h. Cytokines were quantified in cell-free supernatants using mouse IFN- γ DuoSet ELISA kits from R&D or ELISAMAX mouse IL-10 or IL-17A kits from BioLegend as per manufacturer's instructions.

Quantification ag-specific antibodies

Whole blood was collected on day 21. Clotting was allowed overnight at 4°C and serum was recovered after centrifugation for 10 min at 8000 xg. Samples were serially diluted 1:1 in assay diluent and incubated on antigen-medium-binding pre-coated plates (10 µg/mL OVA or 50 µg/mL C1a in sodium carbonate buffer, pH 9.5). Ag-specific serum Ab titers were measured using a standard ELISA protocol and the following antibodies: goat anti-mouse total IgG-HRP from Southern Biotech (1/5000 dilution), biotin rat anti-mouse IgG1 from BD (1/4000 dilution), biotin rat anti-mouse IgG2b from BD (1/4000 dilution), and goat anti-mouse IgG2c-HRP from AbD Serotec (1/2000 dilution) and developed using streptavidin-HRP or OPD substrate directly as required. OD values were obtained using a VersaMax microplate reader at 492nm. Antibody concentrations were expressed as Log10 endpoint titers calculated by regression of a curve of OD values versus reciprocal serum levels to a cutoff point of 4 SD above the blank.

Magnetic sorting of CD8⁺ T cells

CD8⁺ T cells were magnetically sorted through negative selection from the spleen of vaccinated mice using a CD8α⁺ T cell isolation kit from Miltenyi Biotec according to the manufacturer's instructions. Magnetically sorted T cells were then stained according to the instructions above to identify antigen-specific CD8⁺ cells.

Serum antibody neutralisation assay

The presence of neutralising antibodies in the serum was determined by testing the ability of serum from immunised mice to inhibit the adherence of *S. aureus* to fibrinogen. Microtitre plates were coated with fibrinogen (2 µg/mL) overnight at 4°C and were subsequently blocked for 2 h at 37°C with 5% BSA. *S. aureus* PS80 cultures were grown to stationary phase, and were incubated with mouse serum (1:60 dilution) for 30 min at room temperature before being added to the wells of a fibrinogen-coated plate and incubated for 1.5 h at 37°C. After washing with PBS, adherent cells were fixed with formaldehyde (25% v/v), stained with crystal violet and the A570 measured. Adherence was expressed as a percentage of bacterial adherence in the absence of serum and percentage inhibition was determined by subtracting the percentage adherence values from 100.

Quantification of cell death

To quantify cell death, LDH assays were carried out according to the manufacturer's instructions. Firstly, a set of triplicates wells were lysed using addition of 10X lysis buffer and incubation for 45 minutes at 37°C, 95% humidity and 5% CO₂. 600 µL of supplied assay buffer was added to 11.4 mL of supplied substrate stock solution and was mixed to make up the reaction mixture and kept in the dark. Alternatively pre-made-up reaction was thawed. Assay was conducted to fresh cell supernatants to ensure greater accuracy. 50 µL of supernatants were added to a 96 well flat-bottom plate in triplicate samples. 50 µL of reaction mixture was added to each well and the plate was incubated for 30 minutes in the dark at room temperature. Following incubation, 50 µL of supplied stop solution was added to each well, and the optical density (OD) values were determined by measurement of absorbance at 492 nm using a plate reading spectrophotometer. Additionally, the absorbance was read at 680 nm to account for any background absorbance values. This is especially apparent with larger PS nanoparticles (>100 nm). Similarly, the vehicle control of added stimulants was added to a control set of triplicates to account for spontaneous LDH activity. % Cytotoxicity was calculated as follows for each absorbance:

$$\% \text{Cytotoxicity} = \frac{(\text{Compound} - \text{treated LDH activity} - \text{Spontaneous LDH activity})}{100\% \text{ lyse control LDH activity} - 100\% \text{ lyse control LDH activity}} \times 100$$

Then the values obtained for cytotoxicity determined from the 680 nm wavelength were subtracted from this obtained from those measured at the 492 nm wavelength to account for background.

Immunoblotting

Murine BM-DCs were plated in 6-well plates, 2 × 10⁶ cells/well and allowed to adhere. BM-DCs were either left unprimed or primed for 1 h with 10 ng/mL LPS. Following this priming step, cells were stimulated with either media, LPS alone (10 ng/mL), 50 nm PS (1 mg/mL) + LPS (10 ng/mL), or 1 µm PS (1 mg/mL) + LPS (10 ng/mL) for 18 h. Cells primed with LPS (10 ng/mL) and treated with nigericin (10 µM) were used as a positive control. Following stimulation, cells were lysed using Laemmli sample buffer. Samples were then boiled with subsequent SDS-PAGE and immunoblotting. PVDF membranes were probed for full length and cleaved GSDMD protein (G7422, Sigma) and for β-actin (A5316, Sigma).

Cellular ROS quantification

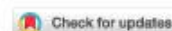
Murine BM-DCs were plated in 12 well plates, 1.5 × 10⁶ cells/well and allowed to adhere. Cells were stimulated with 50 nm, 200 nm or 1 µm PS particles at 3.9, 15.6 and 62.5 mg/mL for 6 or 18 h. Unstimulated cells were used as a control. Cells were then treated with 5 µM of CellROX® Green reagent for 30 minutes at 37°C. After centrifugation at 1200 rpm for 5 min, cells were recovered and stained with BV510 fixable viability stain for 30 min at 4°C. After washing in PBS and resuspension in FACS buffer samples were acquired in a BD FACS CANTO II and analysed in FlowJo software.

Endotoxin quantification

Endotoxin levels were quantified using a Pierce Chromogenic endotoxin quantification kit as per manufacturer's instructions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample size was determined by power analysis based on existing data from pilot studies, where necessary experiments were carried in blocks to achieve the appropriate sample size. One-way ANOVA test was used to determine significant differences between multiple groups. Pairwise comparison of the means was done with the following post-hoc tests: Tukey's test (or Tukey-Kramer for unequal sample sizes) comparison between all groups; Dunnett's for multiple comparison between treatment groups and a pre-selected control group (typically antigen alone); Fisher LSD for pairwise comparisons of selected groups. Variance of the data was tested prior multiple comparison using Levene's test. If the homogeneity of variance was not met, data were transformed to Log10 values prior statistical analysis. Statistical significance for survival analyses determined by Mantel-Cox test. Statistical significance was set at $p \leq 0.05$. p values were represented as **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$; ns $p > 0.05$. In some cases, the symbol "." was used instead of asterisks to discriminate comparisons between treatment groups or against control.



Mucosal vaccines — fortifying the frontiers

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Abstract | Mucosal vaccines offer the potential to trigger robust protective immune responses at the predominant sites of pathogen infection. In principle, the induction of adaptive immunity at mucosal sites, involving secretory antibody responses and tissue-resident T cells, has the capacity to prevent an infection from becoming established in the first place, rather than only curtailing infection and protecting against the development of disease symptoms. Although numerous effective mucosal vaccines are in use, the major advances seen with injectable vaccines (including adjuvanted subunit antigens, RNA and DNA vaccines) have not yet been translated into licensed mucosal vaccines, which currently comprise solely live attenuated and inactivated whole-cell preparations. The identification of safe and effective mucosal adjuvants allied to innovative antigen discovery and delivery strategies is key to advancing mucosal vaccines. Significant progress has been made in resolving the mechanisms that regulate innate and adaptive mucosal immunity and in understanding the crosstalk between mucosal sites, and this provides valuable pointers to inform mucosal adjuvant design. In particular, increased knowledge on mucosal antigen-presenting cells, innate lymphoid cell populations and resident memory cells at mucosal sites highlights attractive targets for vaccine design. Exploiting these insights will allow new vaccine technologies to be leveraged to facilitate rational mucosal vaccine design for pathogens including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and for cancer.

The global burden of mortality and morbidity associated with infectious diseases caused by mucosal pathogens remains unacceptably high. Indeed, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic provides a brutal reminder of the continual threat of novel mucosal infectious challenges, in addition to the threat posed by many widespread mucosal infections for which no or only suboptimal vaccines exist. Now more than ever, there is a clear focus on vaccine requirements for respiratory pathogens but, importantly, new and improved vaccines are also urgently needed for numerous enteric pathogens and other agents such as those causing sexually transmitted diseases (STDs) and oncogenic viruses that gain access through the mucosae.

Respiratory pathogens remain a prominent cause of global mortality, with lower respiratory tract infections constituting the fourth leading cause of death worldwide¹. Lower respiratory tract infections are responsible for approximately 2.4 million deaths per annum, with *Streptococcus pneumoniae*, respiratory syncytial virus (RSV), *Haemophilus influenzae* B and influenza virus taking a particularly high toll on the young (<5 years old) and older people². There is currently no approved vaccine for RSV infection, which is particularly prevalent in children and infants^{3–4}, and

although there are licensed vaccines targeting respiratory pathogens such as *Mycobacterium tuberculosis*, *S. pneumoniae*, *Bordetella pertussis* and influenza virus, improved vaccines for these pathogens are required to enhance suboptimal protection, particularly at the site of infection, and to increase coverage (FIG. 1). There are indications that innovative mucosal vaccine approaches offer promise for these infections. For example, live attenuated influenza vaccines given intranasally are now an integral part of influenza vaccination strategies with particular application to children^{5,6}, intranasally administered *B. pertussis* vaccines have entered phase II trials^{7,8} (Supplementary Table 1) following successful phase I completion, and preclinical data investigating the intranasal delivery of Bacillus Calmette–Guérin for *M. tuberculosis* have yielded promising results⁹. The emergence of SARS-CoV-2 has firmly demonstrated how deadly and disruptive respiratory pathogens can be, with approximately 2.6 million deaths attributed to this pathogen at the time of writing¹⁰ and estimates that the SARS-CoV-2 pandemic will continue to stunt global economic growth in 2021, particularly in low-income countries¹¹. Although an array of effective SARS-CoV-2 vaccines have been designed and implemented, challenges in mass production and deployment

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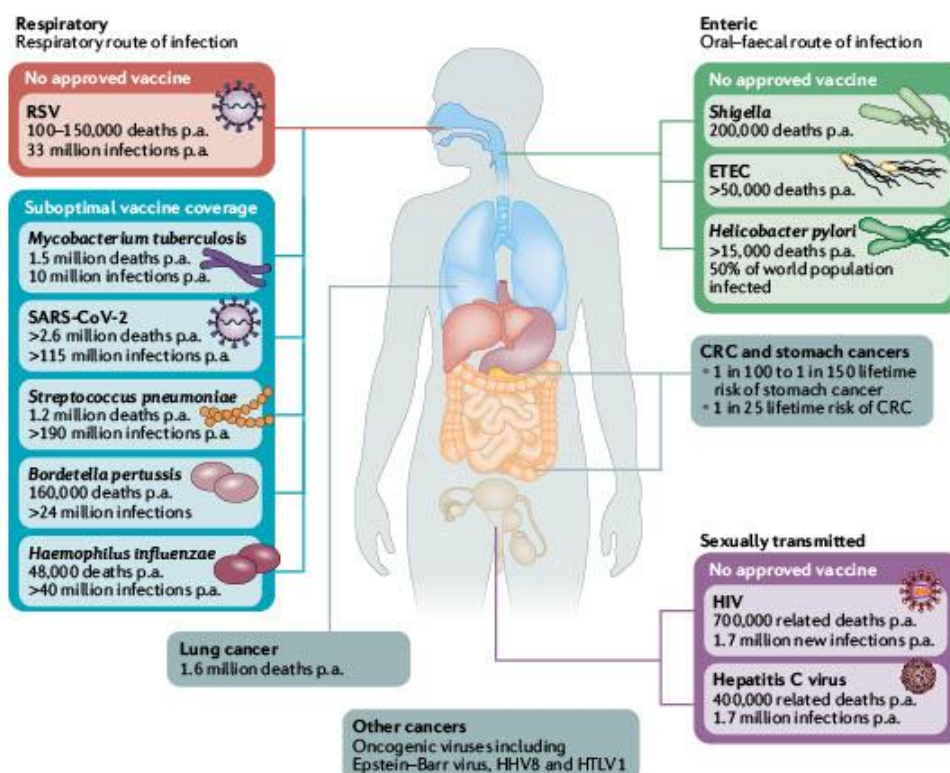


Fig. 1 | Burden of mucosal diseases with unmet vaccine needs. Respiratory, enteric and sexually transmitted infections constitute prominent causes of death worldwide, and this is exacerbated in low-income regions. Aetiological agents shown are vaccine targetable, but there remains an unmet need for new or improved vaccination approaches to address global vaccine coverage. Mucosal vaccination strategies hold promise to address this unmet need, providing more robust mucosal immunity and an alternative to parenteral vaccination. In addition to their centrality in the pathogenesis of infectious disease, mucosal tissues are frequent sites of tumour development and mucosal vaccination strategies may play a role in the prophylactic and therapeutic targeting of these malignancies. CRC, colorectal cancer; ETEC, enterotoxigenic *Escherichia coli*; p.a., per annum; RSV, respiratory syncytial disease; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

still provide an unmet need for global coverage (FIG. 1). New vaccines could help to circumvent these issues. In particular, orally delivered SARS-CoV-2 vaccines would be suited to global vaccination attempts, especially in lower-income countries, as these vaccines will not only allow for enhanced convenience and compliance but also the intestine may represent a viral target organ¹². Indeed, the development of ‘universal’ mucosal vaccines targeting conserved antigens on coronaviruses¹³ and influenza viruses, although challenging, may be a viable option for prevention of future pandemics.

Enteric pathogens causing diarrhoeal disease are the eighth leading cause of death worldwide, with children, in particular, at risk¹⁴. Among these, *Shigella* and enterotoxigenic *Escherichia coli* (ETEC) have an urgent vaccine requirement (FIG. 1). Enteric pathogens and associated acute and chronic infections have a stark impact on the livelihoods of at-risk individuals in lower-income countries. Aside from diarrhoeal disease, the impact of such infections on physical and cognitive development is becoming more apparent¹⁴, not only highlighting the need for vaccine development but also impacting how

we determine vaccine efficacy. Lack of moderate-to-severe symptoms may not be an adequate correlate of protection — prevention of colonization and/or low-grade infection may be the crucial determinant. The World Health Organization (WHO) has endeavoured to end cholera by 2030 through implementation of widespread preventive measures, including vaccination¹⁵, providing a challenge to oral cholera vaccine manufacturers globally. This may be addressed through successful development of lower-cost alternative oral cholera vaccines such as Hillchol, which is currently under clinical evaluation¹⁶.

Mucosal vaccines targeting the genital tract have the potential to combat STDs and local tumours, which is important given that cervical cancer represents the fourth most common cancer in women¹⁷. Clearly, there is an enormous need for an effective HIV vaccine and given the intestinal tropism of the virus¹⁸, mucosal vaccine strategies are warranted¹⁹. Additionally, the emergence of multidrug-resistant STDs is of concern and could be combatted through preventive mucosal vaccine strategies.

Whereas most licensed vaccines are currently administered by injection, mucosal vaccines can outperform parenteral vaccination strategies in eliciting protective mucosal immune responses that block infection or transmission^{20,21}. The nature of the infection must be considered from the outset in designing mucosal vaccines or, indeed, in assessing whether targeting the mucosal route is necessary. For example, in the case of enteric pathogens, the infections may be invasive (as seen in typhoid and polio), partially/locally invasive (as seen in shigellosis) or strictly mucosal (as seen for cholera and ETEC infections)^{22,23}. This will impact on whether a systemic immune response is an appropriate objective, or whether predominantly mucosal or both mucosal and systemic immunity would be more effective. In this context, the nature of the mucosal surface (for example, the uninflamed small intestine versus the lower respiratory tract) will influence the accessibility of circulating antibodies, the nature of the dominant antibody isotype and the transport mechanism governing access of antibodies to mucosal infectious pathogens^{24,25}.

Strong mucosal cellular and humoral immune responses have the potential to induce sterilizing immunity by impeding pathogen binding to and uptake across epithelial surfaces. However, there are significant hurdles to mucosal vaccine development, including incomplete knowledge of the nature of protective mucosal immune responses. Advancing new mucosal vaccines and improving existing vaccines requires innovative adjuvant approaches and delivery strategies, which is the focus of this Review. Given the specific architecture of the mucosal surfaces and their unique cellular composition, vaccine strategies should be specifically tailored for the target site rather than redeploying effective injectable vaccines. In any case, many adjuvants that are effective by injection are not optimal for mucosal delivery.

Lessons from licensed mucosal vaccines

Over recent decades, there has been a broad shift from injectable whole-cell killed and attenuated vaccines towards adjuvanted subunit and, more recently, viral vectored, RNA and DNA vaccines^{26,27}. This can reduce the potential for excessive reactogenicity and is facilitated by advances in antigen discovery, adjuvants and delivery systems. However, the landscape for mucosal vaccines is very different. Of the nine mucosal vaccines approved for use in humans — eight oral and one intranasal — all are either live attenuated or whole-cell inactivated vaccine formulations (FIG. 2). This current dichotomy in approaches is, in part, due to greater tolerability of orally administered whole-cell killed antigens, the susceptibility of unprotected subunit antigens to degradation and clearance, and, crucially, a lack of proven mucosal adjuvants.

Currently, the only subunit antigen included in a licensed mucosal vaccine is cholera toxin B subunit (CTB), included as an additional component of the killed whole-cell *Vibrio cholerae* vaccine Dukoral. CTB cannot be regarded as a 'model' subunit antigen as this is the binding component of cholera toxin — it binds with high affinity to GM1 on epithelial cells and is highly immunogenic^{28–30} (BOX 1). Nevertheless, this does

indicate that potent immune responses can be induced against an orally administered purified protein even if this is in the presence of whole bacteria. The tolerability of oral whole-cell antigens is instructive as, although adjuvanted subunits are preferable for parenteral formulations, leveraging potent mucosal adjuvants with whole-cell antigens may be a more productive approach for mucosal vaccination, especially via oral routes.

Developing whole-cell antigens as a platform offers potential for combination with purified subunits but also, perhaps more encouragingly, for overexpression of antigens on whole cells — ETVAX provides a stellar example of this³¹. Developed at the University of Gothenburg in collaboration with Scandinavian Biopharma, ETVAX comprises four *E. coli* strains engineered to overexpress colonization factor antigens on the bacterial surface, namely CFA/I, CS3, CS5 and CS6, in combination with LCTBA^{31,32} (a CTB and *E. coli* heat-labile enterotoxin B subunit (LTB) hybrid; see BOX 1). Overexpression of antigens on inactivated whole bacteria is thus a promising approach to increase immunogenicity, leveraging the adjuvanticity of inactivated bacteria while helping to minimize the vaccine dose required. This approach may also be applied to inactivated viruses or virus-like particles, taking advantage of their inherent immunogenicity, particulate antigen presentation and well-established expression systems^{33,34}.

Aside from Dukoral, Euvichol and Shanchol, the other licensed mucosal vaccines are all live attenuated bacteria (*Salmonella enterica* subsp. *enterica* serovar Typhimurium) or attenuated and/or reassortant viruses delivered orally (polio vaccine, rotavirus) or nasally (influenza A and influenza B viruses). Overall, this highlights the tolerability and effectiveness of mucosal attenuated and whole-cell vaccines but also points to the key question of why the successful shift to more modern vaccine strategies has not yet occurred for mucosal vaccines.

Vaccine lessons from mucosal tissues

Unique aspects of mucosal immune responses. There are many distinctive features of mucosal immune responses that impact on mucosal vaccine design, ranging from the structure and location of immune inductive sites to the type of effector and memory cells induced and their longevity and location. The mucosal immune system can be broadly categorized into inductive sites where antigen-specific B cell and T cell responses are initiated and effector sites (such as the lamina propria and epithelium) where the adaptive immune responses are mediated. The nature of the inductive sites varies between species and also between different mucosae. In the case of the intestine, the inductive sites are the gut-associated lymphoid tissue and the intestine-draining mesenteric lymph nodes. Gut-associated lymphoid tissue in humans and mice comprises Peyer's patches and isolated lymphoid follicles³⁵. The connection between inductive and effector sites is facilitated by selective expression of integrins and chemokine receptors on B cells³⁶ and T cells³⁷. For example, in the case of the small intestine, imprinting of $\alpha 4\beta 7$ integrin and CC-chemokine receptor 9 (CCR9) expression on lymphocytes is key for tissue-specific homing of

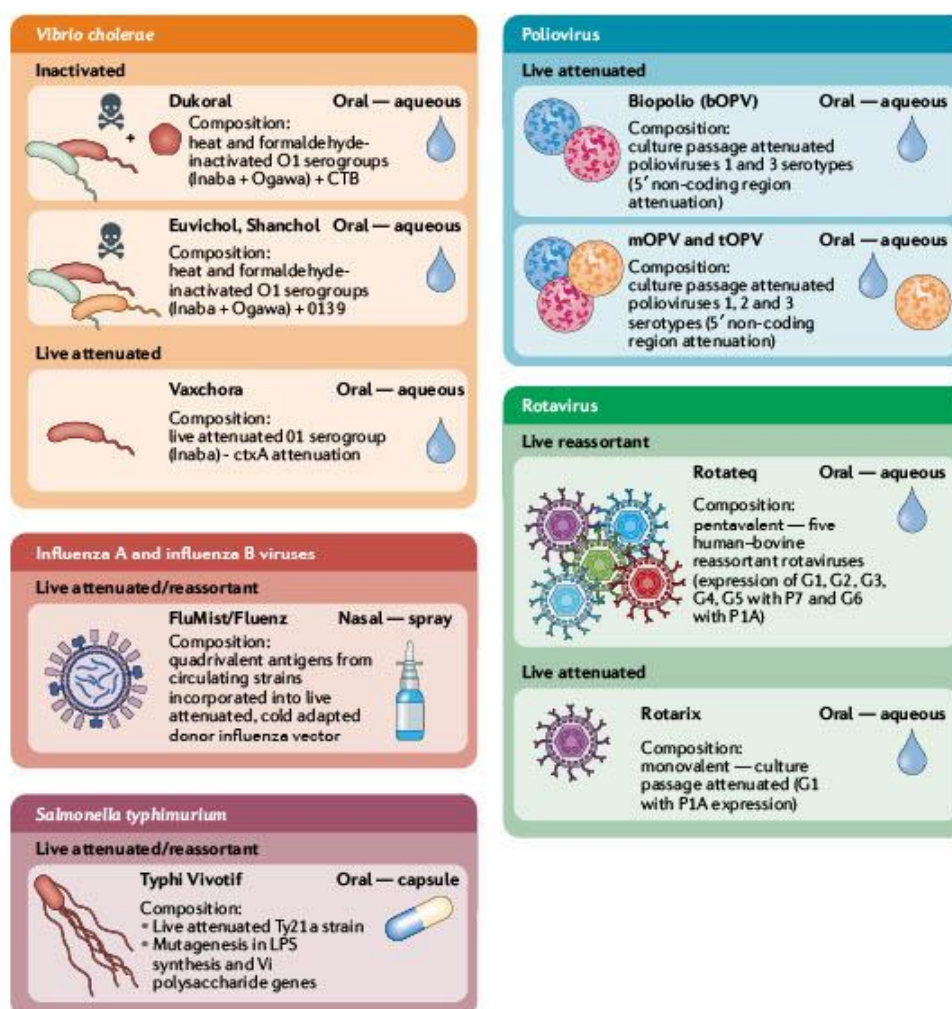


Fig. 2 | Licensed mucosal vaccines. Eight oral vaccines are currently licensed for use against cholera, salmonella, poliovirus and rotavirus. Live attenuated influenza vaccines remain the sole licensed intranasal vaccines. To date, live attenuated and inactivated vaccines have proved the most successful platforms for mucosal vaccine design. CTB, cholera toxin B subunit; LPS, lipopolysaccharide.

cells. Although mucosal immune responses are compartmentalized, there is crosstalk between different mucosae, and it is thus possible to vaccinate at a single mucosal site but also promote immune responses at distant mucosal sites³⁸. Understanding the nature of the signals regulating such homing in a human context is critical to allow design of novel mucosal vaccines that can potentially target mucosae distant from the site of vaccination.

Mucosal sites cover a surface area of 30–40 m² in humans³⁹ predominantly in the gastrointestinal tract, respiratory tract, urogenital tract and ocular cavities, playing a crucial role in homeostasis and interactions with the microbiome, dietary antigens and other environmental material. As a result, they constitute prominent points of pathogen entry and are often sites of tumour development. This high antigenic load and constant exposure to microbes necessitates mucosal

immunoregulatory mechanisms that are vital to maintain homeostasis and prevent damaging chronic inflammatory responses⁴⁰. This has significant implications with regard to vaccine development, for example, many Toll-like receptor (TLR) agonists that are effective adjuvants when injected parenterally have poor efficacy when administered orally. This results, at least in part, from tolerization of intestinal antigen-presenting cells (APCs) to pathogen-associated molecular patterns, particularly TLR ligands to which they are continuously exposed via the microbiome⁴¹, and also as a result of the basolateral rather than luminal expression of TLRs at epithelial surfaces. Detailed knowledge on mucosal responsiveness to pathogen-associated molecular patterns, responsive target cells and their location is critical so that productive target pathways can be identified for adjuvant discovery and optimization.

A recent report demonstrated that proximal intestinal gut-draining lymph nodes preferentially supported regulatory T cell responses whereas distal gut-draining lymph nodes supported the induction of effector T helper cells⁴². These insights into the balance of regulatory and effector responses can inform vaccine design — if antigen uptake in proximal regions of the small intestine preferentially enhances tolerogenic responses, delivery of oral vaccines in solution may not be optimal and targeting of antigens to the distal small intestine or large intestine may be more effective. This preferential induction of regulatory T cells in the proximal intestine could also be affected by the presence of adjuvants or the nature of the orally administered antigen. Vaccines could thus potentially over-ride the tolerogenic environment in the proximal intestine by inducing an inflammatory signature to allow the induction of effector T cell responses, although overall there may be an advantage in targeting the distal intestine.

One such large intestine-targeted oral delivery system was described where vaccine nanoparticles were delivered within pH-dependent microparticles. This oral construct induced protective antiviral immunity in the rectum and vagina comparable with levels seen with colorectal vaccination and protected against rectal and vaginal viral challenge⁴³, providing a potentially productive route for mucosal vaccination for STDs. Although compartmentalization of mucosal immune responses is well established, confirming that the same subdivisions and connections apply from rodents to humans can be a challenge. However, it was shown that oral Dukoral vaccination increased the numbers of circulating IgA⁺ memory B cells with a surface marker expression profile indicative of homing to the large intestine and respiratory tract⁴⁸. Furthermore, translation of vaccine delivery strategies from small animal models to humans can be

challenging owing to differences in parameters including gastrointestinal pH, gastrointestinal tract residence times and intestinal surface area. Some of these factors have been addressed in the case of oral drug delivery, but it is quite clear that in the absence of immunogenic antigens and effective adjuvants, addressing delivery challenges in isolation offers modest benefits and the vaccine components must be optimized for the targeted mucosal pathogen and its site of infection. The nature of the antigen is also a major determinant of responses, whether living or non-living, soluble or particulate. This can dictate the nature of antigen uptake pathways at mucosal sites and should be a principal design feature in the development of mucosal vaccines (FIG. 3). Particulate antigens — whether as whole bacterial cells, attenuated or inactivated viruses, virus-like particles or synthetic particulate formulations — are more immunogenic than purified proteins⁴³ and, in addition to their greater inherent immunogenicity, when delivered mucosally their particulate nature can impact on the site of uptake and APC targeting.

Antigen-presenting cells in mucosal tissues. APC populations at mucosal sites are highly dynamic. In addition to tissue-resident dendritic cells and macrophages, during inflammatory responses or infection, further APCs are recruited that can engage with absorbed antigen and contribute to effector responses⁴⁴. Indeed, there is evidence that during inflammation, monocytes in the gut and lungs can upregulate CCR7, migrate to lymph nodes and prime T cell responses⁴⁵, and recruited monocytes or immature macrophages can produce inflammatory cytokines that contribute to T helper 1 (T_H1) cell and T_H17 cell responses⁴⁴. Local inflammation triggered by mucosal vaccines and/or adjuvants could thus contribute to enhanced adaptive immunity by recruiting APCs. Although tissue-resident macrophages do not migrate to lymph nodes and are thus unlikely to directly prime T cells, antigen sampling CX₃C-chemokine receptor 1 (CX₃CR1⁺) mononuclear phagocytes can transfer antigen to dendritic cells⁴⁶ (FIG. 3) and colonic CX₃CR1⁺ mononuclear phagocytes were shown to be required for induction of T_H17 cell and antibody responses to intestinal fungi⁴⁷.

Two key dendritic cell populations in gut-draining lymph nodes — CD103⁺CD11b⁺ dendritic cells and CD103⁺CD11b⁺ dendritic cells — have been associated with tolerogenic or pro-inflammatory immune responses, respectively⁴⁸. Assigning specific roles to dendritic cell and macrophage populations in the intestine can be challenging as this is context-dependent. A recent study in a model of *S. Typhimurium* infection found that intestinal CX₃CR1⁺ macrophages were superior to conventional type 1 dendritic cell (cDC1) and cDC2 populations in promoting the production of *S. Typhimurium*-specific IgA⁴⁹. Furthermore, these broad categories of dendritic cell populations may not capture the true complexity of responses in the intestine, and subsets of these populations can contribute to the recruitment and activation of T cells and B cells at the site of infection. Differential gene expression profiles in cDC1 and cDC2 populations from different gut regions were reported⁴⁹, indicating that

Box 1 | CTB — mucosal subunit antigen or adjuvant?

The pentameric cholera toxin B subunit (CTB) has been successfully and safely incorporated in recombinant form into the oral cholera vaccine Dukoral since 1991. It represents the only subunit antigen incorporated in a licensed mucosal vaccine to date. CTB binds to the membrane ganglioside GM1 (REF.¹⁴⁷) and fucosylated glycans^{148,149} on cells including enterocytes, microfold (M) cells, macrophages and dendritic cells. As a result, CTB has the potential to promote effective delivery of bound antigens to mucosal antigen-presenting cells (APCs)⁴⁰. Although CTB has in the past been classed as an adjuvant, this definition was complicated by the presence of residual cholera toxin or lipopolysaccharide (LPS) in CTB preparations. Indeed, CTB is now well established as an effective mediator of tolerance to attached or admixed antigens by oral and intranasal routes^{147–149}. It was also proposed that orally delivered CTB can promote intestinal repair and healing responses¹⁵⁰. In human studies, CTB has been shown to also promote induction of antigen-specific local IgA and systemic IgG responses when administered via rectal and intranasal routes^{151,152}. Its inclusion in Dukoral is primarily to induce intestinal and circulating cholera toxin-specific antibodies, which can contribute to short-term protection against cholera¹⁵³ and cross-protection against enterotoxigenic *Escherichia coli* (ETEC) via shared epitopes in *E. coli* heat-labile enterotoxin B subunit (LTB)^{154,155}. Similarly, LCTBA (a CTB and LTB hybrid) is included in the candidate ETEC vaccine ETVAX, expanding the number of antigenic targets and cross-protection¹⁵⁶. Recently, CTB has been shown to promote activation and expansion of polyfunctional T helper 1 (T_H1) cells and T_H17 cells when given intradermally alongside a DEC205⁺ dendritic cell-targeted antigen; notably, this included induction of local and intestinal protective tissue-resident memory T (T_{RM}) cells²⁹. This highlights the potential for incorporation of CTB in parenteral–mucosal push–pull vaccination strategies.

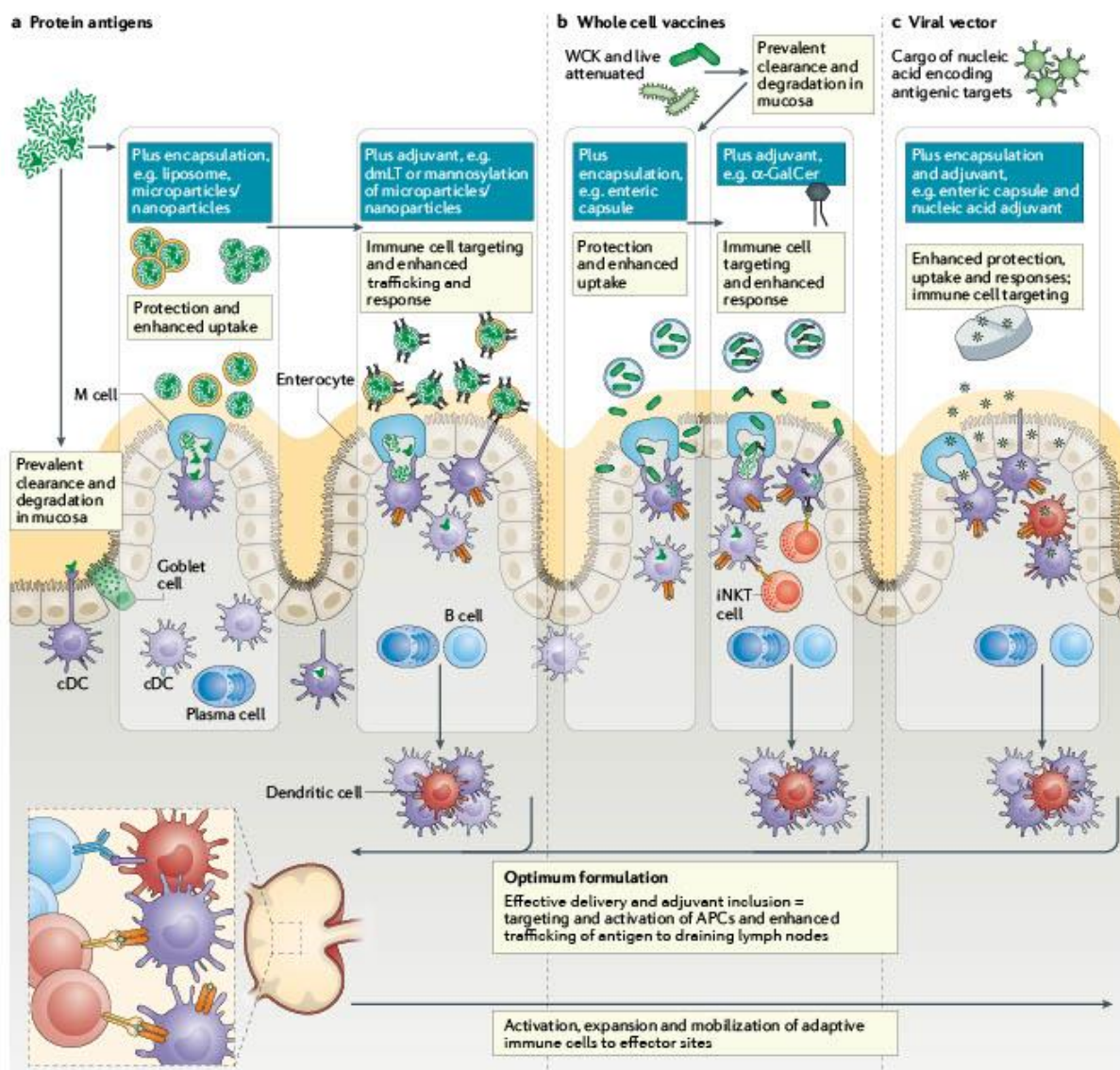


Fig. 3 | Vaccine uptake at mucosal sites. Nature of antigen uptake in the intestine is dependent on the type of vaccine components used, whether soluble or particulate, inert or live. Innovative encapsulation and targeting strategies have the potential to protect antigens while enhancing uptake and delivery to optimal intestinal regions. Inclusion of an effective mucosal vaccine adjuvant can confer multiple benefits from preventing tolerogenic responses to antigens, recruiting and activating antigen-presenting cells (APCs) and engaging other innate immune cells that contribute to protective immunity. Although there are currently few safe and effective adjuvants, cell-targeting adjuvants such as *Escherichia coli* double-mutant heat-labile toxin (dmlT), which binds to gangliosides on microfold (M) cells and dendritic cells, or α -galactosylceramide (α -GalCer), which can promote activation of invariant natural killer T (iNKT) cells locally and in draining lymph nodes via dendritic cell-mediated presentation, offer promise. Optimal formulations will address antigen design, adjuvanticity and antigen protection and targeting to address the unique challenges of intestinal delivery in the case of protein antigens (part a), whole cell vaccines (part b) and viral vector vaccines (part c). cDC, conventional dendritic cell; WCK, whole-cell killed.

not only the type of gut dendritic cell but also its precise tissue location may be key for the outcome of oral vaccination. The latter study also found that, compared with proximal gut-draining lymph nodes, distal gut-draining lymph nodes are more efficient in promoting the

differentiation of T_H17 cells. Given the importance of T_H17 cells for defence against extracellular pathogens and for support of intestinal IgA secretion, this finding may be instructive for delivery of oral vaccines. It is of note that cDC1 and cDC2 frequencies remain relatively stable

Chitosan

A cationic polymer derived from chitin with mucosal adjuvant properties. In addition to its mucoadhesive attributes, chitosan promotes adaptive immunity through activation of the cGAS–STING and NLRP3 inflammasome pathways.

Enchained growth

A process where high-affinity IgA specific for surface antigens cross-links bacteria, preventing daughter cell separation after division and contributing to clearance of mucosal pathogens.

Microfold (M) cells

Specialized antigen sampling epithelial cells generally found in the follicle-associated epithelium overlying organized mucosal lymphoid tissue.

throughout life⁵⁰ (TABLE 1), an important consideration for cDC-targeted vaccine strategies. In the nasal mucosa, resident cDCs are vital for maintaining non-responsiveness to harmless inhaled antigens but viral infection promotes recruitment of monocyte-derived dendritic cells (moDCs) that can mediate T cell priming⁵¹. This study utilized a nasally administered chitosan hydrogel vaccine platform to trigger the inflammatory recruitment of moDCs coupled to the sustained release of antigen, which successfully promoted antigen-specific CD8⁺ T cell activation and expansion. This suggests that in addition to potentially targeting specific populations of mucosal dendritic cells, vaccines can also aim to promote recruitment of monocytes and moDCs to mediate protective mucosal immunity. Indeed, with subunit antigens, adjuvants may be critical to overcome tolerance induction and may also contribute to recruitment of ‘unconditioned’ APCs that prime antigen-specific T cells and B cells. Assessing the capacity of mucosal adjuvants to alter the activation states of tissue-associated or lymph node dendritic cells and to recruit monocytes and other APCs may be a useful indicator of efficacy, certainly more so than in vitro screening of cultured dendritic cells or macrophages that may poorly reflect responses of mucosal APCs following vaccination.

Induction of IgA and other mucosal antibodies. In terms of correlates of immunity following mucosal vaccination, induction of antigen-specific IgA is a crucial consideration. IgA is the dominant antibody at many mucosal sites and mediates protection against a range of enteropathogens. Recently, the importance of dimeric IgA in neutralization of SARS-CoV-2 has been highlighted in patients infected with the virus⁵² and high-avidity IgA can protect against enteropathogens by processes including agglutination and the recently described process of ‘enchained growth’⁵³. For oral vaccination with adjuvanted

subunit antigens, multiple doses (at least three) are needed to induce effective secretory IgA responses²¹. Induction of antigen-specific IgA was observed following two doses of ETVAX, with the addition of the *E. coli* double-mutant heat-labile toxin (dmLT) adjuvant enhancing the kinetics of induction⁵⁴. This suggests that suitable adjuvants may exert a dose-sparing effect in mucosal IgA induction. Vaccines utilizing viral vectors and attenuated viruses may induce IgA responses more efficiently — for example, one dose of intranasal live attenuated influenza vaccine can elicit mucosal IgA⁵⁴ in recipients, and faecal/salivary IgA was observed in recipients of the Vaxart norovirus vaccine candidate, comprising an adjuvanted enterically stable adenovirus type 5 (Ad5) vector⁵⁵. Although detailed assessment of antigen-specific mucosal immunity is more challenging in humans than in preclinical models, recent advances are facilitating novel means of determining vaccine-induced mucosal IgA responses in clinical trials (BOX 2).

Although the importance of mucosal dendritic cell-mediated antigen sampling and trafficking to draining lymph nodes for induction of IgA responses has long been appreciated, Kombar et al. recently uncovered a new layer of antigenic crosstalk between microfold (M) cells and B cells in the subepithelial dome region of Peyer’s patches. CC26⁺CCR1⁺GL7⁺ B cells were shown to be capable of sampling antigen directly from M cells and trafficking to germinal centres where their activation and population expansion occurs, challenging the idea of dependence on cDC-mediated antigen transfer for optimal antigen-specific IgA induction⁵⁶. However, whereas transport of secretory antibody (IgA, IgM) via the polymeric immunoglobulin receptor is an essential and highly efficient process in the intestine, this is not the case at all mucosal sites. In the female reproductive tract, IgG rather than IgA can be critical for protective immunity to viral infection⁵⁷. Likewise, IgG plays an

Table 1 | Frequency of human innate and innate-like immune cell populations at different mucosal sites

Immune cell population	Frequency at mucosal tissue site					Refs
	Upper respiratory tract/oral gingivae	Lungs/lower respiratory tract	Duodenum/jejunum	Ileum	Colon	
ILC ^a	3.5% of total CD45 ⁺ cells (80% ILC3, 20% ILC1, <1% ILC2)	0.19% of total CD45 ⁺ cells (60% ILC3, 30% ILC2, 10% ILC1)	1% of total CD45 ⁺ cells (ILC1 dominant)	0.8% of total CD45 ⁺ cells (ILC3 dominant)	1.1% of total CD45 ⁺ cells (ILC3 dominant)	128–131
cDC1	NA	0.05% of total CD45 ⁺ cells	0.025% of total CD45 ⁺ cells	0.021% of total CD45 ⁺ cells	0.0075% of total CD45 ⁺ cells	50
cDC2	NA	0.25% of total CD45 ⁺ cells	0.1% of total CD45 ⁺ cells	0.07% of total CD45 ⁺ cells	0.06% of total CD45 ⁺ cells	50
pDC	NA	0.03% of total CD45 ⁺ cells	0.0015% of total CD45 ⁺ cells	0.004% of total CD45 ⁺ cells	0.015% of total CD45 ⁺ cells	50
Natural killer cell	NA	10–15% of total CD45 ⁺ cells	1–2% of total CD45 ⁺ cells	0.9% of total CD45 ⁺ cells	1% of total CD45 ⁺ cells	128–131
γδT cell	NA	ND in humans ^b	13% of total IELs	12% of CD3 ⁺ IELs and 35% of CD3 ⁺ LPLs	30–40% of CD3 ⁺ IELs and 5% of CD3 ⁺ LPLs	132–137
NKT cell	NA	ND in humans ^b	0–2% of IELs and LPLs	0–2% of IELs and LPLs	0–2% of IELs and LPLs	138
MAIT cell	NA	2–4% of CD3 ⁺ cells	2–11% of CD3 ⁺ cells	2–11% of CD3 ⁺ cells	0.3–5% of IELs and 1–3% of LPLs	139–142

cDC1, conventional type 1 dendritic cell; IEL, intraepithelial lymphocyte; ILC, innate lymphoid cell; LPL, lamina propria lymphocyte; MAIT, mucosal-associated invariant T cell; NKT cell, natural killer T cell; NA, not available; ND, not defined; pDC, plasmacytoid dendritic cell. ^aExcluding classical natural killer cells. ^bγδT cells have not been defined in human lung tissue but have been measured in bronchoalveolar lavage fluid.

Box 2 | Methodologies and challenges in studying human mucosal immune responses following vaccination

Although mucosal immune responses can be characterized preclinically in great detail in tissues and secretions, this is challenging in a clinical context. Therefore, establishing robust correlates of vaccine-induced adaptive immunity is a priority^{736–738}. Assessing antibody responses in mucosal secretions has been a predominant approach. Indeed, vaccine-induced IgA responses in saliva⁷³⁹, nasal wash⁷⁴⁰ and faecal samples⁷⁴¹ are frequently determined. Salivary IgA sampled from the submandibular/sublingual region has also been shown to correlate well with intestinal IgA responses in an enterotoxigenic *Escherichia coli* (ETEC) challenge study⁷⁴². The potential to determine mucosal cellular immune responses is restricted by access to tissues, and as sampling from mucosal sites such as the lungs and intestines is invasive and unpleasant for trial volunteers, blood collection is predominantly relied upon to identify vaccine-induced migrating effector cells in peripheral blood mononuclear cells. Circulating mucosal effector cell populations can be characterized using lineage and effector markers, alongside mucosal homing marker expression by flow cytometry. ELISPOT assays can be used to measure antibody-secreting cells⁷⁴³ in isolated peripheral blood mononuclear cells; alternatively, supernatants from cultured peripheral blood mononuclear cells can be harvested for evaluation of antibodies in lymphocyte secretion (antibody in lymphocyte supernatant (ALS))⁷⁴⁴ by ELISA or multiplex assays. As the circulation of mucosa-derived lymphocytes in the blood is a dynamic and transient process, optimization of kinetics is critical. Assessing responses 5 days following oral booster vaccination has been suggested as optimal for detecting antibody-secreting cells/ALS responses⁷⁴⁵, which have been shown to correlate with mucosal immune responses in the case of challenge or vaccination trials with ETEC and cholera vaccines^{746–748}. Logistically, ALS methodologies are advantageous as supernatants can be frozen for later analysis. Mottram et al.⁷⁴⁹ recently identified B cell maturation antigen (BCMA) as a biomarker for the induction of vaccine-specific IgA and memory B cell responses to multiple antigens when measured via ELISA in ALS samples following oral vaccination; this simplified assay may prove especially useful when only low blood volumes are available, for example, in paediatric samples.

important role in protective immunity in the lower respiratory tract whereas IgA is relatively more important in the nasal compartment⁵⁸.

Tissue-resident memory T cells in mucosal tissues. Tissue-resident memory T (T_{RM}) cells have been identified at multiple mucosal sites⁵⁹ and are thought to play decisive roles in rapid responses to infection⁶⁰ and cancers (BOX 3), thus providing another important correlate for mucosal vaccine efficacy. The human duodenal CD4⁺ T cell compartment was recently shown to be enriched with a population of polyfunctional T_H1 cells, which survive for at least 1 year⁶¹. This offers significant hope for inducing sustained protective cellular immunity if optimal oral vaccine strategies are designed. There are clearly significant tissue-specific differences in the nature of T_{RM} cell populations so this must be considered in the design of new vaccine approaches. CD8⁺ T_{RM} cells in the lungs are pivotal for protection against respiratory viral infections but these cells are generally relatively short-lived, and this can compromise responses to subsequent infection⁶². The latter study found that systemic vaccination (intravenous administration of *Listeria monocytogenes* expressing influenza virus-associated antigen) could enhance lung T_{RM} cells in mice previously infected with influenza virus by increasing numbers of circulating effector memory T cells. This clearly has implications regarding the potential for systemic booster vaccinations in previously infected or mucosally primed populations to sustain resident memory CD8⁺ T cells in the lungs. A population of lung-resident helper T cells was recently characterized that was required to support

tissue-resident memory B cells and CD8⁺ cells following influenza virus infection^{63,64}. These cells, which are induced locally in the lung, may be key for promoting long-lived cellular and humoral immunity following vaccination in the respiratory tract, so the optimal strategy for their induction should be addressed. Recent evidence indicates that long-term maintenance of lung T_{RM} cells requires airway vaccination and sustained antigen presence in the lungs, which was facilitated by an adenovirus vector vaccine⁶⁵. It was recently shown in mouse models that T_{RM} cells migrate to the mediastinal lymph nodes from the lungs during infection in a process termed ‘retrograde migration’. These cells retained a T_{RM} cell phenotype and provided long-term protection⁶⁶. This may be an important consideration following intranasal vaccination strategies. Further studies from the same group demonstrate that, upon restimulation, T_{RM} cells can undergo retrograde migration and give rise to effector memory T cells and central memory T cells that have a predisposition for homing to their tissue of origin⁶⁷.

Targeting the genital tract. Although oral, sublingual and nasal routes are more convenient and there are currently no vaccines that specifically target the genital tract in clinical use, vaccination in the genital tract could have significant advantages in targeting STDs, even as a vaccine-boosting approach. In mouse models, vaginal immunization with herpes simplex virus 2 (HSV-2) glycoprotein D antigen and the adjuvant α -galactosylceramide (α -GalCer) induced protective immunity against HSV-2 challenge⁶⁸. A combined vaccination approach using recombinant influenza virus–HIV vectors administered via intranasal and intravaginal routes (in mice) resulted in HIV-specific CD8⁺ T_{RM} cells in the vaginal mucosa⁶⁹. Vaginal immunization of mice with an attenuated HSV-2 strain resulted in the induction of a population of IFN γ ⁺CD4⁺ T_{RM} cells, which promoted CXCL9-mediated and CXCL10-mediated recruitment of memory B cells upon secondary challenge⁷⁰. By contrast, primary vaccination did not result in the induction of a tissue-resident population of plasma cells in the female reproductive tract. Thus, vaginal booster vaccination or, possibly, booster vaccination in the large intestine may be an effective strategy following systemic priming to trigger genital tract responses, although these findings must first be confirmed in a human setting. Promising recent data showed that vaginal delivery (by intramucosal vaginal injection or spray) of recombinant glycosylated IL-7 to rhesus macaques acted as an effective mucosal adjuvant, enhancing the induction of antigen-specific IgA/IgG in the vaginal mucosa following subsequent vaginal delivery of diphtheria toxoid⁷¹. This could be a broadly applicable strategy that may overcome hypo-responsiveness to vaginal vaccine delivery.

Immune cell populations targeted by mucosal vaccines. Mucosal adjuvants should aim to activate and target local or recruited APCs (FIG. 3) or populations of immune cells enriched in the mucosa (TABLE 1) in order to mount effective mucosal responses. Innate lymphoid cells (ILCs),

mucosal-associated invariant T cells, natural killer T (NKT) cells and $\gamma\delta$ T cells are abundant in mucosal tissues and can play crucial roles in mediating and shaping mucosal immunity^{72–79}. Adjuvants can also be exploited in parenteral–mucosal push–pull strategies; for example, dmLT has been shown to imprint mucosal homing markers on T cells when injected⁸⁰. Similarly, retinoic acid has been identified as a suitable adjuvant in such strategies, imprinting gut-homing markers on T cells and leading to protective intestinal responses following subcutaneous vaccination⁸¹. There are currently two ongoing trials investigating parenteral–mucosal push–pull strategies for SARS-CoV-2 vaccination: NCT04732468 and IG/VPIN/CVD19/2001. The former trial involves investigating combinations of oral and subcutaneous immunization with a human adenoviral vector expressing modified SARS-CoV-2 spike and nucleocapsid proteins. By contrast, the latter trial involves combinations of intranasal and intramuscular immunization, with the vaccine composed of the receptor-binding domain of SARS-CoV-2 spike protein adjuvanted with hepatitis B virus nucleocapsid protein when given intranasally and with alum when given intramuscularly.

Mucosal adjuvant approaches

Enhancing the efficacy of subunit and inactivated antigens. Toxoid adjuvants are the best-characterized class of mucosal adjuvants and the development of safe yet potent derivatives of *E. coli* heat-labile toxin and cholera toxin (BOX 4) has paved the way for their safe

incorporation in vaccine formulations. Incorporation of dmLT has been shown to improve clinical responses to several whole-cell antigens, as seen with ETVAX and ACE527 (REFS^{31,82}). Excellent overviews of the development and clinical application of dmLT are provided by Clements and Norton⁸⁰ and Qadri et al.³¹. Based on a similar approach, the adjuvant multiple mutated cholera toxin (mmCT) has been proposed as an alternative to dmLT⁸³, and in preclinical studies mmCT has been shown to enhance T_H1 cell and T_H17 cell responses in addition to mucosal and serum antibodies to a whole-cell *Helicobacter pylori* antigen⁸⁴. CTA1DD is a cholera toxin-derived adjuvant that was designed to combine the beneficial immunostimulatory effects of the CTA subunit enzyme with the B cell-targeting properties of a D-domain dimer from *Staphylococcus aureus*, to reduce off-target effects and toxicities^{85,86}. It was recently shown that CTA1DD enhanced the maturation of follicular dendritic cells in lymph nodes following mucosal vaccination in neonatal mice and that oral priming with a construct incorporating the influenza virus M2e antigen (CTA1-3M2e-DD) induced protective immunity in neonates against influenza challenge⁸⁷. Combination of lipid nanoparticles and CTA1-3M2e-DD generated a highly effective nasal vaccination system that conferred protective immunity against influenza virus infection in mice⁸⁸. This combination adjuvant was particularly effective in promoting respiratory tract IgA, T_H1 cell and T_H17 cell responses, holding promise for universal influenza vaccination applications⁸⁸. However, the efficacy and safety of CTA1DD remains to be determined clinically. The use of toxoid adjuvants intranasally has been somewhat marred by the clinical emergence of Bell's palsy in some recipients of influenza vaccines adjuvanted with wild-type *E. coli* heat-labile toxin⁸⁹ or LTK63, a genetically detoxified *E. coli* heat-labile toxin derivative⁹⁰. An alternative is sublingual vaccination, which has shown significant promise as a means of promoting protective immunity in animal models^{91,92} although immune responses to sublingual dmLT were modest in a clinical trial⁹³. There may be scope to enhance such responses by formulation with agents such as chitosan to enhance antigen and adjuvant residence times (BOX 2).

These studies would suggest that nasal delivery of ganglioside-targeting toxoid adjuvants is inadvisable. However, results from a phase II clinical trial on a trivalent influenza vaccine, composed of haemagglutinin and adjuvanted with LThaK, a detoxified *E. coli* heat-labile toxin derivative, were recently reported^{94,95} (NCT03784885). An acceptable safety profile was reported following two nasal vaccinations, which induced higher antigen-specific nasal IgA responses than the non-adjuvanted antigen⁹⁴. LThaK is reported to have no ribosylating activity, correlating with enhanced retention in the nasal passages and the enhanced safety profile. Phase III trials will investigate efficacy in a challenge setting and will further elucidate the safety of LThaK for intranasal incorporation in a larger patient cohort. Importantly, nasal delivery of CTA1DD did not result in trafficking to the olfactory bulb, indicating its safety as a nasal vaccine adjuvant⁸⁸. In summary, toxoid adjuvants are the most advanced mucosal adjuvants,

Box 3 | Vaccine approaches for mucosal cancers

Malignancies commonly emerge at the mucosae, providing a rationale for mucosal vaccine targeting. Incidences of mucosal cancers are increasing, with cancers of the head and neck and the reproductive, respiratory and digestive tracts estimated to cause 8.52 million deaths per annum by 2040 compared with a current estimate of 5.15 million deaths per annum¹⁷⁰. Effective tumour immunosurveillance and elimination relies on tumour-specific CD8⁺ T cells. Therefore, mucosal vaccine strategies that effectively mobilize cell-mediated immunity with generation of sentinel tissue-resident memory T cells (T_{RM} cells) are required^{71,172}. Mucosal vaccination strategies have proved more effective than parenteral vaccination routes^{171,173,174}. Nizard et al. demonstrated that an intranasal dendritic cell-targeted vaccine was more protective than parenteral vaccination in an orthotopic head and neck tumour model (HPV16 E6⁺ and E7⁺ expressing TC-1), and this effect was dependent on the presence of mucosal antigen-specific CD8⁺ T_{RM} cells¹⁷¹. Optimal targeting of dendritic cell subsets is vital for such approaches: conventional type 1 dendritic cell (cDC1) populations including CD103⁺ non-lymphoid dendritic cells (analogous to CD141⁺ dendritic cells in humans) and CD8a⁺ lymphoid dendritic cells are efficient at cross-priming cytotoxic T lymphocytes¹⁷⁵ and in imprinting mucosal homing receptors, displaying antitumour functionality^{175,177}. Furthermore, the proficiency of CD103⁺ dendritic cells in trafficking intact antigens from tumours to tumour-draining lymph nodes and their importance in the context of checkpoint blockade responses has been highlighted^{176,178,179}. Vaccines/adjuvants that effectively target these subsets and/or strategies to expand their number prior to vaccination should prove most successful. With the exception of virally induced cancers where viral antigens are vaccine targetable, antigen selection is problematic in prophylactic vaccination. Targeting tumour neoantigens is an attractive concept yet it is unlikely to ever be a 'one size fits all approach' as the degree and composition of mutational burden is highly patient-specific and tumour-specific^{180,181}. Therapeutic mucosal cancer vaccines can circumvent these issues via personalized medicine-based vaccine design^{182,183} or, possibly, through local antigen release for a more general approach¹⁸⁴. Mucosal cancer vaccines not only have potential to prevent and treat mucosal tumours, but also to prevent infection with viruses linked to non-mucosal malignancies, such as Epstein–Barr virus and hepatitis viruses.

Box 4 | Enterotoxin-derived mucosal adjuvants: dmlT and mmCT

Cholera toxin and *Escherichia coli* heat-labile toxin are the gold-standard mucosal adjuvants. However, their toxicity necessitated strategies to enhance safety whilst retaining adjuvanticity, culminating in generation of *E. coli* double-mutant heat-labile toxin (dmlT)¹⁰⁵ and multiple-mutated cholera toxin (mmCT)¹⁰³. The introduced mutations target the ADP ribosyltransferase activity of the toxin A subunit^{105,106} and both molecules are powerful mucosal adjuvants that enhance mucosal IgA and serum IgG responses in addition to CD4⁺ T cell responses, particularly T helper 17 (T_H17) cells^{105,106}. Interaction of cholera toxin with GM1 on gut dendritic cells is required for its oral adjuvanticity¹⁴⁰, although the precise mechanisms of action are not fully resolved. NF- κ B activation was required for the adjuvanticity of mmCT, with cyclic AMP–protein kinase A (cAMP–PKA) signalling proposed to be required for NF- κ B activation in mmCT-stimulated dendritic cells *in vitro*¹⁰⁵, although PKA may be dispensable for dendritic cell activation by dmlT¹⁰⁷. Both dmlT and mmCT required cAMP–PKA-dependent inflammasome activation to promote human T_H17-type responses¹⁰⁸. There has been extensive evaluation of dmlT in clinical trials as an oral vaccine adjuvant, with results indicating an acceptable safety profile and strong adjuvanticity^{11,12,13,109,110}. Furthermore, a National Institute of Allergy and Infectious Diseases (NIAID)-sponsored trial investigating the safety and adjuvanticity of three doses of dmlT by oral, sublingual and intradermal routes has begun recruitment in an enterotoxigenic *E. coli* (ETEC) endemic area of Bangladesh (NCT03548064). The inclusion of toxoid-derived adjuvants in mucosal vaccines may improve responses in low-responding demographics, such as older people and young children¹¹. Toxoid-derived adjuvants may also potentially help in addressing the lower responses to oral vaccines that are often seen in endemic regions (known as the ‘tropical barrier’) compared with the higher income countries where early-stage clinical trials are frequently conducted. Although existing antibodies to dmlT do not appear to impair its adjuvanticity upon booster vaccination¹⁰, different outcomes have been observed in trials between Swedish and Bangladeshi recipients^{11,37} that require further evaluation on the potential impact of previous exposure.

having demonstrated impressive efficacy in clinical trials for oral whole-cell killed vaccines.

Aside from toxoid adjuvants, there are a small number of mucosal adjuvants with demonstrated safety and efficacy. The invariant NKT cell activator α -GalCer is a promising mucosal adjuvant and potentially an indicator of the potential for targeting innate-like lymphocytes to produce a new generation of mucosal adjuvants. We have demonstrated that oral delivery of a whole-cell killed *H. pylori* antigen adjuvanted with α -GalCer induced protective immunity from gastric bacterial challenge, characterized by induction of local IgA and T_H1 cell immunity, comparable with a cholera toxin adjuvanted vaccine⁹⁶. The induction of antigen-specific T_H1 cell responses was dependent on CD1d, IL-1R1 and IL-17R signalling; therefore, α -GalCer provides a proof of principle for targeting the relatively abundant mucosal invariant NKT cell populations for effective adjuvanticity. We have further characterized α -GalCer as an effective adjuvant with oral whole-cell killed ETEC and cholera antigens including the CFA/I overexpressing JT-49 ETEC vaccine combined into enterically stable smPill mini-spheres. Potent induction of intestinal CFA/I-specific IgA was observed in addition to serum IgG responses⁹⁷. Oral vaccination with Dukoral and α -GalCer induced stronger intestinal IgA and serum IgG responses than Dukoral alone and was comparable with cholera toxin adjuvanted Dukoral⁹⁸. Finally, incorporation of α -GalCer in a novel multi-antigen cholera vaccine composed of whole-cell killed double-lipopolysaccharide (LPS) antigen expressing cholera vaccine with CTB promoted robust mucosal immunity with concomitant systemic antibody production, outperforming Dukoral

and the whole-cell killed cholera alone⁹⁸. Our preclinical data provide a rationale for the inclusion of α -GalCer in future whole-cell oral vaccines, which may lead to more durable protection, addressing shortcomings in immunity and response rates.

Chitosan is well established as a mucosal adjuvant/delivery system given its mucoadhesive properties and immunostimulatory effects⁹⁹. We have demonstrated the effectiveness of chitosan as an intranasal adjuvant in mouse models. Intranasal vaccination with chitosan and pneumococcal surface protein A (PspA) led to the induction of lung PspA-specific IFN γ and IgG1, IgG2c and IgA responses that were dependent on STING signalling¹⁰⁰. STING-activating cyclic dinucleotides have been trialled as mucosal adjuvants. An intranasal synthetic cyclic dinucleotide (cyclic diguanylate) adjuvanted subunit vaccine induced protective immunity against *M. tuberculosis* in mice, correlating with potent induction of T_H17 cells¹⁰¹. Other cyclic dinucleotides — including cyclic di-AMP and cyclic di-GMP — have also shown promise as mucosal adjuvants^{102,103}. Mansouri et al. recently highlighted roles for two lung cDC2 populations in intranasal cyclic di-GMP adjuvanticity. Antibody responses were dependent on activation of moDCs by TNFR2⁺ cDC2 populations, with subsequent T follicular helper cell and germinal centre B cell activation, whereas induction of T_H1 cell and/or T_H17 cell responses was dependent on their TNFR⁺ cDC2 counterparts¹⁰⁴. These studies provide a strong rationale for further development of mucosal adjuvants targeting the STING pathway. In this context, chitosan has specific advantages in its record of clinical use and mucoadhesive properties in addition to STING-dependent adjuvanticity.

Whereas emulsion-based adjuvants have been highly successful in injectable vaccines, such approaches have not reached clinical application mucosally. Bluewillow Biologics currently have a phase I trial underway (NCT04148118) utilizing an intranasally administered nanoemulsion (oil in water emulsion) adjuvanted recombinant protein vaccine against anthrax (BW-1010). Preclinically, this vaccine has previously been shown to be protective in guinea pig models of infection, correlating with induction of systemic and local antibody induction¹⁰⁵. The nanoemulsion adjuvant has also been shown to promote T_H1 cell immunity and T_H17 cell immunity in anthrax and *M. tuberculosis* vaccine formulations, respectively^{105,106}. Kimoto et al. recently reported a promising mucosal adjuvant with possible applications in oral and intranasal vaccination routes. Two oral doses of HAv (haemagglutinin-based vaccine) adjuvanted with SF10 (a synthetic surfactant adjuvant) led to induction of antigen-specific mucosal IgA and protection from influenza in a preclinical model, outperforming cholera toxin¹⁰⁷. These recent studies highlight the promise of adjuvanted mucosal vaccines, with many taking inspiration from bacteria-derived virulence factors and showing promise for inclusion not only in subunit but also whole-cell formulations.

Mucosal nucleic acid and viral vectored vaccines. Until very recently, there were no licensed nucleic acid vaccines for clinical use. However, mRNA vaccines against

Polymersomes

Hollow vesicles generally comprising block copolymers that can incorporate vaccine antigens and adjuvants in the aqueous phase.

SARS-CoV-2 have now been successfully trialled and rolled out for parenteral vaccination, displaying impressive efficacy and paving the way for others to follow¹⁰⁸. Mucosal vaccination utilizing nucleic acids poses a greater challenge, as successful candidates must penetrate the mucus layer, translocate into target cells and evade extracellular and intracellular degradation. Vaccination via the oral route poses an added challenge with the low gastric pH and difficulty in ensuring release of the nucleic acid payload at the appropriate location. Innovative protective delivery strategies for nucleic acids have been developed using nanocarriers and biomaterials^{109–111}, and in particular the complexing of nucleic acids with polycationic materials including chitosan and polyethylenimine (PEI) and encapsulation of the nucleic acid cargo utilizing liposomes and polymersomes are showing potential. Lipidoid nanoparticles have been shown to effectively deliver small interfering RNA molecules to intestinal epithelial cells in the lower small intestine and colon following oral administration¹¹². Additionally, intranasal delivery of chitosan nanoparticles encapsulating mRNA with a viral protein coating elicited protection from avian influenza in chickens¹¹³. The coming years are likely to see great activity in this space, particularly around mobilizing solid lipid nanoparticles for mucosal RNA vaccine development.

Viral vectors are among the most promising strategies for mucosal vaccination, owing to their capacity for intracellular delivery, versatility and intrinsic immunogenicity. Viral vector strategies are applicable to oral vaccination when protection from conditions of the gastrointestinal tract and effective release are addressed. This is exemplified by the technology from Vaxart, whose oral influenza vaccine candidate VXA-A1.1 utilizes an enterically stable tableted delivery system, carrying a cargo of haemagglutinin encoding adenoviral vectors and a double-stranded RNA adjuvant. Data from phase I (NCT01688297) and phase II (NCT02918006) clinical trials demonstrated that VXA-A1.1 is well tolerated¹¹⁴ and, crucially for future adenoviral vector strategies, is not hindered by pre-existing adenoviral immunity when given orally¹¹⁴. Oral vaccination with VXA-A1.1 induced superior protection from influenza A virus challenge compared with the conventional intramuscularly delivered FluZone vaccine¹¹⁵. Whether this platform can be used to develop an effective oral quadrivalent influenza vaccine remains to be demonstrated. Additionally, orally administered RSV vaccines are in preclinical development (VXA-RSV-f)¹¹⁶, and an oral Norovirus vaccine (VXA-G1.1-NN) showed favourable safety and immunogenicity in a phase 1 trial (NCT02868073)¹¹⁵. More recently, an orally administered SARS-CoV-2 vaccine (VXA-CoV2-1) was described that uses the same formulation and is currently in phase I trials (NCT04563702). The efficacy of the double-stranded RNA adjuvant included in this platform is also very promising as an alternative to the canonical toxoid-based adjuvants in various stages of development and broadens the range of PRR targets that can be exploited for oral vaccination. This double-stranded RNA adjuvant will likely effectively target dendritic cell populations

for activation owing to their high TLR3 expression. This capacity to successfully adjuvant viral vectors may be critical as they will likely be less effective when given mucosally compared with parenteral routes. Further to this point, it has been recently shown that responses to viral vector (MVA) antigens can be enhanced by the saponin-containing adjuvant matrix M following subcutaneous vaccination¹¹⁷.

Viral vector approaches also hold potential for vaccination in the respiratory tract. Intranasal vaccination of mice with an adenoviral vector encoding influenza virus nucleoprotein induced a population of CD8⁺ T_{RM} cells in the lungs that was sustained for longer than 1 year⁶⁵. This was dependent on respiratory vaccination and sustained antigen expression, and contrasted with the situation following parenteral influenza virus infection, where the local CD8⁺ T_{RM} cell population was rapidly lost. The authors suggested that induction of robust local cellular immunity may address issues surrounding the reliance on systemic antibody responses to haemagglutinin associated with parenteral influenza vaccination⁶⁵. Nasal delivery of chimpanzee adenoviral (ChAd) vectors may also have potential in SARS-CoV-2 vaccines. Nasal delivery of ChAd-SARS-CoV-2 expressing homotrimeric spike antigen induced promising results in a murine infection model (K18-hACE2 mice). A single dose provided protection from upper and lower respiratory tract infection, correlating with induction of neutralizing antibody titres in the serum and bronchoalveolar lavage alongside the induction of IFN γ ⁺ and granzyme B⁺ CD8⁺ T cells¹⁸. Whether efficacy would be sufficient clinically with viral vectors alone is unclear but, as with oral delivery, there may be scope to enhance responses with appropriately targeted mucosal adjuvants. With viral (or bacterial) vectored vaccines, the capacity of vaccine-induced secretory antibody responses to compromise responses to booster vaccination must be considered¹¹⁹. However, data from preclinical models have shown that pre-existing intestinal immunity did not compromise efficacy of an oral experimental viral vectored rabies vaccine¹²⁰. Furthermore, compelling recent clinical data found no detrimental effect of pre-existing influenza-specific nasal IgA responses on the efficacy of nasal live attenuated influenza virus vaccination in children¹²¹, and recently Janssen reported no clear impact of pre-existing immunity to their Ad26 vectored vaccine platform on efficacy following priming or boosting vaccinations¹²². This must be ascertained for each specific vaccine but, moving forwards, the availability of numerous mucosal viral vectors and adjuvant strategies would allow a heterologous prime-boost approach to overcome pre-existing immunity if required.

Concluding remarks

We are currently in the midst of a revolution in vaccine research and development. Cutting-edge research and advances into nucleic acid and viral vector vaccine technologies allowed SARS-CoV-2 vaccines to be developed and produced in an unprecedented short period of time. These advances are yet to impact on clinically used mucosal vaccines, but this will likely change in the near future. Mucosal vaccines offer the significant

benefit of triggering immune responses at the principal sites of infection, offering scope for sterilizing immunity achieved by local secretory antibody responses and resident populations of CD4⁺ and CD8⁺ T cells. Thus, the outstanding obstacles to mucosal vaccine development are worth the effort as they are far outweighed by the potential immunological and logistical benefits in terms of ease of delivery. One of the major challenges that requires innovative solutions is the 'tropical barrier', where responses to oral vaccines in low- and middle-income countries can be lower than those seen throughout clinical trials in high-income countries. Interventions to address this problem are urgently required¹²³ and may include implementation of probiotic supplements prior to or during vaccination¹²⁴. The potential for adjuvants to overcome suboptimal responses must be addressed as this and increased antigen doses may have a greater impact than other proposed strategies. Indeed, the most advanced mucosal adjuvant, dmLT, has demonstrated efficacy in both high-income and low-income countries^{11,32}. Identifying whether other candidate adjuvants can also increase efficacy of existing oral vaccines as well as facilitating the development of novel vaccines is a priority. Targeting mucosally abundant cellular populations such as ILCs, mucosal-associated invariant T cells and NKT cells has significant promise but clinical validation of these

approaches is required. A recent study in mice found that intestinal ILCs can migrate via the lymph to the mesenteric lymph nodes, and in response to infection with *S. Typhimurium* these migrating ILCs exhibited greater levels of activation and cytokine production. Mobilizing this population using ILC-targeting adjuvants may have significant potential to bolster mucosal immune responses²⁵. In addition to stand-alone mucosal vaccine approaches, parenteral mucosal prime-boost strategies offer promise. These may be enhanced with injectable vaccines that imprint a degree of mucosal homing, for example, with dmLT¹²⁵ or retinoic acid⁸¹, and their relative ability to enhance tissue-resident T cell responses may be key to success. In some cases, antigen alone may be sufficient for mucosal boosting¹²⁶ although this will depend on the nature and immunogenicity of the antigen and it is likely, in most cases, that an effective adjuvant will be required. In summary, although the leaps forward in injectable vaccine strategies have not yet been seen with mucosal vaccines, this is likely to change in the near future. Advances in our understanding of mucosal protective immunity, developments in measuring human mucosal immunity¹²⁷ and antigen and adjuvant discovery offer hope that novel mucosal vaccines for infectious diseases and cancer are on the horizon.

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Author contributions

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Competing interests

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