Chitin-derived STING Activators as Adjuvants and Therapeutics

Presented by

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Supervised by

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Declaration

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Joanna Turley
Permission

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Electronically signed by Joanna Turley
Acknowledgement

Ed, I can’t thank you enough for affording me the opportunity to work in this Lab. It has been an experience of a lifetime. No doubt I had a few wobbly moments but your constant encouragement, advice and time has kept me going. Your passion for science is truly contagious and I hope to never lose it.

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Finally, to Martha, thank you for all the invaluable advice you gave me throughout life. You are an absolute legend and this PhD is dedicated to you.
Abstract

A significant challenge in the advancement of vaccine research is a lack of adjuvants that can safely drive potent cellular immunity against intracellular pathogens and cancer, a problem only exacerbated by our limited knowledge of how adjuvants work. As such, there has been an increasing focus on purified pathogen-derived or synthetic agonists for well-defined pathogen recognition receptors as adjuvants. Since the discovery that the cGAS-STING pathway occupies a central role in the activation of tumour-targeting immune responses, there has been a surge in research aimed at identifying natural and synthetic CDNs and non-nucleotidyl STING agonists for use in cancer settings. Despite pre-clinical evidence of efficacy, no widely applicable, clinically effective and safe agonist has been identified or completed phase III trials. The primary barriers to clinical translation are low cellular uptake and intracellular accessibility, poor pharmacokinetics, and STING variability, necessitating personalised STING agonists.

This work has identified C100, a chitosan polymer with no acetyl groups, as an attractive alternative to conventional STING agonists. C100 polymers were demonstrated to promote potent STING and IFNAR-dependent cellular immunity against the TB antigen H56 and tumour growth suppression upon intratumoral injection in B16 melanoma models. Unlike most licensed adjuvants, the study delineated the mechanism and physiochemical properties required for C100-induced immune activation. In a cellular uptake-independent manner, C100 polymers triggered mitochondrial stress that was pivotal for both cGAS-STING and NLRP3 inflammasome activation and subsequent Th1 immunity. Mechanistically, extensive mitochondrial stress damaged nuclear DNA, triggering its accumulation in the cytosol of DCs and activation of STING-dependent type I IFNs. Complete deacetylation of the chitosan backbone was critical for optimal adjuvanticity, as addition of acetyl groups reduced the degree of mitochondrial stress, nuclear damage, IFNAR-dependent DC maturation, NLRP3 activation, Th1 responses and protective anti-tumour immunity. The ability of acetylation to diminish the adjuvanticity of C100 addresses a longstanding mystery of how chitosan polymers can achieve both inert and inflammatory properties. Altogether, these results reveal an effective anti-tumour STING-dependent adjuvant with unique properties that sidestep common limitations of existing STING therapeutics.
Publications


Joanna L Turley., 1Hannah BT Moran., 1McEntee, C.P., 1Flood, R., 1Liddicoat, A., 1Katie O’Grady., 1Natalia Muñoz-Wolf., 2Lei, J., 3Peter L Andersen, 4Mats Andersson and 1,5Ed C. Lavelle. **Chitin-derived polymer deacetylation regulates reactive oxygen species dependent cGAS-STING and NLRP3 inflammasome activation** (Manuscript under review by Biomaterials, see appendix)

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Abbreviations

2-ABP 2-Aminoethoxydiphenyl borate
actb β-actin
Ag Antigen
AMPK 5’ AMP-activated protein kinase
APAF1 Apoptotic peptidase activating factor 1
APC Antigen presenting cell
ARF ADP-riboseylation factor
ASC Apoptosis-associated speck-like protein containing CARD
ASK Apoptosis signal-regulating kinase 1
ATM Ataxia-telangiectasia mutated
ATP Adenosine triphosphate
ATF Activating transcription factor
ATR ATM- and RAD3-related
ATG Autophagy related protein
BafA Bafilomycin A
BCG *Bacille Calmette-Guerin*
BCL2 B cell lymphoma-2
BCL6 B cell lymphoma-6
Bip Binding-immunoglobulin protein
BLM Bloom syndrome protein
BMDM Bone marrow-derived macrophage
BrdU Bromodeoxyuridine
BrefA Brefeldin A
BTK Bruton’s tyrosine kinase β2m
β2m β2 microglobulin
CARD Caspase recruitment domain
Ca2+ Calcium
CD Cluster of differentiation
CDN Cyclic Dinucleotide
CLR C-type lectin receptor
COX-2 Cyclooxygenase 2
cGAS Cyclic GMP-AMP synthase
cGAMP Cyclic GMP-AMP
CHOP CCAAT-enhancer-binding protein homologous protein
CTT Carboxy-terminal tail
CTL Cytotoxic T lymphocyte
CTLA4 Cytotoxic T-lymphocyte-associated protein
CytC Cytochrome C
DAMPS Danger-associated molecular pattern
DAG Diacylglycerol
DAP10/12 DNAX-activating protein of 10/12kDA
DCs Dendritic cell
DD Degree of deacetylation
DDR DNA damage response
DDS Drug delivery system
DDX DEAD box protein
DFCP1 Double FYVE-containing protein 1
Dloop Mitochondrial D-loop region
DNA-PK DNA-dependent protein kinase
dNTP Deoxyribonucleotide triphosphate
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>DRP1</td>
<td>Dynamin-related protein 1</td>
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<tr>
<td>DSB</td>
<td>Double-strand break</td>
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<td>Epstein Bar virus</td>
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<td>Ecto-nucleotide pyrophosphatase/phosphodiesterase-1</td>
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<td>Excision repair cross-complementing group 1</td>
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<td>ERK</td>
<td>Extracellular Receptor Kinase</td>
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<td>Eukaryotic translation initiation factor 2</td>
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<td>ER</td>
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<td>ERGIC</td>
<td>ER-golgi intermediate compartment</td>
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<td>ERAD</td>
<td>ER-associated protein degradation</td>
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<td>ETC</td>
<td>Electron transport chain</td>
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<td>Ethidium bromide</td>
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<td>FcRy</td>
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<td>FIK200</td>
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<td>Mitochondrial Fission 1</td>
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<td>FOXP3</td>
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<td>Gamma-aminobutyric acid receptor-associated protein</td>
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<td>GADD34</td>
<td>Growth arrest and DNA damage-inducible protein</td>
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<tr>
<td>GC</td>
<td>Germinal center</td>
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<td>Grp94</td>
<td>Glucose regulated protein 94</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>High mobility group box 1</td>
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<td>Homologous recombination</td>
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<td>Histone H2A variant</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IDO</td>
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<tr>
<td>IFNAR</td>
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<td>iNOS</td>
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<td>IP3R</td>
<td>Inositol-1,4,5-triphosphate receptor</td>
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<td>IBMM</td>
<td>Immortalised bone marrow-derived macrophage</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motif</td>
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<td>IkB</td>
<td>Inhibitor of NF-κB</td>
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<td>IKK</td>
<td>κB kinase</td>
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<td>IFI16</td>
<td>Interferon γ -inducible protein 16</td>
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<td>ILCs</td>
<td>Innate lymphoid cell</td>
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<td>IMS</td>
<td>Inner mitochondrial space</td>
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<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K+</td>
<td>Potassium</td>
</tr>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>KSHV</td>
<td>Kaposi’s Sarcoma-associated Herpesvirus</td>
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<td>LIRs</td>
<td>LC3-interacting motif</td>
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<td>LAP</td>
<td>LC3-associated phagocytosis</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>Laboratory of genetics and physiology 2</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>Latrunculin B</td>
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<td>Leucine-rich repeat</td>
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<td>LRRC8</td>
<td>Leucine-rich repeat-containing 8</td>
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<td>LBD</td>
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<td>LAG3</td>
<td>Lymphocyte-activation gene 3</td>
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<td>LMP</td>
<td>Lysosomal membrane permeabilization</td>
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<td>MAL</td>
<td>MyD88 adaptor-like</td>
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<td>MAVS</td>
<td>Mitochondrial antiviral-signalling protein</td>
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<td>MALT1</td>
<td>Mucosa Associated Lymphoid Tissue Lymphoma Translocation 1</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCU</td>
<td>Mitochondrial calcium uniporter</td>
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<td>MD2</td>
<td>Myeloid differentiation factor 2</td>
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<td>MDA5</td>
<td>Melanoma differentiation-associated gene 5</td>
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<td>MCP-1</td>
<td>Monocyte chemotactic protein</td>
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<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>MFN1</td>
<td>Mitofusin 1</td>
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<tr>
<td>MFN2</td>
<td>Mitofusin 2</td>
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<td>MIDD49-51</td>
<td>Mitochondrial dynamics protein of 49 and 51 kDa</td>
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<td>Mincle</td>
<td>Macrophage inducible Ca(^{2+})-dependent lectin receptor</td>
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<td>MOA</td>
<td>Mechanism of action</td>
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<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
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<td>MOMP</td>
<td><em>Chlamydia trachomatis</em> major outer membrane protein</td>
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<td>MPLA</td>
<td>Monophosphoryl Lipid A</td>
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<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
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<td>MRE11</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>Myeloid differentiation primary response 88</td>
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<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<td>mtROS</td>
<td>Mitochondrial reactive oxygen species</td>
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<td>mTOR1</td>
<td>Mammalian target of rapamycin 1</td>
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<td>NACHT</td>
<td>Nucleotide-binding and oligomerization</td>
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<td>Neutrophil extracellular trap</td>
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<td>NEK7</td>
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<td>NF-(\kappa)B</td>
<td>Nuclear factor Kappa B</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T-cell</td>
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<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<td>NK</td>
<td>Natural Killer</td>
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<td>NLR</td>
<td>NOD-like receptor</td>
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<td>NOD2</td>
<td>Nucleotide oligomerization domain 2</td>
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<td>non-numt</td>
<td>mtDNA that is not inserted into nuclear DNA</td>
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<td>OMM</td>
<td>Outer mitochondrial membrane</td>
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<td>Opa1</td>
<td>Optic atrophy 1</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PARP1</td>
<td>Poly (ADP-ribose) polymerase 1</td>
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<td>pDC</td>
<td>Plasmacytoid DC</td>
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<tr>
<td>PKD</td>
<td>Partial knock down</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<td>PERK</td>
<td>Protein kinase R-like endoplasmic reticulum kinase</td>
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<td>PI3P</td>
<td>Phosphatidylinositol 3-monophosphate</td>
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<td>Phosphoinositide 3-kinase</td>
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<td>Phospholipase C</td>
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<td>Protein phosphatase 1</td>
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<td>pTreg</td>
<td>Peripheral T regulatory cell</td>
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<td>Pyrin domain</td>
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<td>Double strand break repair protein</td>
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<td>Retinoic acid</td>
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<td>RAF1</td>
<td>Proto-oncogene, serine/threonine kinase</td>
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<td>RET</td>
<td>Reverse electron transport</td>
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<td>RIDD</td>
<td>iRE1α-dependent decay</td>
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<td>Receptor Interacting Protein 2</td>
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<td>Superfamily 2</td>
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<td>Src Homology Phosphatase 2</td>
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<td>SNARE</td>
<td>soluble NSF attachment receptor</td>
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<td>SOCE</td>
<td>Store-operated Ca(^{2+}) entry</td>
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<td>SRBC</td>
<td>Sheep red blood cell</td>
</tr>
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<td>STIM1</td>
<td>Stromal Interaction Molecule 1</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>SYK</td>
<td>Tyrosine kinase or Spleen tyrosine kinase</td>
</tr>
<tr>
<td>S1P</td>
<td>Site 1 protease</td>
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<tr>
<td>S2P</td>
<td>Site 2 protease</td>
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<td>TAB1/2</td>
<td>TAK1-binding protein</td>
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<td>Transforming growth factor beta-activated kinase 1</td>
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<td>Tuberculosis</td>
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<td>TANK-binding kinase 1</td>
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<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<td>tert</td>
<td>Nuclear DNA telomerase reverse transcriptase</td>
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<td>TFEB</td>
<td>Transcription factor E Box</td>
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<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<td>TGN</td>
<td>Trans-Golgi network</td>
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<td>Tfh</td>
<td>T follicular helper</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Th</td>
<td>T Helper lymphocyte</td>
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<td>TIR</td>
<td>Toll-IL-1-receptor</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
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<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester</td>
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<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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1. Introduction

1.1. Vaccination

Vaccination is the most successful and efficient means of preventing and controlling infectious diseases and pandemics. Through it smallpox has been eradicated, polio nearly abolished and childhood vaccination has reduced the mortality and morbidity resulting from a plethora of infectious diseases such as measles, mumps, rubella, diphtheria, tetanus and yellow fever. The fundamental concept behind vaccination is to mimic natural infection and induce an adaptive response that will be both specific and long lasting, without causing disease. It involves administration of a live attenuated or killed pathogen, or a component thereof, to generate a strong and specific immune response that will protect against subsequent encounter with that pathogen [1].

Early vaccines were developed using attenuated live organisms (e.g. smallpox, adenovirus) or whole killed pathogens (pertussis, plague). While hugely successful, the demand for new vaccines remains high as there are safety concerns regarding reactogenicity and reversion of organisms back to virulent states when using attenuated pathogens. Furthermore, these methods have not generated optimal vaccines for diseases such as tuberculosis (TB), human immunodeficiency virus (HIV), malaria and cancer [1]. Improved subunit, DNA or RNA vaccine strategy approaches offer a means to significantly improve vaccine safety, enable the development of vaccines where traditional methods have failed (TB and HIV) and expand vaccine targets (cancer) [1]. However, despite desirable qualities these vaccines often incorporate poorly immunogenic antigens and fail to convey long lasting immunity requiring multiple boosters [2].

1.2. Adjuvants

Adjuvants were first described by Ramon as "substances used in combination with a specific antigen that produce more immunity than the antigen alone" [3]. They can be incorporated into any vaccine to enhance the magnitude and functionality of an immune response to antigen [2]. It is widely accepted that adjuvants such as aluminium salts or oil-in-water emulsions do not just increase overall antibody titre but can enhance induction of functional antibodies with high affinity for vaccine antigens. With the current
global COVID-19 pandemic in mind, additional adjuvant benefits are becoming increasingly relevant; (1) Not only can adjuvants enhance the immunogenicity of weak antigens, they enable the use of lower antigen doses increasing global vaccine supply, (2) they can accelerate immune responses, minimising the need for booster shots, as exemplified by the addition of AS04 (synthetic TLR4 ligand adsorbed to aluminium hydroxide) to GSK’s Hepatitis B vaccine Fendrix (which enabled the reduction of a three-dose regimen to two) and (3) they enable antibody response broadening, a crucial feature for vaccines against pathogens that display substantial antigenic drift, strain variations or both [2], [4].

Despite the variety of compounds with adjuvant properties, few are licensed for use in humans [5], [6]. Moreover, the majority of commercially available vaccines incorporate alum, oil-in-water emulsions (MF59, ASO3, and AF03) or virosomes that largely promote Th2-biased antibody-mediated responses (Table 1). While this response is appropriate for infections where neutralising antibodies can protect, it is unsuitable for complex pathogens that require additional features of the immune response beyond antibodies, such as cell-mediated responses that result in the activation of effector CD4⁺ Th1- biased and CD8⁺ T cells (Table 2). This represents a major bottleneck in tuberculosis, malaria, HIV, Leishmaniasis, dengue and cancer vaccine design.

Adjuvants have applications outside prophylactic vaccine settings. Since the discovery that the immune system can be harnessed to subjugate cancer, adjuvants have been administered intratumorally in an attempt to stimulate the activation pathways of tumour-infiltrating effector T cells. However accumulating evidence from clinical trials and animal studies highlights a need for better adjuvants than those currently licensed, as they fail to induce potent anti-tumour immunity or induce undesired immunity that facilitates tumour growth [7], [8].

Although it is known that the innate immune system is pivotal to adjuvant function, even in response to non-microbial compounds such as alum and MF59, we remain largely ignorant of the precise molecular mechanisms by which these licensed, empirically-derived compounds promote adaptive immunity (Table 1) [2]. There is a clear need to molecularly decipher the mechanisms behind the success and shortcomings of current adjuvants and to develop new adjuvants with defined mechanisms of promoting durable cellular immunity.
<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Description</th>
<th>Mechanism of action</th>
<th>Immune response</th>
<th>Licensed use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium Salts</td>
<td>Insoluble particulates of aluminium hydroxide, phosphate or hydroxyphosphate sulfate salts.</td>
<td>Unknown, but several DAMPS have been implicated- uric acid, ATP, HMGB-1 (high mobility group box 1) protein, host DNA, IL -1α, IL-33 and NETs</td>
<td>Humoral immunity.</td>
<td>Routine childhood vaccines and many others.</td>
</tr>
<tr>
<td>MF59</td>
<td>Oil dispersed nanoemulsions (Mainly squalene), stabilised with polysorbate 80, sorbitan trioleate &amp; citrate buffer</td>
<td>Unknown. Likely trigger cell injury or death that lead to DAMP release – ATP has been implicated</td>
<td>Humoral Immunity and Th2-prone CD4+ T cell response.</td>
<td>Seasonal influenza, Pandemic influenza, avian influenza.</td>
</tr>
<tr>
<td>AS03</td>
<td>Oil dispersed nanoemulsions (mainly squalene) stabilised with α-tocopherol, polysorbate-80, &amp; phosphate buffered saline.</td>
<td>Unknown- Involves activation of ER stress sensor IRE1α</td>
<td>T Follicular helper cells and Humoral Immunity.</td>
<td>Pandemic influenza. Avian influenza</td>
</tr>
<tr>
<td>AF03</td>
<td>Oil dispersed nanoemulsions (mainly squalene) polyoxyethylene-cetylstearylether, sorbitan, oleate, mannitol &amp; phosphate buffered saline.</td>
<td>Unknown. Likely triggers cell injury or death that lead to DAMP release</td>
<td>Humoral Immunity.</td>
<td>Pandemic influenza.</td>
</tr>
</tbody>
</table>

[9]–[13], [14], [15], [16], [17], [18]
<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Description</th>
<th>Mechanism of action</th>
<th>Immune response</th>
<th>Licensed use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virosomes (often referred to as liposome)</td>
<td>Dispersed lipid vesicles</td>
<td>Unknown. Involves mimicking viruses to assist antigen uptake</td>
<td>Humoral Immunity, Th1 and Th2 CD4+ T cells.</td>
<td>Pandemic Influenza and Hepatitis A Virus.</td>
</tr>
<tr>
<td>AS01 (liposome derivative)</td>
<td>Dispersed lipid vesicles containing cholesterol, TLR4 ligand and saponin QS-21.</td>
<td>-TLR4 signalling -Saponin component activates NLRP3 inflammasome in vitro and promotes CD8+ T cells in mice through an unknown mechanism.</td>
<td>Humoral Immunity, Th1-biased and CD4+ T cells.</td>
<td>Malaria, Herpes Zoster Virus.</td>
</tr>
<tr>
<td>AS04</td>
<td>Synthetic TLR4 ligand adsorbed to aluminium hydroxide</td>
<td>-TLR4 signalling Aluminium component - unknown</td>
<td>Humoral Immunity, Th1-biased and CD4+ T cells.</td>
<td>Hepatitis B Virus, Human papillomavirus</td>
</tr>
<tr>
<td>CpG ODN (1018 ISS)</td>
<td>Soluble TLR9 ligand (oligonucleotide)</td>
<td>TLR9 Signalling</td>
<td>Humoral Immunity, Th1-biased and CD4+ T cells, CD8+ T cells</td>
<td>Hepatitis B Virus</td>
</tr>
</tbody>
</table>

Abbreviations: IL, Interleukin; ATP, Adenosine triphosphate; NETs, Neutrophil extracellular traps; HMGB-1, high mobility group box 1; IRE1α, Inositol-requiring enzyme-1α; TLR, Toll like receptor; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3.
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Correlate of protection</th>
<th>Vaccine candidates (licensed and pipeline) and Immunogen type</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TB</strong></td>
<td>Uncertain</td>
<td>• Licensed vaccine is Live attenuated BCG administered by injection.</td>
<td>BCG Vaccine</td>
</tr>
<tr>
<td></td>
<td>Thought to involve:</td>
<td>• 16 distinct candidates in clinical trials (Phase I-III).</td>
<td>- Moderate efficacy in children</td>
</tr>
<tr>
<td></td>
<td>Th1/Th17 CD4+ T cell responses</td>
<td>- 8 are subunit (virally vectored or adjuvanted recombinant protein candidates)</td>
<td>- Poor efficacy against adult pulmonary TB</td>
</tr>
<tr>
<td></td>
<td>IgA or IgG antibody response</td>
<td>- Remaining 8 are live attenuated, inactivated or lysates</td>
<td>• M72/AS01E</td>
</tr>
<tr>
<td></td>
<td>Trained immunity</td>
<td>- Front runner is currently M72/AS01E*, (Phase II trial in Latent TB infected, HIV negative adults) adjuvanted recombinant protein candidate.</td>
<td>-54 % efficacy in adults (NCT01755598)</td>
</tr>
<tr>
<td><strong>Malaria</strong></td>
<td>Uncertain</td>
<td>• Only licensed vaccine is adjuvanted recombinant protein candidate, RTS,S/AS01</td>
<td>RTS,S/AS01:</td>
</tr>
<tr>
<td></td>
<td>Thought to involve:</td>
<td>• 10 distinct candidates currently in clinical trial (Phase I-II)</td>
<td>- Moderate protection - up to 50 %.</td>
</tr>
<tr>
<td></td>
<td>CD8+ T cells</td>
<td>- 7/10 are subunit</td>
<td>- Antibody levels wane over time, and are age dependent (protection is 25 % in infants 6–12 weeks of age.</td>
</tr>
<tr>
<td></td>
<td>IgG antibody response</td>
<td>- Remaining 3 are live attenuated</td>
<td>- Requires 4 injections</td>
</tr>
<tr>
<td></td>
<td>γδ T cells</td>
<td></td>
<td>- Not recommended for babies between 6-12 weeks of age due to low efficacy and safety concerns (meningitis, cerebral malaria &amp; febrile convulsions).</td>
</tr>
</tbody>
</table>

\[26\], \[22\], \[27\]
<table>
<thead>
<tr>
<th>HIV</th>
<th>Uncertain Thought to involve:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD8+ T cells Neutralising antibodies</td>
</tr>
<tr>
<td></td>
<td>- No Licensed vaccine</td>
</tr>
<tr>
<td></td>
<td>- Frontline candidates in clinical trials include:</td>
</tr>
<tr>
<td></td>
<td>- Adenovirus vector prime-adjuvanted protein boost vaccine. Adjuvant: aluminium phosphate Protein: Clade C gp140 and Mosaic gp140.</td>
</tr>
<tr>
<td></td>
<td>- Adjuvanted DNA vaccine. Two-arm regimen of DNA/AIDSVAX (adjuvanted with Alum) and DNA/CN54gp140+ MVA/CN54gp140 (adjuvanted with MPLA).</td>
</tr>
</tbody>
</table>

|     | Phase II & III |
|     | NCT03964415 NCT02935686 |
|     | Phase II |
|     | NCT04066881 |

[28], [29]
1.3. Innate Immunity

Innate immunity relies on germline encoded pattern recognition receptors (PRRs) expressed by cells, notably professional phagocytes, to sense conserved microbial molecular structures, known as pathogen-associated molecular patterns (PAMPs), or endogenous danger signals known as danger-associated molecular patterns (DAMPs) and initiate signalling pathways leading to inflammation and host defence [30]. Dendritic cells (DCs) are uniquely well-equipped professional antigen presenting cells (APCs) fundamental in the initiation and regulation of immune responses during natural infection and vaccination. They are sparsely but widely distributed in the peripheral tissues where they capture and process antigens [31]. Upon activation, via PRR stimulation, DCs migrate from the peripheral site of infection to secondary lymphoid organs where they display elevated levels of co-stimulatory molecules such as cluster of differentiation (CD)40, CD80 and CD86, and present processed antigen in complex with Major histocompatibility complex (MHC) to antigen (ag)-specific T cells [32]. The combination of these two mechanisms along with the secretion of specific cytokines from DCs and other cells in the environment, provide T cells with signals required to become polarized and activated [32], [33]. Given the pivotal role of these sentinels as a bridge between innate and adaptive immunity, understanding how PRR pathways regulate DC activation is crucial for optimal adjuvant design.

1.4. PRRs

There is an increasing focus on purified pathogen-derived or synthetic agonists for well-defined PRRs as adjuvants. PRR families expressed by DCs, include the transmembrane Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) which scan the extracellular and endosomal compartments for PAMPs and DAMPs and RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA sensors which cooperate to provide cytosolic surveillance [2].
TLRs are the best characterized class of PRRs in mammalian species and are heavily investigated as adjuvant targets. Although the exact number of TLR genes may differ between species, it is likely that most mammalian species have 10 to 15, with 10 functional TLRs found in human and 12 found in mice [34]. The family are characterised by their ectodomains containing leucine rich repeats (LRRs) that mediate recognition of ligands, transmembrane domain, and intracellular toll-interleukin 1 (IL-1) receptor (TIR) domains that initiate downstream signalling [35]. TLRs are subdivided into two groups based on their cellular localisation and respective ligands. Cell surface TLRs principally recognise microbial cell wall components, such as lipopolysaccharide (LPS) of gram-negative bacteria (TLR4), lipoteichoic proteins (TLR1/2 and TLR2/6), and flagellin (TLR5). The other group primarily detect microbial nucleic acids, such as double stranded (ds)RNA (TLR3), single stranded (ss)RNA (TLR7) and unmethylated CpG dsDNA of bacteria and viruses (TLR9) [34]. As detection of host-nucleic acid ligands may lead to autoimmunity, these nucleic acid-sensing TLRs are sorted into intracellular compartments concealed from host DNA [36].

Following PAMP recognition, TLRs recruit specific adaptor proteins, including Myeloid differentiation primary response 88 (MyD88), MyD88 adaptor-like (MAL), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRAM (TRIF-related adaptor molecule) to activate distinct signalling pathways tailored to the infecting microbe. MyD88 is recruited by all TLRs except TLR3, and activates the transcription factor Nuclear factor Kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) to induce pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNF-α), IL-6, IL-12p40, inducible Nitric Oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). In contrast, TRIF is only used by TLR3 and TLR4, induces alternative signalling pathways that lead to activation of the transcription factors Interferon regulatory factor 3 (IRF3) and NF-κB resulting in the induction of type I interferons (IFNs), as well as IFN-dependent genes, such as the chemokines, monocyte chemotactic protein (MCP-1) and IFN-γ induced protein 10 (IP10 or CXCL10). The two additional adaptor proteins, TRAM and MAL, function as scaffolds to recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively. TLR4 is unique in that it uses all four adaptors and activates both the MyD88- and TRIF-dependent pathways [35]. In response to LPS, TLR4 forms a complex with myeloid differentiation factor 2 (MD2) and CD14 leading to the recruitment of MAL and subsequently MyD88 to trigger initial activation of NF-κB and MAPK. TLR4 is then
internalised by dynamin-dependent endocytosis, where it forms a signalling complex with TRAM and TRIF, to terminate MyD88 signalling and facilitate TRIF-dependent IRF3 and late-phase NF-κB and MAPK activation [37]. Similar to TLR4 signalling, endosomal localisation is critical for MyD88 activation downstream of TLR7 and TLR9 [36]. However, unlike TLR4, MyD88 is responsible for the induction of type I IFNs (Figure 1.1).

Purified pathogen-derived or synthetic TLR agonists can act as adjuvants to promote Th1 and CD8\(^+\) effector responses to co-administered antigens [38]. Notably, the TLR7/8 agonist, imiquimod is in clinical use for the topical treatment of basal cell carcinoma [33], *Bacillus Calmette-Guérin* (BCG)- a live attenuated vaccine and agonist of TLR2 and TLR4- is used in the treatment of early stage bladder cancer and CpG 1018 is the adjuvant contained in the FDA approved hepatitis B vaccine (HEPLISAV-B®). Of the 14 current trials investigating TLR agonists as adjuvants against infectious disease, 8 contain CpG. In fact, CpG derivatives are under evaluation in four distinct recombinant SARS-Co-V-2 subunit vaccines (Table 3). Furthermore, CpG derivatives are heavily investigated in prophylactic and therapeutic murine cancer models [39]. Despite this, CpG has had limited translational success, particularly as a standalone cancer adjuvant (NCT02254772) with a discrepancy between preclinical and clinical efficacy at least partly due to low TLR9 expression in humans (only in plasmacytoid DCs (pDCs) and B cells) compared to rodents (myeloid cells) [40]. Some hope has been restored by the discovery that CpG enhances the efficacy of immune activator and checkpoint inhibitor therapies. Intratumoral injection of CpG increases OX40 expression on CD4\(^+\) T cells in mice and humans and synergises with agonistic anti-OX40 antibody to activate intratumoral T cells and eradicate spontaneous malignancy [39]. Additionally, CpG generates durable T cell responses when combined with anti-PD-1 treatment in murine colon carcinomas and synergises with CTLA-4 or PD-1 antibodies in murine bladder cancer, resulting in improved long-term survival [41]–[43]. These approaches are in clinical development (Table 4).

A limitation for all TLR agonists as adjuvants is that in addition to driving Th1 differentiation, they also promote the expression of IL-10, transforming growth factor beta (TGF-β), indoleamine dioxygenase (IDO) and COX-2, which are immunosuppressive molecules and promote the induction of antigen-specific Treg cells or induce peripheral CD25\(^-\)Foxp3\(^+\) Treg cells [44]. Interestingly, combined administration of anti-IL10 receptor antibody with CpG enhanced the therapeutic efficacy of a DC vaccine in a mouse tumour model [45]. Similarly, p38 MAPK inhibition which targets TLR induced IL-10, improved
the therapeutic efficacy of a CpG-activated DC immunotherapy against tumours and protective immunity induced with a vaccine against *Bordetella pertussis* [46]. Furthermore, *in situ* vaccination with CpG is currently being investigated in a phase II trial for the treatment of advanced solid tumours and lymphoma in combination with radiotherapy and IDO blockade (NCT03322384).

Figure 1.1 Overview of TLR signalling

Upon TLR activation (except TLR3), MyD88 recruits IRAK4, IRAK1 and IRAK2. IRAK4 is initially activated and leads to the sequential phosphorylation of IRAK1 and IRAK2. TRAF6 is then recruited and phosphorylated leading to the dissociation of the IRAK4-TRAF6 complex from the cell membrane. TRAF6 forms oligomers and this oligomerisation activates its ubiquitin E3 ligase activity, leading to K63 polyubiquitination of target proteins including TRAF6 itself. Ubiquitinated TRAF6 recruits a complex of TAK1, TAB1 and TAB2/3, and activates TAK1, which then phosphorylates and activates the IKK complex. The IKK complex phosphorylates IκB proteins and this phosphorylation targets IκB for polyubiquitination and subsequent degradation of IκB by the proteasome,
thereby releasing the NF-κB dimer to let it enter the nucleus in which it regulates pro-inflammatory genes. Ubiquitin-activated TAK1 also phosphorylates and activates MKKs, which in turn activate the JNK and p38, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. In the case of TLR7, TLR8, and TLR9, in addition to NF-κB and MAPK activation, MyD88 activates IRF7. Upon activation, the complex comprised of MyD88, IRAK4, IRAK1, IRAK2 and TRAF6 is formed. IRAK1 then phosphorylates IRF7 at multiple serine clusters in the C-terminus, and this phosphorylation triggers its dimerization and nuclear translocation to induce type I IFN expression. The activation of TLR3-induced pathway and TLR4-induced MyD88-independent pathway relies on TRIF as an adapter. TRIF associates to TBK1 through TRAF3. TBK1 and IKKε are the crucial kinases for IRF3 activation and subsequent induction of the IFN-β gene. After TRIF engagement, there are two pathways to activate NF-κB: N-terminus of TRIF binds TRAF6 to activate NF-κB, but not IRF3; and C-terminus of TRIF recruits RIP1 to activate NF-κB. In the TLR4 signalling, the MyD88-dependent pathway leads to an early-phase activation of NF-κB and MAPKs, whereas the TRIF-dependent pathway induces a late-phase activation of NF-κB and MAPKs. TRIF- and MyD88-dependent activation downstream of TLR4 is sequential and compartment-specific, with the MAL–MyD88 pathway being engaged by TLR4 on the plasma membrane, whereas the TRAM–TRIF pathway is engaged at early endosomes. Figure taken from [47].

Abbreviations TLR, Toll like receptor; MyD88, Myeloid differentiation primary response 88 ;IRAK, interleukin-1 receptor-associated kinase 4 ; TRAF, TNF receptor (TNFR) associated factor; E3, enzyme 3 ;K63, Lysine 63 ; TAK1, Transforming growth factor beta-activated kinase 1 ;TAB, TAK1-binding protein ; IκB, inhibitor of NF-κB; IKK, IκB kinase; NF-κB, Nuclear factor Kappa B; MAPK, mitogen-activated protein kinase; MKKs, mitogen-activated MAPK kinases; JNK, Jun N-terminal Kinase ;AP-1, Activator protein 1; IRF, Interferon regulatory factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.
<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>TLR</th>
<th>Disease</th>
<th>Immunogen type</th>
<th>Phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-ICLC</td>
<td>TLR3</td>
<td>HIV</td>
<td>DCVax001 recombinant protein vaccine</td>
<td>I</td>
<td>NCT01127464</td>
</tr>
<tr>
<td>Poly-ICLC</td>
<td>TLR3</td>
<td>HIV</td>
<td>DCVax001 recombinant vaccine &amp; MVA recombinant vaccine</td>
<td>I</td>
<td>NCT01889719</td>
</tr>
<tr>
<td>MPLA liposomes</td>
<td>TLR4</td>
<td>HIV</td>
<td>Adjuvanted native-like HIV-1 envelope vaccine.</td>
<td>I</td>
<td>NCT03961438</td>
</tr>
<tr>
<td>GLA-SE</td>
<td>TLR4</td>
<td>Tuberculosis</td>
<td>Peptide ID93 vaccine</td>
<td>II</td>
<td>NCT02465216 NCT01927159</td>
</tr>
<tr>
<td>R848 (topical)</td>
<td>TLR7/8</td>
<td>Influenza</td>
<td>Supplementing influenza vaccine with adjuvant</td>
<td>I</td>
<td>NCT01737580</td>
</tr>
<tr>
<td>MEDI9197</td>
<td>TLR7/8</td>
<td>HIV</td>
<td>HIV-1 BG505 SOSIP.664 gp140</td>
<td>I</td>
<td>NCT04177355</td>
</tr>
<tr>
<td>CpG 7909</td>
<td>TLR9</td>
<td><em>Streptococcus pneumoniae</em></td>
<td>Supplementing Prevnar with adjuvant</td>
<td>II</td>
<td>NCT00562939</td>
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<tr>
<td>CpG 1018</td>
<td>TLR9</td>
<td>HIV</td>
<td>Broadly Neutralising antibody vaccine</td>
<td>II</td>
<td>NCT03837756</td>
</tr>
<tr>
<td>CpG 1018</td>
<td>TLR9</td>
<td>HIV</td>
<td>HIV-1 BG505 SOSIP.664 gp140 recombinant vaccine</td>
<td>I</td>
<td>NCT04177355</td>
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</table>
Table 3 continued.

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>TLR</th>
<th>Disease</th>
<th>Immunogen type</th>
<th>Phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplivax</td>
<td>TLR9</td>
<td>HIV</td>
<td>HIV-1 immunogen peptide vaccine</td>
<td>III</td>
<td>NCT02366026</td>
</tr>
<tr>
<td>CpG 1018</td>
<td>TLR9</td>
<td>COVID-19</td>
<td>Recombinant Coronavirus-Like Particle Vaccine</td>
<td>I</td>
<td>NCT04450004,</td>
</tr>
<tr>
<td>CpG 1018</td>
<td>TLR9</td>
<td>COVID-19</td>
<td>SARS-CoV-2 spike subunit vaccine</td>
<td>I</td>
<td>NCT04487210</td>
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<tr>
<td>CpG 1018</td>
<td>TLR9</td>
<td>COVID-19</td>
<td>Recombinant SARS-CoV-2 Trimeric S protein subunit vaccine</td>
<td>I</td>
<td>NCT04405908</td>
</tr>
<tr>
<td>Advax-SM</td>
<td>TLR9,</td>
<td>COVID-19</td>
<td>Recombinant spike protein vaccine</td>
<td>I</td>
<td>NCT04453852</td>
</tr>
<tr>
<td></td>
<td>formulated with delta inulin polysaccharide</td>
<td></td>
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</table>
Table 4 Combinational CpG immunoadjuvant therapies in clinical trials

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>TLR</th>
<th>Combination</th>
<th>Indication</th>
<th>Clinical phase &amp; status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Iplimumab</td>
<td>Low grade B-cell lymphoma</td>
<td>II- Recently Completed</td>
<td>NCT02254772</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 % CR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.3 % PR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.3 % SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85.7 % PD</td>
<td></td>
</tr>
<tr>
<td>MGN1703</td>
<td>TLR9</td>
<td>Iplimumab</td>
<td>Advanced solid malignancies</td>
<td>I-Active</td>
<td>NCT03007732</td>
</tr>
<tr>
<td>Double loop CpG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Pembrolizumab</td>
<td>Prostate cancer</td>
<td>II-Active</td>
<td>NCT03007732</td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Pembrolizumab</td>
<td>Metastatic melanoma or Head and neck squamous cell carcinoma</td>
<td>II- Recently Terminated</td>
<td>NCT02521870</td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Pembrolizumab + Radiotherapy</td>
<td>Prostate cancer</td>
<td>II-Active</td>
<td>NCT03007732</td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Nivolumab and Radiation Therapy</td>
<td>Metastatic Pancreatic Cancer</td>
<td>I-Active</td>
<td>NCT04050085</td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Nivolumab and Irreversible electroporation</td>
<td>Metastatic Pancreatic Cancer</td>
<td>I-Active</td>
<td>NCT04612530</td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Anti-OX40 antibody</td>
<td>Low grade B-cell lymphoma</td>
<td>I- Suspended (Business decision)</td>
<td>NCT03831295</td>
</tr>
<tr>
<td>SD101 CpG-C</td>
<td>TLR9</td>
<td>Anti-OX40 antibody + Radiotherapy</td>
<td>Low grade B-cell lymphoma</td>
<td>I-Active</td>
<td>NCT03410901</td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
Members of other PRR families are potential targets for adjuvants. Among the PRRs, the NLR family are the most diverse in terms of function, structure and the signals they respond to [48]. The NLR family are generally considered to perform cytoplasmic surveillance of PAMPs or DAMPs. They are characterised by their central nucleotide-binding and oligomerization (NACHT) domain which enables adenosine triphosphate (ATP)-dependent oligomerisation, C-terminal LRRs, which are believed to function in ligand sensing and autoregulation, and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains that mediate homotypic protein-protein interactions for downstream signalling [48]. While specific ligands for the majority of NLR family members are poorly characterised, two members, nucleotide oligomerization domain (NOD)1 and NOD2 are known to recognise bacterial cell wall breakdown products. Upon ligand binding, the proteins oligomerize into signalosomes and recruit the RIP2 serine/threonine kinase via CARD-CARD interactions, ultimately leading to NF-κB activation and inflammatory gene expression. Alternatively, NOD2 can sense ssRNA and via mitochondrial antiviral signalling protein (MAVS) activate IRF3, resulting in type I IFN induction [49].

Additionally, some NLR members are components of macromolecular complexes called inflammasomes. Inflammasomes are composed of three different types of proteins: a sensor (most often a member of the NLR family), an adaptor (apoptosis-associated speck-like protein containing CARD (ASC)), and an effector protein, the cysteine protease pro-caspase-1. To date, only NLRP1, NLRP3 and NLRC4 in the NLR family have been demonstrated to self-oligomerise and trigger caspase-1 auto-activation [50]. Caspase-1 then processes the cytokine precursors pro-IL-1β and pro-IL-18 into mature and biologically active IL-1β and IL-18 and induces a pro-inflammatory form of cell death called pyroptosis. IL-1β is a powerful pro-inflammatory cytokine, which notably elicits the recruitment of innate immune cells to sites of infection and controls adaptive immune cells. IL-18 promotes the activation of Natural killer (NK) cells and T cells [51].

Despite human NLRP1 being the first inflammasome-forming sensor characterised, it’s direct ligand was only established this year as dsRNA. Infection with a positive-strand RNA virus leads to the formation of dsRNA, and the generated dsRNA recruits human NLRP1 via the LRR domain. This results in the activation of NLRP1, which leads to the formation of an inflammasome complex [52]. In contrast, NLRC4 requires neuronal apoptosis inhibitory proteins (NAIPs) to detect bacterial products such as flagellin and
proteins from type III and IV bacterial secretion systems [53], [54]. Ligand binding leads to structural changes in NAIPs that allow recruitment and oligomerization of NLRC4, which acts as an adaptor to recruit and activate caspase-1 [55].

NLRP3 is the most characterised and unique inflammasome due to its ability to respond to highly diverse stimuli from microbial pathogens and toxins to UV light and crystalline particles. Its activity depends on two functionally distinct steps, priming and activation [56]. Priming comprises the direct engagement of TLRs resulting in NF-κB activation, pro-IL-1β synthesis, increased expression of NLRP3 [50] and potentially enhanced mitochondrial DNA (mtDNA) synthesis [57]. Activation involves homotypic interactions between the N-terminal PYD domain of NLRP3 and the PYD domain of ASC, as well as between the respective CARD domains of ASC and pro-caspase-1 [58]. Given the variety of factors detected by NLRP3 [56], it is likely that activation (i.e inflammasome formation) occurs in response to a cellular alteration triggered by all activating agents. Among these, potassium efflux [59], mitochondrial reactive oxygen species (mtROS) production [60], oxidised mtDNA release [57], [61], leakage from lysosomes and cathepsin release [62], cardiolipin externalisation [63], NLRP3 translocation to the mitochondria [64] calcium (Ca^{2+}) mobilisation [65], [66] have been proposed. However, these processes do not occur in response to all NLRP3 inducers, and contradictory findings show that their involvement is still a matter of debate. A more recent study suggests dispersion of the Trans-Golgi network (TGN) as an early and common stress event necessary for NLRP3 activation by diverse agonists. Five structurally unrelated NLRP3 agonists (nigericin, ATP, gramicidin, imiquimod and CL097) triggered disassembly of the TGN, exposing phosphatidylinositol-4-phosphate (PtdIns4P) microdomains that recruit NLRP3 through its KKKK motif [52]. Future work must address how NLRP3 activators initiate the dispersion of the TGN.

Several adjuvants have been reported to activate inflammasomes (Table 5) and growing evidence continues to implicate inflammasomes and their products in adjuvant function, but the effect is complicated and context dependent. Intranasal administration of IL-1β and IL-18 enhances antigen IgG and IgE responses when administrated with an antigen and IL-1α/β-deficient mice have impaired antibody production in a sheep red blood cells (SRBC) antigen vaccination model [67]–[70]. The NLRP3 inflammasome acts in conjunction with the cGAS-STING pathway to drive chitosan-induced Th1 responses, but not humoral immunity [71]. Similarly, in addition to TLR7/8 ligation, imiquimod activates the NLRP3 inflammasome and these combined processes contribute to its antiviral and anti-tumour properties [72]. Despite several reports showing that alum and
MF59 induce NLRP3 activation and IL-1β secretion in vitro, NLRP3 is dispensable for their adjuvanticity in vivo [13], [73]–[76]. Furthermore, QS-21-induced humoral and cellular responses are negatively regulated by NLRP3 in mice immunized with HIV-1 gp120 [20]. The inability to pinpoint a universal role of inflammasomes in adjuvanticity is likely due to crosstalk with other PRR signalling pathways. For example, depending on the availability of IL-12, IL-18 can support Th1 or Th2 responses [77]. Further studies are required to address the molecular mechanisms behind the production, activation, and modulation of inflammasomes before we can optimally manipulate NLRs for adjuvant function.

Finally, extensive care must be taken with inflammasome activating adjuvants in therapeutic cancer settings. Despite the ability of IL-1β to induce Th1 and Th17 responses and thus anti-tumorigenic effects, several lines of evidence support a dominant pro-tumour role across all cancer types. Solid tumours such as breast, colon, lung, head and neck cancers and melanomas commonly upregulate IL-1β and patients with IL-1β producing tumours generally exhibit a poorer prognosis [78]. Administration of inhibitory antibodies targeting IL-1β augments anti-tumour immunity and even synergises with anti-PD-1 therapy in a murine model of breast cancer [79]. In line with this, a completed phase III clinical trial investigating the role of IL-1β inhibition in atherosclerosis serendipitously demonstrated the ability of an inhibitory antibody targeting IL-1β (canakinumab) to reduce the incidence and mortality of lung cancer (NCT01327846) [80]. Replication of this protection is needed in formal settings of cancer screening and treatment. Several studies have also investigated the role of IL-18 in cancer and revealed both pro- and anti-tumorigenic functions. Increased levels of IL-18 in the serum of cancer patients is associated with poor prognosis [81]–[84]. Suppression of IL-18 expression in gastric cancer cells regressed primary tumour mass and suppressed metastasis in vivo [84]. The pro-tumour role of IL-1β is likely dependent on its ability to promote expression of pro-tumorigenic cytokines such as IL-6, TGF-β, TNF-α, endothelial growth factor (EGF) and IL-22 [85]. Similarly, IL-18’s pro-tumorigenic functions are likely related to its induction of the angiogenic factor, vascular endothelial growth factor (VEGF) [86], suppression of tight junction proteins, supporting metastasis [87], and upregulation of PD-1 expression on NK cells, converting them into a subset with immunosuppressive characteristics [88].
### Table 5 Role of inflammasomes in efficacy of adjuvants in licensed human vaccines or at advanced clinical stages

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Components</th>
<th>IL-1 family cytokines and adjuvant MOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum</td>
<td>Mineral salt</td>
<td>Aluminium oxyhydroxide or aluminium hydroxyphosphate</td>
<td>NLRP3-independent IL-1β and IL-33 promote innate recruitment. Not required for adjuvanticity.</td>
</tr>
<tr>
<td>AS04</td>
<td>Mineral salt + TLR agonist</td>
<td>Alum-aborbed MPLA</td>
<td>Not fully addressed; NLRP3-derived IL-1β seems dispensable.</td>
</tr>
<tr>
<td>MF59</td>
<td>Oil-in-water</td>
<td>Squalene, polysorbate 80, sorbitan trioleate, citrate buffer</td>
<td>NLRP3-derived IL-1β and IL-18 not required.</td>
</tr>
<tr>
<td>AS02</td>
<td>Oil-in-water</td>
<td>AS03 + MPLA + QS-21</td>
<td>NLRP3 shown to negatively regulate QS-21 adjuvanticity. IL-1 family cytokine role in AS02 MOA remains unknown.</td>
</tr>
<tr>
<td>GLA-SE</td>
<td>Oil-in-water</td>
<td>Glucopyranosyl lipid A in a stable squalene emulsion</td>
<td>NLRP3-independent secretion of IL-18 required for IFN-γ and CD8^+ T cells</td>
</tr>
<tr>
<td>CAF01</td>
<td>Liposomes (cationic)</td>
<td>Two-component liposomal suspension with DDA and TBD</td>
<td>IL-17 and IFN-γ production but not for antibodies</td>
</tr>
<tr>
<td>ISCOMATRIX</td>
<td>Self-assembled nanoparticles</td>
<td>Quillaja saponins, cholesterol and phospholipids at a molar ratio ~ 1:1:1</td>
<td>NLRP3, NLRC4-independent IL-18 required for IFN-γ and IgG2c.</td>
</tr>
</tbody>
</table>

Taken from [89]

Abbreviations: MOA, Mechanism of action; MPLA (Monophosphoryl Lipid A)
1.4.3. RLRs

RLRs are a family of cytosolic pattern recognition receptors composed of RIG-I, Melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) that are essential in detecting signatures of virus replication within the cytosol of infected cells. The family are characterised by their common central DEAD box helicase/ATPase domain that alongside the downstream C terminal domain, is required for RNA binding. Upstream of the RNA helicase domain, RIG-I and MDA5 have two N-terminal CARDs that promote the IRF3-, IRF7-dependent expression of type I and type III interferons (IFNs) and NK-κB-dependent expression of inflammatory genes via mitochondrial antiviral-signaling protein (MAVS) [90]. The receptors differ slightly in ligand specificity; while RIG-I recognises blunt ended 5’triphosphate double stranded RNA (dsRNA) of 20-300 base pairs [91], MDA5 recognises both single stranded RNA (ssRNA) and dsRNA organised into web-like structures [92]. LGP2 senses dsRNA but has no N-terminal CARD domains and consequently cannot interact with MAVs [90]. Ongoing research aims to unravel a regulatory role in promoting and inhibiting MDA5 and RIG-I receptor signalling respectively [93]. In response to certain viruses, RIG-I mediates IL-1β production independently of the NLRP3 inflammasome. This is a novel pathway involving RIG-I, ASC and strikingly does not require MAVS [94].

Delineating RLR signalling has led to the development of a novel adjuvant, N’-CARD polypeptide. N’-CARD polypeptide is a MAVS CARD-fusion protein that drives type I IFN production, DC maturation, and subsequent antigen-specific Th1 responses in influenza and HPV tumour models. Coadministration with Flu vax or E7 antigen protects mice from lethal influenza infection and from outgrowth of transplanted tumours respectively [95]. Synthetic analogues of dsRNA (Poly IC) target both MDA5 and TLR3 and have been used as adjuvants with soluble proteins, DC targeting constructs and inactivated viral vaccines [2]. Dual activation of both pathways prime a much more potent cellular immune response than either PRR pathway alone [96]. mRNA vaccines were initially designed to induce expression and presentation of antigens in APCs, however, due to inherent features they additionally activate RLR pathways to promote high innate immune activation and thus adjuvanticity [97]. These vaccines have several advantages- (1) the antigen and adjuvant stem from the same RNA entity (2) they are a non-integrating platform and so pose no potential risk of infection or insertional mutagenesis. (3) They are degraded by normal cellular processes with half-life adjustable depending on the use of delivery systems and RNA modifications. (4) Delivery systems additionally facilitate
rapid uptake, expression and PRR detection in the cytosol (5) The inherent immunogenicity of mRNA can be reduced by modifying RNA sequences. This allows adequate activation of the innate immune system while averting toxic overactivation that results in OAS- and PKR-dependent blocks on mRNA translation and protein synthesis. (6) mRNA vaccines allow the potential for rapid, inexpensive and scalable vaccine development owing to the high yields of in vitro transcription reactions [98].

This field is developing extremely rapidly; a large body of preclinical data has accumulated over the last few years, and multiple human clinical trials have been initiated. mRNA vaccines are so far the most popular and successful vaccine approach for tackling the current COVID-19 global crisis. As of February 2021, 2 of 3 licensed vaccines in Ireland against SARS-CoV-2 use mRNA technology. Pfizer’s mRNA-based vaccine candidate, BNT162b2 was found to be more than 90% effective in preventing COVID-19 in participants without evidence of prior SARS-CoV-2 infection in the first interim efficacy analysis (NCT04368728) [99]. Similarly, Moderna’s mRNA-1273 vaccine against SARS-CoV-2 provided 94.5 % vaccine efficacy in phase III clinical trials (NCT04470427) [100].
1.4.4. CLR

CLR receptors (CLRs) are a large transmembrane receptor family characterised by the presence of a Ca²⁺-dependent carbohydrate binding domain. The family recognises a diverse range of carbohydrates from various microorganisms such as viruses, bacteria and fungi. CLRs activate intracellular signalling either via cytoplasmic ITAM-like motifs or via adaptors harbouring an ITAM domain, such as Fc receptor gamma chain (FcRγ), DNAX-activating protein of 10KDa (DAP10) or DNAX-activating protein of 12KDa (DAP12), leading to the consecutive recruitment of Src Homology Phosphatase 2 (SHP2), the tyrosine kinase SYK, and the CARD9-BCL-MALT1 scaffold complex. The CARD9 complex subsequently activates MAP kinases, nuclear factor of activated T-cells (NF-AT), and NK-κB, resulting in the induction of pro-inflammatory cytokines [101].

The prototypic CLR, Dectin-1 recognises β-glucans from fungi. Upon ligand binding, it signals through its cytoplasmic ITAM-like motif, leading to the consecutive recruitment of SHP2, Syk, and the CARD9-BCL-MALT1 scaffold complex, that ultimately results in both canonical (p65-p50 & rel-p50) and non-canonical (RELβ-p52) NK-κB activation [102]. Canonical and non-canonical NK-κB activation trigger the expression of IL-6 and IL23p19 that are necessary for Dectin-1 mediated Th17 induction [103][104]. A syk-independent Dectin-1 signalling pathway through RAF1 additionally drives pro-IL-1β and IL12p40 induction [103]. Fungi appear to have an array of mechanisms that activate cellular processing of pro-IL-1β to its active form. While some fungi activate the cytosolic NLRP3 inflammasome, others can trigger canonical caspase 1 activation via Dectin-1. This process is dependent on pathogen internalisation, ROS production and potassium efflux. In contrast, Dectin-1 signalling can drive non-canonical caspase 8 inflammasome activation that is dependent on β-glucan sensing rather than uptake [105]. Why certain fungi activate different inflammasomes awaits further clarification. Irrespective, it is clear that Dectin-1 mediated pro-IL-1β cleavage in conjunction with SYK and RAF1 signalling leads to potent anti-fungal Th1 and TH17 responses [106].

CLRs have long been studied as vaccine targets to improve antigen presentation rather than their ability to induce immunity [102]. However, there are several possible means of triggering CLR signalling, such as dead or attenuated pathogens, carbohydrate ligands or particles containing carbohydrates, increasing interest in their use as vaccine adjuvants. For instance, β-glucan sensing and mycobacterial cord factor-macrophage inducible Ca²⁺-dependent lectin receptor (Mincle) ligation have successfully been used
to enhance anti-tumour [reviewed in 36] and anti-bacterial responses [108] in vivo, respectively. Similarly, a synthetic ligand for Mincle formulated in liposomes (CAF01) shows great promise as an adjuvant for a TB vaccine [109]. A drawback of using a single carbohydrate motif is that many CLRs have overlapping binding specificities and may potentially induce distinct and potentially contrasting immune responses. For instance, Dectin-1 and Dectin-2 both recognise β-glucan and in addition to driving Th17 responses, can stimulate Th1 and Th2 responses respectively [reviewed in 38].
1.4.5. DNA sensors

1.4.5.1. cGAS-STING signalling

In eukaryotic cells, DNA is contained in the nucleus and mitochondria, leaving the cytosol largely free of self-DNA. Multitudes of pathogens use the cytosol for replication, and so heavy PRR surveillance is in place to detect their presence. Of these, the sensor/enzyme cGAS has emerged as a widely expressed, essential molecule for cytoplasmic DNA recognition. cGAS contains nucleotidyltransferase domain and two major DNA binding domains (denoted A and B) that recognises B form dsDNA independently of its sequence by binding the sugar phosphate backbone [110]. This bestows an ability to recognise DNA from both endogenous and microbial sources. DNA binding induces a global conformational change that relieves auto-inhibition, forms an active site and subsequently catalyses the synthesis of non-canonical 2'-5'-linked cyclic GMP-AMP (cGAMP) from ATP and guanosine triphosphate (GTP). cGAMP then functions as a second messenger which binds to and activates the endoplasmic reticulum (ER)-resident transmembrane receptor STING [111], [112].

Initial localisation studies detected small amounts of cGAS in nuclear and perinuclear regions, suggesting cGAS was a cytoplasmic protein and that this compartmentalisation dictates specificity of GAS towards non-self DNA [111]. However, recent reports challenge this simplistic view showing that cGAS is additionally, and perhaps even preferentially localized within the nucleus tethered to chromatin. How cGAS that does reside in the nucleus is kept inactive despite interacting with DNA is not yet clear. It is possible that the structural organization of chromatin or alternative intrinsic biophysical DNA properties restrain cGAS activity toward endogenous DNA [113]–[115].

Not all DNAs activate cGAS although they bind to cGAS. For full activation, cGAS must form a stable homodimer that sandwiches two DNA strands forming a 2:2 complex, with each DNA molecule engaged by site A of one cGAS protomer and to site B of the respective cGAS protomer [110]. This is an allosteric process that is coupled to active site formation and is enhanced by increased cGAS clustering, highlighting why long DNA elements are the optimal drivers of cGAS signalling [116], [117]. Inside cells, DNA and cGAS complexes form punctate structures with liquid-like behaviour. These structures appear to function as “microreactors” to facilitate cGAMP synthesis and are highly sensitive to concentrations of cGAS and DNA, suggesting a threshold response in which cGAS is activated only when cytosolic DNA reaches a certain level [118]. Oxidation of
DNA bases, such as that caused by ultraviolet irradiation, does not modulate DNA's ability to activate cGAS, but the oxidized DNA is more resistant to degradation which leads to greater production of type I IFNs [119].

cGAMP is structurally related to bacterial cyclic dinucleotides (CDNs) (for example, c-di-GMP and c-di-AMP), revealing a role for these molecules as second messengers across phylogenetic kingdoms. However, mammalian cGAMP contains an unusual 2′–5′ phosphodiester bond between the 2′O of guanosine and the 5′O of adenosine that is specific to metazoans. For this reason, the cGAS product is sometimes referred to as 2′3′-cGAMP to distinguish it from bacterial cGAMP (3′3′-cGAMP). In bacteria, 3′3′-cGAMPs play diverse roles in regulating cellular physiology and gene expression. In vertebrates, 2′3′-cGAMP activates STING, with the major outcome being TANK-binding kinase 1 (TBK1)/IRF3-dependent type I IFNs. Of note, Bacterial CDNs can activate mammalian STING to drive type I IFNs, albeit with much lower affinity than 2′3′-cGAMP. This is a natural phenomenon, with the best characterised example being the activation of STING by the intracellular bacterium *Listeria monocytogenes* upon secretion of c-di-AMP. The significance of this is complicated and context dependent (reviewed in [120] and [121]).

The ability of cGAS-dimers to produce multiple copies of cGAMP provides a mechanism by which detection of small amounts of cytosolic DNA can produce a rapidly amplified anti-viral signalling response. In addition, cGAMP can trigger anti-viral responses in bystander cells through gap junctions or enter distant cells through incorporation into viral capsids [122]. Damage to the cell membrane can release cGAMP into the microenvironment (for example, upon cell damage or necrotic cell death) and this extracellular cGAMP can enter other cells, most likely via ligand- specific or voltage-gated channels. For example, solute carrier family 19 member 1 (SLC19A1), is a direct importer for cGAMP in U937 and THP-1 cells and LRR-containing 8 (LRRC8) volume-regulated anion channels can function as a conduit for cGAMP efflux and influx in multiple human and murine cell types [123], [124]. Of note, extracellular cGAMP is rapidly degraded by the ecto-nucleotide pyrophosphatase/ phosphodiesterase-1 (ENPP1), thereby limiting cGAMP uptake by nearby cells. In contrast, cGAMP is highly stable in the cytosol of mammalian cells [125]. No cytosolic phosphodiesterase has yet been reported and its metabolic fate is unclear.

STING is anchored to the ER membrane by 4 transmembrane domains at the amino terminus, while the large carboxy- terminal domain containing the carboxy-terminal tail
(CTT) and ligand binding domain (LBD) project into the cytoplasm [126]. Under steady-state conditions, STING forms a dimer on the ER membrane with both LBDs juxtaposed to form a V-shaped ligand binding pocket for one cyclic dinucleotide (CDN) ligand. cGAMP then binds to the central crevice of the STING homo-dimer via hydrogen bonds and hydrophobic interactions [127]. The interaction induces a 180° clockwise rotation of the LBDs in relation to the transmembrane portion and allows the closure of a lid structure around cGAMP and the lateral oligomerisation of several STING molecules in a side-by-side configuration [128]–[130]. STING oligomerisation, like cGAS condensation, is a prerequisite and cooperative function for optimal STING activation. Indeed, mutations in residues encoding the STING oligomerisation interface prevent STING exit from the ER and TBK1 activation [130], [131]. Upon ligand binding, TBK1 phosphorylates the CTT of an adjacent STING dimer at several serine and threonine residues, such as Ser366. This forms the docking site for IRF3, which is then brought into close proximity to the catalytically active TBK1 molecules [130], [132]–[134]. Once phosphorylated, IRF3 homodimerizes and enters the nucleus to induce IFN expression.

In addition to oligomerisation, STING translocation to the perinuclear regions is critical for type I IFN responses. Inhibition of the trafficking of STING with brefeldin A, an inhibitor of ADP-ribosylation factor (ARF) GTPase, blocks activation of the downstream pathway. Similarly, the shigella effector protein IpaJ, which inactivates the ARF family of GTPases inhibits the induction of IFNs by STING [135]. Furthermore, palmitoylation of STING at Cysteine 88 and Cysteine 91 is necessary for type I IFNs and ubiquitination at lysine 224 and 288 is required for STING trafficking from the ER to the ER-Golgi intermediate compartment (ERGIC), where it elicits type I IFN production [136]. ER exit depends on canonical COPII coat complex-dependent anterograde transport, but it remains largely unclear what signal within STING is sensed to initiate this transport [137]. A recent advancement in understanding came from the discovery that stromal interaction molecule 1 (STIM1) moonlights as an anchor for STING on the ER to limit STING signalling. Upon STIM-1 or STING activation, via ER Ca^{2+} depletion or cGAMP binding respectively, the proteins dissociate from one another and STING traffics to the ERGIC. Interestingly, STING was shown to reciprocally regulate STIM1, ultimately limiting store operated Ca^{2+} entry (SOCE), and implicating STING as a regulator of Ca^{2+} signalling [138]. Supporting this notion, stimulation of RAW264.7 macrophages with two different STING agonists, DMXAA and exogenous cGAMP triggers rapid Ca^{2+} bursts from the ER [19] and STING-associated vasculopathy with onset in infancy (SAVI) patients with mutations at N154S (causing STING translocation in the absence of cGAMP) have disrupted ER Ca^{2+} homeostasis and high levels of ER stress [139].
Despite being the best studied, IRF3 activation is the most recent evolutionary function of STING [120]. A less understood primordial function of STING is mediating a pro-inflammatory cytokine response (TNF-α, pro-IL-1β, and IL-6) through the activity of NF-κB [140]. It is unclear how STING activates NF-κB signalling. Initial studies on TBK1-deficient and TBK1 RNAi-silenced MEFs suggested that NF-κB activation [141], like IRF3 was dependent on TBK1 activity, but recent data disputes these claims; Drosophila melanogaster and Nematostella vectensis STING, which lack TBK1 docking sites, can induce pathways homologous to NF-κB and removal of a part or the entire CTT of human STING ablates IFN activity, but not NF-κB. Furthermore, the HIV-2/simian immunodeficiency virus (SIV) protein Vpx selectively suppresses the activation of NF-κB, but not IRF3, by interacting with residues 329-334 that precede the CTT [142]. A likely explanation for the discrepancies is that NF-κB responses are mediated via a TBK1/IKKe redundant mechanism. Interestingly, STING-induced NF-κB activation is much less dependent on the kinase activities of TBK1 and IKKe that occur at the ERGIC than IRF3 [143]. This suggests a spatial uncoupling of type I IFN and NF-κB signalling downstream of STING. In support of this, ubiquitination of lysine residues on STING that are necessary for translocation are required for type I IFN but not NF-κB signalling [136], [144]. The MCMV protein M152 that perturbs STING translocation from the ER to the Golgi compartment, blocks anti-viral type I IFN production while keeping the early pro-viral NF-κB response intact [144]. Finally, Etoposide-induced STING-dependent IRF3 but not NF-κB responses are blocked in keratinocytes by forced retention of STING at the ER [145].

STING agonists are highly successful adjuvants for promoting both humoral and cellular immune responses in experimental models of vaccination and have emerged as promising therapeutic adjuvants for cancer immunotherapy. Intratumoral administration of cGAMP or CDN derivatives successfully reduce tumour volume and growth in mouse models of colon, brain, skin, pancreatic, breast and B cell malignancies and chemotherapeutic agents drive non-canonical STING activation via DNA damage and cytosolic leakage, promoting immune responses that enhance tumour cell death and rejection [reviewed in 99]. Indeed, a number of STING agonists have entered clinical testing. However, like CpG, translational progression has been disappointing for a variety of reasons. Similar to TLR agonists, STING signalling can promote tolerogenic immune responses in tumours bearing low antigenicity through the induction of IDO [147]. Human STING is a polymorphic protein with implications for the selection of a single STING agonist [148]. Small molecule STING agonists can disseminate rapidly through the
bloodstream, resulting in cytokine storms, tissue toxicity and promotion of tumour growth [149]. While it is well established that STING-dependent type I IFNs play beneficial roles in priming anti-tumour responses, the role of STING-mediated NF-κB activation in antitumour immunity is unclear. It is plausible that NF-κB signalling diminishes the full potential of STING agonists as therapeutic adjuvants, given that all hallmarks of cancer involve NF-κB activation [150], [151]. Furthermore, STING signalling has multiple stringent auto-regulatory mechanisms in place to prevent chronic activation. These include lysosomal degradation of STING upon translocation from the ER, degradation of DNA by DNAases, degradation of cGAMP by ENPP1 and autophagic clearance of cytosolic DNA [137], [152]–[154]. While these mechanisms are critical for the prevention of overt inflammation, they dramatically narrow the therapeutic window of STING agonists.

Table 6 Limitations in the clinical development of STING agonists for Immunotherapy

<table>
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<tr>
<th>Limitation</th>
<th>Consequence</th>
<th>Solution</th>
<th>Currently in Clinical trials NCT</th>
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<tr>
<td>CDNs are large, electronegative and hydrophilic.</td>
<td>• Poor membrane permeability</td>
<td>• Incorporate in delivery systems; ▪ Liposomes, ▪ Polymers, ▪ Hydrogels • non-CDN small molecules STING agonists; ▪ DMXAA ▪ GSK3745417</td>
<td>Not yet reached clinical trials</td>
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<tr>
<td>STING LBD faces the cytosol</td>
<td>• Agonists must gain entry to the cytosol</td>
<td>• Use compounds that drive the endogenous release of DNA (Chemotherapy, radiation therapy and chitosan) • Incorporate into delivery systems</td>
<td>Variety Licensed</td>
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<tr>
<th>CDNs are susceptible to enzymatic digestion</th>
<th>Poor bioavailability in tumour tissues</th>
<th>Narrow therapeutic window</th>
<th>Synthetic CDNs 2'3'-cG^3^A^3^MP,</th>
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<td>Non-CDN agonists</td>
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<td>ENPP1 inhibitors (MAVU-104)</td>
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<td>Protect in delivery systems</td>
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<td>STING is highly polymorphic</td>
<td>Inability of CDNs to bind all STING alleles</td>
<td>Use compounds that drive the endogenous release of DNA</td>
<td>Identify pan-genotypic agonists (E7766 -targets 7 haplotypes)</td>
<td>Variety Licensed</td>
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<tr>
<td>Lysosomal degradation of STING</td>
<td>Transient immune signalling</td>
<td>Block lysosomal degradation [152]</td>
<td>Not clinically applicable</td>
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<td></td>
<td>Narrow therapeutic window</td>
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<td>NF-kB signalling</td>
<td>Pro-tumour</td>
<td>Cotreatment with NFKB Inhibitors</td>
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<td>Identify STING agonists with exclusive Type I IFN profiles</td>
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<td>Autophagy induction</td>
<td>Unclear</td>
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Abbreviations: CDN, Cyclic dinucleotide; LBD, Ligand binding domain; STING, Stimulator of interferon genes; ENPP1, Ecto-nucleotide pyrophosphatase/phosphodiesterase-1.
1.4.5.2. IFI16, AIM2 & DDX41

While cGAS appears to be is the predominant DNA sensor in cells, others have been identified that converge on the adaptor STING. Interferon γ -inducible protein 16 (IFI16) was first identified in a mass spectrometry analysis of cytosolic proteins coprecipitated with immunostimulatory DNA, but not RNA. It is a member of the p200 family of proteins, possessing an N-terminal pyrin domain and two C-terminal HIN domains, which bind DNA in a sequence-independent manner [155]. Upon nuclear damage in keratinocytes, IFI16 can cooperate with ataxia-telangiectasia mutated (ATM), recognise nuclear DNA damage and trigger the formation of a cytosolic multi-protein signalling complex on STING to promote NF-κB-biased activation [145]. In addition, upon integration of Kaposi’s Sarcoma-associated Herpesvirus (KSHV), Epstein Bar virus (EBV) and Herpes Simplex virus (HSV) genomes into the nuclei of infected fibroblasts, IFI16 can bind breast cancer type 1 (BRCA1) and form an inflammasome complex consisting of ASC and caspase 1. The IFI16 complex can then migrate to the cytosol to cleave IL-1β into its mature form [156]. Alternatively, IFI16 can cooperate with cGAS to promote optimal canonical STING activation [157]. The extent of cooperation/redundancy between cGAS and IFI16 is unclear. While IFI16 plays a cooperate and /or synergistic role with cGAS during \textit{Francisella novicida} infection in murine RAW264.7 monocytic cells [158], \textit{Listeria monocytogenes} infection in human myeloid cells [159], and HSV-1 infection in primary human primary foreskin fibroblasts [156], [160], it does not cooperate with cGAS upon nuclear damage in keratinocytes and p204, the murine homologue of IFI16, is dispensable altogether for IFN responses to DNA in mice [145], [161]. Future work must address these inconsistencies and determine whether IFI16 cooperation with ATM, BRCA1 and cGAS are cell-specific functions.

Another p200 protein, AIM2 (absent in melanoma 2), is a sensor for cytosolic DNA that exclusively activates caspase-1 leading to release of IL-1β and IL-18. Like IFI16, it recognises long dsDNA fragments through the HIN domain in a sequence-independent manner. Electrostatic binding between the DNA backbone and HIN domain of AIM2 releases autoinhibition, allowing subsequent oligomerisation, recruitment of adaptor ASC via PYD-PYD interaction and nucleation of the ASC protein. Pro-caspase 1 is then recruited via CARD-CARD interaction with ASC molecules. Similar to the role of RLRs in the efficacy of RNA vaccines, the AIM2 inflammasome has been implicated in the success of DNA vaccines. Optimal antigen-specific antibody responses to a DNA vaccine expressing influenza haemagglutinin required AIM2 and the adaptor molecule ASC [162]. Despite this, AIM2 has additional T cell-intrinsic, inflammasome-independent
roles in promoting the stability of regulatory T cells and while T cells are crucial in preventing autoimmune disorders, they can diminish the strength vaccine-induced immunity [163], [164].

DEAD box protein DDX41 is a member of the superfamily 2 (SF2) helicases that acts as a sensor for both cytosolic DNA and c-di-GMP. Its sensing capacity is dependent on the phosphorylation of Y414 by bruton's tyrosine kinase (BTK). Once activated, it can directly bind DNA and c-di-AMP to trigger STING-dependent TBK1-IRF3 signalling [165]. Of note, many members of the DNA repair pathway are reported to interact with cytoplasmic DNA and converge on the adaptor STING. These are reviewed in section 1.5.5.
1.5. The role of cell autonomous responses in PRR signalling

The above literature encompasses decades of research highlighting the pivotal role of PRR signalling in innate immunity. Each of these receptors initiate specific signalling cascades that alter gene expression to create an appropriate immune response to the particular threat encountered. Beyond this well characterised PAMP engagement of PRRs, it is only now becoming clear that constitutive cell-autonomous responses (mechanisms that are activated through pre-existing molecules to directly eliminate danger), such as autophagy, ER stress, mitochondrial stress and the DNA damage response (DDR) can modulate, initiate or block PRR signalling pathways. There is no doubt that understanding how constitutive cell processes regulate innate immune responses will provide considerable insights into the design of future vaccine adjuvants. The following section will describe the currently defined roles of cellular organelles and processes in PRR signalling.

1.5.1. Autophagy

Autophagy is a highly evolutionarily conserved mechanism in eukaryotic cells that maintains cell homeostasis by recycling and degrading cytoplasmic constituents, such as defective organelles, protein aggregates and intracellular pathogens. The process begins with the formation of the characteristic double membrane structure, referred to as the autophagosome, which engulfs portions of the cytoplasm containing the autophagy target and finally fuses with lysosomes, leading to the degradation and recycling of autophagosome contents. Autophagy relies on the activity of at least 30 conserved autophagy-related proteins (ATGs) that act sequentially through three main stages; initiation, elongation and maturation, on their way to fusing with lysosomes. The earliest stage of initiation is activation of the ULK1 macromolecular complex. Activation of the ULK1 complex is controlled through phosphorylation by the low energy sensor 5’ AMP-activated protein kinase (AMPK) and metabolic integrator mammalian target of rapamycin 1 (mTOR1). Once activated, ULK1 phosphorylates Beclin-1, an essential component of the PI3K nucleation complex which increases the local concentration of phosphatidylinositol 3-monophosphate (PI3P) required for membrane nucleation. Membrane elongation begins with the involvement of ATG12-ATG5 system which recruits ATG16L1, specifying the site of microtubule-associated protein 1A/1B-light chain 3 (LC3) lipidation. This system displays an E3-ligase-like function that will eventually
conjugate cytosolic LC3 to phosphatidylethanolamine (PE), forming membrane bound LC3 which enables further elongation and recruitment of autophagy targets. Finally, the transcription factor EB (TFEB) couples lysosome generation and autophagy induction to drive lysosome-autophagosome fusion. Lysosomal acid hydrolases then degrade the autophagosomal contents (Figure 1.2) [166], [167].

The impact of autophagy on innate immune responses is PRR dependent (Figure 1.3). The most widely reported effect of autophagy on innate signalling is regulation of the NLRP3 and AIM2 inflammasomes. Autophagy limits NLRP3 and AIM2 inflammasome activation and thus subsequent production of IL-1β and IL-18 in response to a variety of activators. The current theory surrounding this regulation is that inflammasome components and pro-IL-1β are degraded by autophagy, thereby reducing the number of functional inflammasomes [168], [169]. Similarly, autophagy appears to play a negative role in RLR signaling. Autophagy removes dysfunctional ROS-producing mitochondria that play a key role in potentiating RLR signaling [170]. In addition, The ATG12-ATG5 conjugate complex regulates RLR signaling through direct binding to RIG-I and MAVS, preventing their CARD-CARD interaction and blocking type I IFN production [171]. RLR activation also induces autophagy [172], highlighting a complex relationship that on the one hand could shuts down cytokine responses, while at the same time, promotes direct virus restriction via autophagy. Although a controversial topic in DCs, TLR signaling (with the exception of TLR5) induces autophagy in macrophages [173]. The role of autophagy in TLR signalling is only clear in the case of TLR9; Activation of TLR9 stimulates ATG5-dependent recruitment of LC3 around phagosomes which is critical for IKKα recruitment and IRF7 phosphorylation [174], [175]. An important flip side of the relationship between TLRs and autophagy is that in addition to autophagy being an effector function downstream of PRR activation, at least in the case of TLR7, it also facilitates the delivery of cytosolic PAMPs to their cognate endosomal receptors so that TLR signaling can be initiated [173], [176].

Similar to RLR and TLR signaling, autophagy is an effector function of both cGAS and STING activation. Upon DNA binding, cGAS binds to beclin-1, stimulating the release of rubicon, a negative regulator of autophagy from the beclin1 complex, leading to the induction of autophagy. Not only does the direct interaction between cGAS and beclin-1 drive autophagy mediated degradation of DNA, it additionally suppresses the cGAS NTase activity to stop 2’3’-cGAMP production [177]. STING activation drives a non-conventional form of autophagy, whereby STING-containing ERGIC vesicles activate LC3 lipidation in WIP12 and ATG5-dependent manner. It’s unclear how STING
contributes to autophagy induction, but a conserved motif located in STING’s LBD seems to be critical to the function [137]. Intriguingly, in the context of HSV-1 infection, it seems that STING-induced autophagy, but not type I IFN induction, is required for controlling the virus [178].

It would appear unlikely that autophagy is beneficial to cGAS-STING agonists in adjuvant settings given that it acts as a feedback-negative regulatory mechanism to prevent excessive activation. The impact of autophagy on STING-induced IFN and NF-κB signaling is unclear; Some studies suggest that autophagy and IFN functions are uncoupled, as ATG5-deficient cells that are defective in LC3 lipidation maintain TBK1 and IRF3 activity. However contradicting this theory, another study has demonstrated that ER-phagy, a form of autophagy that selectively targets the ER, is required for STING trafficking to phagosomes rich in molecules conductive for IFN signaling [179]. Given the number of STING agonists currently under investigation as therapeutic anti-cancer adjuvants, future studies must delineate the impact of autophagy on STING agonist responses.

There are potential benefits of adjuvants triggering autophagy. Autophagy has been linked to the induction of CD4+ and CD8+ T cell responses through increased MHCcl and MHCcll antigen processing and presentation [180]–[183]. The use of recombinant beclin-1 as an autophagy inducing adjuvant in the Human Papilloma Virus 16 (HPV16) E7 DNA vaccine increased autophagy-mediated antigen presentation and led to superior lymphocyte proliferation and cytotoxicity when compared to the DNA vaccine alone [184]. Overexpression of the autophagy inducing and TLR2 activating C5 peptide in the BCG vaccin drove robust LC3-dependent autophagy in macrophages which increased antigen presentation to CD4+ T cells in vitro and enhanced effector and central memory T cell responses in vivo compared to the BCG vaccine alone [183], [185]. Accordingly, a recombinant BCG vaccine with enhanced AIM2 inflammasome activation and autophagy was more efficient against TB in preclinical animal models than BCG alone [186].
Figure 1.2 Schematic depicting the process and machinery involved in autophagy

Autophagy is a multistep process that can be divided into 5 sequential steps; (1) initiation, (2) double membrane nucleation and phagophore formation, (3) phagophore elongation and uptake of cytoplasmic cargo, (4) fusion of the autophagosome to a lysosome and finally (5) degradation of sequestered cargo in the autolysosome. mTOR and AMPK are the main regulators of autophagy, with mTOR acting as an inhibitor and AMPK as an activator. Initiation of autophagy requires ATGs to assemble into two complexes, the ULK1 initiation complex and class III PI3K nucleation complex. Elongation proceeds with two ubiquitin-like conjugation pathways; ATG12 and LC3/GABARAPs (for simplicity only LC3 is noted in the figure). In the ATG12 conjugation system, ATG12 is attached to ATG5, which is then attached to ATG16L1, followed by dimerisation (not shown) and interaction with the PI3P-binding complex (formed by WIPIs and DFCP1). The ATG12–ATG5–ATG16L1 complex then promotes conjugation of LC3, whereby LC3 is cleaved by the protease ATG4 to form LC3-I, which is then conjugated with the help of ATG7 and ATG3 to PE to form LC3-II. LC3-II is incorporated into phagophore membranes, where it interacts with cargo receptors that harbour LIRs such as p62 that recognises ubiquitylated proteins/organelles targeted for degradation and BNIP3 that recognises dysfunctional mitochondrial. Membranes for phagophore expansion are delivered. Once fully formed, the autophagosome fuses with lysosomes resulting in cargo degradation. Taken from [187].

Abbreviations; ATGs, autophagy related proteins; mTOR, mammalian target of rapamycin 1; AMPK, 5’ AMP-activated protein kinase; PI3P, phosphatidylinositol 3-monophosphate; LC3, microtubule-associated protein 1A/1B-light chain 3; PE, phosphatidylethanolamine; GABARAP (Gamma-aminobutyric acid receptor-associated protein) PI3K, Phosphoinositide 3-kinase; WIPI,
WD-repeat phosphatidylinositol 3-monophosphate effector protein; DFCP1, Double FYVE-containing protein 1; LIRs, LC3-interacting motifs.

Figure 1.3 The relationship between autophagy and PRR pathways
(Left to right) Autophagy is an effector function downstream of TLR signalling and facilitates the delivery of TLR ligands to cognate receptors in endosomal compartments.
LAP downstream of TLR9 activation initiates IKKα, TRAF3 and IRF7 recruitment that is necessary for type I IFN production. Canonical autophagy negatively regulates AIM2 and NLRP3 inflammasome activation, presumably through the degradation of core signalling components. Autophagy is an effector function of RLR signalling and functions to limit excessive signalling through the removal of dysfunctional mitochondria that amplify RLR signalling and through direct association of the autophagy complex ATG12-ATG5 with RIG-I and MAVS. Autophagy is an effector function of both cGAS and STING activation that is important for the clearance of DNA and hence limits activation of the pathway. Association of cGAS with Beclin-1 initiates canonical autophagy and dysregulates cGAS activity to block cGAMP production. STING activation drives a form of autophagy that is dependent on WIP12 and ATG5 but independent of ULK and VPS34-beclin kinase complexes. (Blue: Positive regulation, Red: Negative regulation)
Abbreviations; LAP, LC3-associated phagocytosis, ATGs, autophagy related proteins, TRAF3, TNF receptor associated factor 3; IKK, Inhibitor of NF-κB kinase; IRF7, Interferon regulatory factor 3; WIPI, WD-repeat phosphatidylinositol 3-monophosphate effector protein.
1.5.2. ER stress

The ER is an elaborate endomembrane compartment present in all eukaryotic cells that is central to the folding and maturation of newly synthesized secretory and transmembrane proteins, which constitute approximately one-third of all proteins made in the cell. Protein folding is highly sensitive to a variety of extracellular and intracellular stimuli that alter Ca\textsuperscript{2+} levels, redox levels and energy stores. Under conditions of ER stress, the organelle signals, via ER-resident UPR sensors, inositol-requiring enzyme (IRE)\textsubscript{1α}, activating transcription factor (ATF)\textsubscript{6} and Protein kinase R-like ER kinase (PERK), to mobilise a coordinated process known as the unfolded protein response (UPR) that acts to restore homeostasis [188].

Under normal conditions in which the ER is presumably “stress free”, the protein chaperone binding-immunoglobulin protein (BiP) physically interacts with the intraluminal domains of the UPR sensors to keep them inactive. However, BiP has a higher affinity for misfolded proteins and so in circumstances of ER stress, BiP dissociates from the sensors, permitting their activation. On release from BiP, ATF\textsubscript{6} moves from the ER to the Golgi, where it is cleaved by two proteases, site 1 protease (S1P) and site 2 protease (S2P), to produce an active transcription factor (ATF\textsubscript{6p50}) that migrates to the nucleus. ATF\textsubscript{5p50} then drives the transcription of genes that function to increase ER capacity and folding, such as X-box binding protein 1 (XBP-1), ER chaperones BiP and glucose regulated protein (GRP)\textsubscript{94} as well as components of the ER-associated protein degradation (ERAD) machinery, which removes misfolded proteins from the ER to the cytosol for subsequent degradation by the ubiquitin–proteasome system. In contrast, IRE\textsubscript{1α} and PERK are activated by oligomerisation and autophosphorylation. IRE\textsubscript{1α} is the most evolutionarily conserved UPR sensor and possesses two enzymatic activities; a serine/threonine kinase domain and an endonuclease (RNase) domain. Upon release from BiP, dimerization of IRE\textsubscript{1α} elicits an intrinsic RNase domain, leading to an unconventional splicing reaction that removes 26 nucleotides from xbp\textsubscript{1} mRNA. This event eliminates a premature stop codon inducing the expression of a functionally active, potent transcription factor (XBP-1s). XBP-1s induces an array of transcriptional programs that enhance the expression of protein chaperones, foldases, ERAD components, as well as lipid and hexosamine biosynthesis that together increase ER size and function. Furthermore, IRE\textsubscript{1α} decreases the entry of newly translated proteins to the ER by degrading mRNA and miRNA substrates through a process known as IRE\textsubscript{1α}-dependent decay (RIDD). As with IRE\textsubscript{1α}, dissociation of PERK from BiP activates its cytoplasmic kinase domain. Global mRNA translation is then transiently attenuated.
through PERK-dependent phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2). This event selectively promotes the translation of ATF4, a transcription factor that regulates the expression of genes involved in antioxidant responses, amino acid metabolism and autophagy. ATF4 additionally upregulates growth arrest and DNA damage-inducible protein (GADD34) which controls eIF2α dephosphorylation by protein phosphatase (PP)1, to restore protein translation. In circumstances where ER stress prevails, ATF4 induces the expression of C/EBP homologous protein (CHOP) to activate pro-apoptotic programmes [188].

The UPR has increasingly been shown to have crucial functions in immunity and inflammation. Not only does an effective immune response depend on the capacity of immune cells to generate an array of transmembrane and secreted effector molecules such as ligands, receptors, cytokines and chemokines, under specific circumstances, the UPR can directly activate or intersect with innate inflammatory pathways leading to activation of NF-κb, JNK and IRF3 signaling pathways [189]. For example, simultaneous chemical activation of ER stress with TLR ligand signaling has been shown to amplify type I IFNs, IL-6, IL-8, TNF-α and IL-1β production in macrophages and type I IFNs and IL-23 production in dendritic cells [190]–[194]. The UPR-target gene XBP-1 is suggested to amplify TLR ligand-induced type I IFN production through an enhancer element 6kb downstream of the \textit{ifnb} gene [191]. The mechanism behind ER stress enhanced IL-6, IL-8, TNF-α and IL-23 production is not yet clear but is likely to involve IRE1α; ER stress induced by infection with \textit{Brucella abortus} or chemical compounds can activate IRE1α to initiate inflammatory responses and IL-6 production via TRAF2 recruitment to the ER membrane and subsequent engagement of the NOD1/2-RIP2-NF-κB pathway [195]. The IRE1α-TRAF2 complex can, in addition, recruit ASK1, and subsequently activate JNK, resulting in enhanced expression of inflammatory genes via AP1 activity [196]. Additionally, ER stress induced by \textit{Brucella abortus} or the chemical compounds tunicamycin and thapsigargin drive a non-canonical NLRP3 inflammasome activation. IRE1α activation leads to NLRP3-mediated crosstalk between the ER and mitochondria, resulting in the release of mitochondrial contents through activation of a caspase 2-bid-signalling axis [197]. It remains unclear how IRE1α leads to NLRP3-dependent stimulation of caspase 2.

ER stress and metabolites are implicated in the efficacy of several adjuvants. Activation of IRE1α by the vaccine adjuvant AS03 contributes to its immunostimulatory properties. Treatment with 4BPA, a chemical chaperone or deletion of IRE1α in myeloid cells
decreased AS03-induced cytokine production and its ability to drive high affinity antigen-specific antibodies in vivo [16]. The role of NLRP3 in AS03 signalling has not been defined, but given the vital function of IRE1α, it is plausible that NLRP3 plays a role. The adjuvant c-di-AMP from Listeria monocytogenes triggers a STING-dependent ER stress response that appears to be necessary for type I IFN secretion. At the cellular level, ER stress inactivates mTOR and induces canonical autophagy. Autophagic removal of stressed ER membranes protects APCs from infection-induced death and localises STING to membranes conductive to IFN-I signalling [179]. It is unclear how STING regulates ER stress, whether it be through direct Ca\textsuperscript{2+} regulation or Ca\textsuperscript{2+} sensing proteins like STIM-1. However recent work has implicated a novel motif located in the helix aa322-343 called “the UPR motif” [139]. Finally, STING-mediated ER Ca\textsuperscript{2+} release in monocytes and macrophages promotes gasdermin D cleavage by caspase1/11 or caspase 8 in response to E. coli or S. pneumoniae respectively [198]. Given that gasdermin D restrains type I IFN signalling in response to dsDNA [199], it is possible that STING-induced ER Ca\textsuperscript{2+} efflux is an intrinsic negative-feedback mechanism to limit excessive STING signalling in myeloid cells.

The ER protein, calreticulin is a DAMP that has adjuvant properties when exposed on the cell membrane [200], [201]. Indeed, chimeric constructs encoding calreticulin and tumour antigens are more immunogenic than tumour antigens alone when incorporated into viral vectors and gene guns [202]–[204]. However, ER stress does not elicit the appearance of calreticulin on the cell surface and toxicity provoked by ER stress is actually non-immunogenic. Rather, inhibitors of ER stress attenuate the ER stress response, promote calreticulin cell surface exposure and immunogenic cell death [201].

Critically, ER stress plays a negative role in DC cross presentation. Hyperactivation of IRE1α and RIDD in cDCs drives degradation of components of the cross-presentation machinery, such as tapasin [205]. Consistent with this finding, several others have demonstrated that ER stress affects the cell surface expression of MHC class I molecules [206]–[208]. Notably, DCs in the tumour environment exhibit ER stress and robust IRE1α activation [209], ER-stress imprinted DCs promote tumour growth in mice [208], [209] and relieving ER stress in DCs extends host survival by enhancing T cell anti-tumour immunity [209].

Lastly, ER stress is harmful to T cell viability and responsible for T cell cytopenia in SAVI patients. SAVI (STING-N154S) mice have chronically elevated ER stress through disrupted Ca\textsuperscript{2+} homeostasis that renders T cells hyperresponsive to T cell receptor
(TCR)-signalling-induced ER stress and tips the balance toward cell death. Importantly, pharmacological inhibition of ER stress with the chemical chaperone TUDCA or point mutation of residues R331 and R334 in the UPR motif protects STING-N154S mutants from T cell cytopenia [139]. Despite typically lacking type I IFN machinery, cGAS expression is an established characteristic of many tumour cells, and as a result have high levels of cGAMP production. It is possible that tumour derived cGAMP has evolved as a method of tumour evasion, whereby cGAMP uptake within the tumour drives stress and death in T-lymphocytes. These findings emphasise the caution which must be taken with ER-stress-inducing adjuvants, particularly when being used for in situ tumour vaccination.
Figure 1.4 ER Stress and UPR signalling pathways

Three ER transmembrane proteins, IRE1α, PERK, and ATF6, sense ER stress in the ER lumen and become activated, regulating a cascade of signalling pathways collectively termed the Unfolded Protein Response (UPR). IRE1α splices XBP-1 mRNA leading to the translation of an active transcription factor, PERK phosphorylates eIF2α blocking global mRNA translation and activated ATF6α regulates the transcription of many UPR target genes. Additionally, IRE1α decreases the entry of newly translated proteins to the ER by degrading mRNA and miRNA substrates through a process known as RIDD. High levels of chronic ER stress can lead to the recruitment of TRAF2 by IRE1α and the activation of ASK1-JNK axis, which plays a role in apoptosis by regulating the BCL2 family of proteins. Phosphorylated eIF2α prevents formation of ribosomal initiation complexes leading to global mRNA translational attenuation and reduced ER workload. Additionally, eIF2α phosphorylation permits ATF4 activation, a transcription factor that regulates ER stress-mediated apoptosis such as CHOP. Figure taken from [210]

Abbreviations: IRE1α, inositol-requiring enzyme a; PERK, Protein kinase R-like endoplasmic reticulum kinase; ATF, activating transcription factor; XBP-1, X-box binding protein 1; RIDD, IRE1α-dependent decay; ASK, Apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; BCL2, B-cell lymphoma 2; miRNA, microRNA; EIF2α, eukaryotic translation initiation factor 2 alpha; CHOP, C/EBP homologous protein.
Figure 1.5 Crosstalk between PRR pathways and ER stress

STING activation drives the release of Ca\(^{2+}\) from the ER in monocytes, macrophages, dendritic cells and T cells resulting in ER stress. Elevated cytosolic Ca\(^{2+}\) drives Gasdermin D cleavage by caspase 1,11 or 8 in monocytes and macrophages. This may function to restrain STING signalling by depleting intracellular K\(^{+}\). In addition, in macrophages, STING-induced ER stress promotes mTOR inactivation and subsequent removal of stressed ER membranes via autophagy (ER-phagy). The removal of damaged ER organelles promotes cell survival and allows prolonged IFN signalling. ER-phagy may additionally promote IFN signalling by facilitating STING translocation to autophagosomes-derived sites conductive to IFN signalling. ER stress activates the UPR regulator IRE1\(\alpha\) leading to slicing of XBP-1. XBP-1s can bind an enhancer element upstream of the \(ifnb\) transcription site. In addition, activated IRE1\(\alpha\) can bind the adaptor protein TRAF2, which can associate with RIP2 and NOD1/2 or ASC to activate NF-\(\kappa\)B or JNK pathways respectively. IRE1\(\alpha\) induces ROS-dependent NLRP3 translocation to mitochondria. NLRP3 stimulates the caspase-2-Bid mitochondrial damage pathway,
leading to release of mitochondrial danger signals that activate the inflammasome. (Blue: ER promotes/initiates PRR signalling. Red ER limits PRR signalling). (Dotted line-Unproven theories)

Abbreviations mTOR, mammalian target of rapamycin 1 IRE1α; inositol-requiring enzyme 1a; XBP-1, X-box binding protein 1; TRAF2, TNF receptor associated factor 2; RIP2, Receptor Interacting Protein 2; NOD, nucleotide oligomerization domain; ASC, apoptosis-associated speck-like protein containing CARD; Bid, BH3 Interacting Domain Death Agonist; JNK, c-Jun N-terminal kinase.
Mitochondria are dynamic double membrane organelles with complex architecture and high degrees of compartmentalisation which are vital for their wide range of functions. The outer mitochondrial membrane (OMM) contains multiple proteins such as voltage-dependent anion channels, that facilitate the diffusion of small molecules across the OMM, MAVS, involved in virus recognition, regulators of mitochondrial dynamics such as mitofusin (Mfn)1 and 2 and regulators of apoptosis such as B-cell lymphoma 2 (BCL-2). The inner mitochondrial membrane (IMM) is a highly folded, impermeable structure that functions as the site of the electron transport chain (ETC). Indeed, transport across the IMM requires the use of specific transporters. The mitochondrial matrix contains enzymes, its own 16 kB circular genome that encodes 37 mitochondrial genes (13 proteins of ETC machinery, 2 ribosomal (r)RNAs and 22 transfer (t)RNAs required for translation), as well as nucleotides for mtDNA replication [65]. ETC complexes I-IV encoded by both mitochondrial and nuclear genomes are embedded in specialised invaginations of the inner membrane, termed cristae, where they form supramolecular complexes that work cooperatively. Transfer of electrons through the supramolecular complexes creates a proton motive force across the inner membrane. This is critical for ATP production via the turbine action of ATP synthase (complex V), organelle biogenesis via protein import and Ca\(^{2+}\) buffering (Figure 1.6) [211]. Beyond their well appreciated role in ATP production via oxidative phosphorylation, mitochondria are gaining increasing recognition for their involvement in innate immune pathways [212].

Mitochondria adopt specific shapes referred to as ‘mitochondrial dynamics’ to regulate proper function, quality, and distribution in response to changing cellular environments. Mitochondrial fission is regulated by the GTPase dynamin-related protein 1 (Drp1) that drives division at specific points along mitochondria. These sites are pre-marked by actin filaments and ER Ca\(^{2+}\) that drive mitochondrial Ca\(^{2+}\) uptake, leading to IMM constriction. IMM constriction precedes OMM division and allows Drp1 to oligomerise into rings encircling the OMM and pinch the mitochondrial apart. Actin mediates increased mitochondrial Ca\(^{2+}\) levels by enhancing ER–mitochondrial interaction, allowing disproportionate amounts of Ca\(^{2+}\) to flow from the ER to the mitochondrion [213]–[215]. Independently, actin stimulates Drp1 oligomerization through direct binding [214]. Finally, OMM separation proceeds via cooperation between Drp1 and dynamin 2 [216]. Of note, Drp1 is a cytosolic protein and recruitment requires the aid of specific adaptors. To date, the mitochondrial fission factor (Mff), mitochondrial fission 1 protein (Fis1), and
Mitochondrial dynamics protein of 49 and 51 kDa (Mid49-51) have been described to regulate Drp1 recruitment, with partially overlapping functions [211]. Mitochondrial fusion is a two-step process whereby OMM fusion by Mfn1/2 is followed by IMM fusion by optic atrophy 1 (Opa1). Precisely how Mfns mediate OMM fusion is unclear, but several reports suggest a mechanism whereby Mfn1 and Mfn2 interact with their C-termini in trans (that is, between adjacent mitochondria), thus promoting tethering and subsequent fusion of the OMM [217]. Opa1 is located on the IMM and in the mitochondrial intermembrane space. There are two forms of Opa-1; long Opa-1 (L-Opa1) and short Opa1 (S-Opa1) formed by cleavage with two IMM peptidases. The relative levels of both forms dictate the fusion competence of mitochondria; L-Opa1 promotes fusion while S-Opa1 promotes fission. Opa1 plays additional roles in regulating cristae morphology, mtDNA replication and supercomplex formation [211].

It is clear that mitochondrial dynamics plays critical roles in regulating adaptive T cell functions [reviewed in 87], for instance, fission facilitates repurposing of the ETC away from ATP production and toward ROS production, but virtually no research has focused on the effects of mitochondrial fission and fusion on innate immune cell functions (Figure 1.6). Most evidence for a role of mitochondria in innate immunity stems from studies highlighting mitochondrial processes that generate/stimulate the release of matrix DAMPs and mitochondrial membranes acting as signalling platforms for PRRs.

Mitochondria are a rich source of DAMPs that can activate PRR pathways (Figure 1.7). mtROS is generated in the mitochondrial matrix and lumen as a consequence of ETC activity due to proton leakage of complex I and III, and a lesser extent complex II. ROS production is regulated by a variety of factors such as ETC flux, membrane potential and ETC architecture (Figure 1.6) [219]. Depending on the generated amount, it can either function as a cellular signalling cue or lead to damage of lipids, proteins and DNA. Mitochondrial DNA is a circular loop that contains a substantial number of hypomethylated CpG islands [220]. It can be released from the inner mitochondrial matrix into the cytosol upon mitochondrial outer membrane permeabilization (MOMP). MOMP occurs in response to mitochondrial stress and allows the non-selective release of soluble intermembrane space proteins, such as cytochrome C, from the inner mitochondrial space (IMS). Over time, pores expand, enabling IMM extrusion through the OMM, whereupon the IMM herniates and ruptures allowing the release of mtDNA [221]–[223]. The signals and mechanism required for herniation are unknown.
Egress of mtDNA from the mitochondria can potently activate both TLR9 and STING via cGAS activity [220]. It is unclear how mtDNA is trafficked into the endolysosomal compartment to engage TLR9, although autophagy is the most likely route. In addition, oxidised mtDNA is suggested to directly bind NLRP3 to promote oligomerisation and activation [57]. mtROS is also implicated in NLRP3 activation, with the most convincing evidence stemming from work on the adjuvant imiquimod. Imiquimod blocks the quinone oxidoreductase 2 (NQO2) and complex I of the ETC leading to mitochondrial stress and subsequent NEK7-dependent NLRP3 activation [72]. Signalling via a subset of TLRs (TLR1, TLR2 and TLR4) drives mtROS production in macrophages [224], which in the case of TLR4, drives hypoxia-inducible factor (HIF)-1α-dependent expression of a range of pro-inflammatory gens [225]. TLR4-induced mtROS emanates from complex I and requires succinate oxidation by succinate dehydrogenase (SDH) and increased mitochondrial membrane potential [225]. This process is termed reverse electron transport (RET) and is intrinsically regulated by the LPS enhanced mitochondrial metabolite itaconate. The vaccine adjuvant chitosan is proposed to drive mtROS-dependent MOMP and subsequent release of mtDNA that triggers cGAS activation [71]. MOMP is better known for its role in apoptosis, whereby release of cytochrome c, an essential component of the ETC, binds apoptotic peptidase activating factor 1 (APAF1), forming the apoptosome, which in turn binds to and activates apoptotic caspases. Apoptotic caspases can shut down PRR signalling by cleaving signalling components such as MAVS, cGAS and IRF3, inhibiting protein translation to prevent the secretion of inflammatory cytokines and eliciting rapid cell death and clearance (reviewed in [226]). It is unclear how MOMP elicits both pro- and anti-inflammatory effects or when/how they interplay with each other. For instance, alum has also been shown to stimulate mtROS production and mtDNA release, which have been linked to its ability to activate the NLRP3 inflammasome and promote IL-1β maturation but it fails to stimulate type I IFN production [13].

Once in the cytosol, mtROS can cause spontaneous MAVS oligomerisation on the OMM leading to the induction of type I IFN in the absence of RNA as well as potentiate PolyI:C-induced RLR signalling [227]. Depleting fusion machinery reduces MAVS-driven innate antiviral signalling in a mitochondrial membrane potential-dependent manner. Because only a limited number of MAVS molecules are present on each mitochondrion, it is believed that fusion facilitates MAVS aggregation by supplying new molecules from different mitochondria. Mitochondria additionally appear to serve as signalling platforms for the NLRP3 inflammasome. MAVS associate with NLRP3 to promotes its activation and depletion of Mfn2 reduces NLRP3 activity [228]. However, it must be noted that
recent reports have challenged the scaffolding role of mitochondria in PRR signalling. For RIG-I signalling, activation has been proposed to occur at ER-derived membranes [229] and convincing evidence suggests the dispersed TGN is the scaffold for NLRP3 recruitment, assembly and activation [230].

A lot of work is needed to understand the impact of mitochondrial dynamics on innate immune cell function. For instance, immediately following MOMP, extensive mitochondrial fragmentation occurs at mitochondria–ER contact sites and the function this serves is not yet clear. In addition, future work must delineate the role of MOMP and mitochondrial danger signals in the stimulation of adaptive immunity and circumstances in which MOMP is inflammatory, apoptotic or both. Only then will mitochondrial targeting adjuvants reach their full potential.
Figure 1.6 Generation of ATP and reactive oxygen species from the electron transport chain

The ETC, which is composed of five multi-enzyme complexes and the freely mobile electron transfer carrier's ubiquinone and cytochrome c, exist in the folded inner mitochondrial membrane. The complexes must be assembled into a specifically configured supercomplex to prevent electron leakage and allow optimal ATP production. There are two electron transport pathways in the ETC: Complex I/III/IV, with NADH as the substrate and complex II/III/IV, with succinic acid as the substrate. As electrons pass through complex I, III and IV, protons are translocated from the mitochondrial matrix across the inner membrane into the intermembrane space thus setting up a proton electrochemical gradient across the mitochondrial inner membrane. The flow of protons back across the membrane through ATP synthase (Complex V) drives the synthesis of ATP from ADP and inorganic phosphate. Leakage of electrons from the respiratory chain account for the background levels of ROS observed under normal conditions. Electron leakage can occur at complex I at sites IF and IQ, complex II at site IIF and complex III from site IIIQo. Superoxide formed from complex I and II is released into the matrix. Superoxide from complex III can be released on either side of the membrane. Under certain stress or metabolic conditions, more electrons can exit the respiratory chain to augment ROS generation.

The black arrows indicate substrate reactions. The red arrows indicate electron pathways. The broken red arrows indicate potential sites of superoxide formation. The blue arrows represent the proton pumping across the IMM. Abbreviations: The complexes I-V are marked as I, II, III, IV, V, respectively. Q, ubiquinone; C, cytochrome c; IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane.
Figure 1.7 Role of mitochondria in PRR pathways
TLR4 induced succinate oxidation by SDH triggers complex I-dependent ROS production by RET. RET activates HIF-α gene expression, promoting pro-inflammatory cytokines and limiting anti-inflammatory cytokines. To limit excessive inflammation, TLR signalling boosts aconitate decarboxylation by Irg1 to itaconate, which can inhibit SDH activity. Mitochondrial stress signals, such as Ca^{2+} overload, excessive ROS production or depolarisation can trigger MOMP. MOMP usually commits a cell to die through the release of CytC. CytC binds the APAF1, forming a complex called the apoptosome which in turn binds to and activates caspase 9. Caspase 9 then cleaves and activates caspase 3 and 7 resulting in a halt of protein translation that prevents the secretion of PRR-induced cytokines and the induction of cell death that shuts down PRR pathways. Apoptotic caspases can additionally regulate PRR signalling through the cleavage of inflammatory signalling components. In some instances, MOMP facilitates the activation of PRR pathways. mtDNA release can trigger TLR9 and cGAS-STING activation. Oxidised mtDNA can directly bind and activate the NLRP3 inflammasome, as can NEK7.
upon activation by mtROS. Once mtROS has reached the cytosol it can cause spontaneous MAVS oligomerisation and trigger RIG-I-MAVS signalling. The mitochondria outer membrane functions as a scaffold for MAVS in RIG-I signalling and for the NLRP3 inflammasome. Mitochondrial fission negatively regulates RIG-I and NLRP3 activation by preventing MAVS oligomerisation.

Abbreviations: RET, reverse electron transport; HIF-α, Hypoxia-inducible factor 1α; irg1, Immune-Responsive Gene 1; SDH, Succinate dehydrogenase; MOMP, mitochondrial outer membrane permeabilization; CytC, Cytochrome C; APAF1, apoptotic peptidase activating factor 1; NEK7, NIMA-related kinase 7.
1.5.4. Lysosomes

Lysosomes are membrane bound compartments that function as the terminal hub of multiple trafficking routes that carry cargo destined for degradation. They are defined by their single phospholipid bilayer and acidic internal pH (ranging between 4.5 and 5.5) that is established by the v-ATPase and aided by a counterflux of other ion species such as Cl\(^{-}\), Na\(^{+}\) and K\(^{+}\). The acidic environment is required for the function of roughly 60 resident luminal hydrolases that digest all classes of macromolecules leading to the production of amino acids, monosaccharides, and free fatty acids. Examples of pathways that require the lysosomal degradation system include endocytosis and autophagy. Hence lysosomal processing is essential for the efficient removal of extracellular and intracellular pathogens, damaged organelles and toxic cellular components. Ultimately, the digested contents are actively or passively transported by integral membrane proteins of the lysosomal membrane to the cytoplasm, where they are utilized in biosynthetic reactions or stored in the lysosome until required [231], [232].

In addition, lysosomes play critical roles in the shutdown of PRR pathways and the induction of cell death. Activation of the STING signalling cascade begins with the formation of STING-containing foci within the Golgi compartment and is terminated by movement to recycling endosomes and subsequently endolysosomes for degradation [137]. Indeed, chloroquine and bafilomycin A1 treatment prevent STING degradation and bafilomycin A1 treatment enhances STING-dependent signalling [152], [233]. The exact molecular mechanism underlying STING trafficking from endosomes to lysosomes is not clear. Furthermore, it is unclear whether the autophagic process contributes to STING degradation [137]. Similarly, termination of TLR signalling requires lysosomal degradation. Absence of Vsp33B, a member of the Sec1/Munc18 family of proteins that directly interact with soluble NSF attachment receptors (SNAREs) and mediate fusion with lysosomes, causes an accumulation of activated internalized TLR3, TLR4 and TLR9 receptors in aberrant endosomal compartments resulting in exaggerated pro-inflammatory cytokine responses [234].

In addition to terminating STING signalling, in human myeloid cells, STING trafficking to the lysosomes drives membrane permeabilisation. This triggers a cell death pathway that is compatible with lysosomal cell death (LCD). STING-induced LCD is distinct from canonical IRF3 and NF-\(\kappa\)B STING signalling and instead functions to drive K\(^{+}\) efflux-dependent, secondary NLRP3 inflammasome activation [235]. Of note, MEFs and
murine macrophages solely utilise lysosomes for terminating STING signalling, thus, it appears that cell-type specific properties dictate the involvement of lysosomes in STING signalling.

Several lines of evidence implicate lysosomal membrane permeabilisation (LMB) in the activation of the NLRP3 inflammasome. (1) Crystalline compounds, chemical compounds, nanomaterials and rare earth materials that rupture lysosomal membranes drive NLRP3 activation [236]. (2) The lysosomal hydrolase cathepsin B can bind and activate NLRP3 when released into the cytosol [237]. (3) ROS production and K⁺ efflux are apparent downstream events of lysosomal rupture [238]. In addition, LMB and cathepsin B leakage is involved in various inflammatory cell death pathways. During pyroptosis, cathepsin B release acts as an initiator by promoting the assembly of the NLRP3 inflammasome or pyroptosome [239]. In cells undergoing necroptosis, LMP and cathepsin B release are downstream consequences of MLKL-induced ROS and Ca²⁺ signalling and can directly cause cell death via mitochondrial and plasma membrane permeabilization [236]. In ferroptosis, lysosomes act in autophagy dependent manners to control the degradation of ferritin and cause iron accumulation [240]. Although there is a consensus that free iron is indispensable in the initiation of ferroptosis, the precise role of iron still remains elusive. Altogether it appears that targeting lysosomes may be a valuable approach for enhancing adjuvant-induced immunity, either by sustaining PRR signalling, or preventing cell death that can shut down PRR pathways.
1.5.5. DNA damage response

Cellular DNA is constantly under attack by exogenous and endogenous factors. For instance, exposure to ultraviolet light, ionizing radiation and chemotherapeutic drugs inflict dsDNA breaks as can cell intrinsic factors, such as metabolically derived ROS and aldehydes. The DDR is fundamental to counteracting these threats and maintaining genome stability. Of the various forms of damage inflicted by mutagens, double-strand breaks (DSB) of DNA are the most toxic form. Three related kinases, ATM, ATM- and RAD3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK) control the DDR and orchestrate DSB repair [241]. However, in cases where DNA damage is beyond repair, they trigger the induction of senescence or apoptosis, thus acting as a barrier to tumorigenesis [242].

In addition to regulating genome integrity, the DDR also regulates inflammatory responses. A hallmark of senescence is the senescence-associated secretory phenotype (SASP), characterized by the secretion of various cytokines, chemokines, growth factors and proteases that trigger a pro-inflammatory programme to clear senescent cells by the immune system. IL-6 and IL-8, two key products of the SASP, enforce senescence growth arrest in neighbouring cells and attract immune cells, leading to the elimination of these senescent cells [243], [244]. Several studies have identified cGAS as a regulator of senescence. MEFs from cgas<sup>-/-</sup> mice display reduced SASP gene expression and senescence and undergo faster spontaneous immortalisation compared to WT cells [245].

In addition, compelling evidence has accumulated demonstrating that abrogation of DNA replication and/or repair factors results in the activation of the cGAS-STING DNA sensing pathway. This is distinct from, and complementary to, the role of cGAS in senescence. For example, loss of ATM or excision repair cross-complementing group 1 (ERCC1), a structure-specific endonuclease critical in excision repair, can trigger DNA damage and cytoplasmic DNA accumulation, which in turn activates cGAS-STING and type I IFN responses [246], [247]. Similarly, inhibiting SAMHD1, a dNTP triphosphohydrolase or mutation of bloom syndrome protein (BLM), a DNA helicase acting at the interface between replication and repair required for replication fork progression causes replication failure, cytosolic DNA accumulation and cGAS-STING activation [248],[249]. The clinical relevance of these observations is exemplified by breast cancer patients with loss of function mutations in BRCA1/2- known to cause replication failure and DSB
accumulation. These patients exhibit enhanced cGAS-STING-dependent type I IFN responses and CD4+ and CD8+ T cell tumour infiltration [250]. Functional DDR proteins can also promote anti-tumour immunity; DNA-PK and Ku70 in response to free DNA can drive STING-independent IRF3 and IRF1/7-dependent responses respectively [251], [252]. The DNA damage sensor meiotic recombination 11 homolog 1 (MRE11) recognises endogenous, but not viral cytosolic dsDNA leading to the induction of type I IFNs via STING [253]. Moreover, the DNA damage sensor RAD50 forms a signalling complex with CARD9 in DCs upon accumulation of cytosolic DNA resulting in NF-κB activation and pro-IL-1β generation [254].

How DNA, normally confined to the nuclei can be evicted to the cytoplasm upon nuclear damage remains an open question. Its speculated that DSB and stalled replication forks can directly release DNA “speckles” into the cytoplasm [248], [255], [256]. In addition, it is suggested that physiological breakdown of the nuclear compartment during mitosis or senescence provides the means for cytosolic DNA exposure and the generation of micronuclei- fragile nuclear envelope-like structures that contain fragments of chromosomes. These chromatid fragments arise as a result of dsDNA breaks, mitotic errors, problems in DNA replication and chromosome missegregation. Due to structural defects in the underlying lamina, more than half of micronuclei undergo envelope collapse, enabling cGAS to access DNA and trigger anti-tumour immunity [243], [257], [258].

It is unlikely the detection of self-DNA by DNA sensors is solely prevented through confinement to compartments or organelles. For instance, cGAS constitutively resides in the nucleus [115], [259], [260], cGAS recognises viral DNA in the nucleus [113], IFI16 detects foreign and damaged DNA in the nucleus [261] and the DDR protein ATM can activate STING in response to nuclear DNA damage independently of cGAS [262]. Instead, more recent work would suggest that self-DNA avoids PRR detection by sequestration to structures such as chromatin [113], [114].

There is huge potential in targeting the DDR for medicinal purposes. Indeed, studies have revealed that the efficacy of radiotherapy and chemotherapy, in addition to the induction of tumour-cell death, is dependent on cGAS activation in premalignant and cancer cells that leads to the induction of anti-tumour responses [244], [255], [258], [263], [264]. Such responses may be actionable in prophylactic and intratumoral adjuvant settings and hence require investigation.
Figure 1.8 The role of DNA damage and repair proteins in PRR signalling

DNA damage in the nucleus results in activation of the DDR, for instance via NHEJ or HR. If unsuccessful or damage is too extensive, DNA can accumulate in the cytosol. Genetic deficiencies that compromise DDR functions or degradation and packaging of DNA can additionally lead to the accumulation of cytosolic DNA. Once in the cytosol, DNA can be sensed by cGAS resulting in two functionally independent events, the induction of senescence and activation of canonical cGAS-STING signalling. Both H2AX and DNA-PK are suggested to facilitate cGAS sensing of cytoplasmic DNA. cGAS regulates senescence through the SASP, whereby cells produce cytokines in a cGAS-STING dependent manner. These cytokines, namely IL-8 and IL-6 signal in an autocrine and paracrine manner to enforce senescence. Alternatively, DNA triggers canonical activation of cGAS and STING characterised by potent activation of IRF3 and to a lesser extent NF-κB. cGAMP can also be released to the extracellular space or transferred to neighbouring cells through gap junctions or by incorporation into viral capsids. Nuclear cGAS can inhibit DNA repair by HR in a non-catalytic, STING-independent manner. Multiple proteins of the DDR sense cytosolic DNA (in bold) and trigger immune
pathways. Ku70, DNA-PK and the RAD50-CARD9 complex act as cytosolic DNA sensors for activation of IRF1/7, IRF3 and NF-κB respectively. Alternatively, RAD50 can form a complex with the DNA sensor MRE11 and activate STING and downstream IRF3. MRE11 possesses additional nuclease activity which potentially functions to limit excessive signalling. ATM and PARP-1 detect dsDNA breaks in the nucleus and signal inside out to activate the TRAF6 and p53-IFI16 complex. TRAF6, P53 and IFI16 then migrate to the ER, where IFI16 promotes TRAF6-mediated assembly of K63-linked ubiquitin chains on STING, resulting in the activation of NF-κB and to a lesser extent IRF3.

Abbreviations: DDR, DNA damage response; NHEJ, non-homologous end joining; HR, homologous recombination; H2AX, histone H2A variant; DNA-PK, DNA-dependent protein kinase; SASP, senescence-associated secretory phenotype; RAD50, double strand break repair protein; CARD9, caspase recruitment domain 9; MRE11, meiotic recombination 11 homolog 1; IRF, Interferon regulatory factor; NF-κB, Nuclear factor κB; ATM, ataxia-telangiectasia mutated kinase; PARP-1, Poly ADP-ribose polymerase 1; TRAF6, TNF receptor associated factor; IFI16, Interferon γ -inducible protein 16.

The above cellular organelles and responses play a central role in maintaining cell homeostasis and it is becoming clear that they can dramatically alter innate signalling, in some cases antigen presentation and thus, adaptive responses. There is no doubt that understanding the intertwining connections between constitutive processes and PRR pathways will improve our understanding of how certain adjuvants work as well as our ability to optimise them to target specific PRR pathways.
1.5.6. PRR-induced cytokine responses shape T cell immunity

As mentioned above, DCs direct the adaptive immune system by providing a cytokine milieu in response to PRR stimulation that shapes T cell activation and differentiation. The following section focuses on how cytokines shape T cell differentiation, with emphasis on cytokines that promote Th1 and CD8+ T cell responses given the need for adjuvants that can elicit these responses.

T cell activation mediated by APCs, typically DCs, requires three signals; Signal 1 is delivered through the engagement of a TCR to an appropriate antigen-MHC complex on the surface of the APC membrane. Signal 2 and 3 require PRR stimulation and involve the upregulation of co-stimulatory molecules on the cell surface and the secretion of cytokines that will dictate T cell polarisation. Co-stimulation is essential for the induction of T cell responses, as in its absence T cells become anergic (unresponsive) and undergo apoptosis. Activated T cells produce two major types of effector cells; Helper (Th) and cytotoxic T lymphocytes (CTL) derived from CD4+ and CD8+ T cells respectively. CD4+ T cells recognise peptides derived from extracellular pathogens/antigens in complex with MHCcII on APCs. In contrast, CD8+ T cells recognise endogenously-derived peptide–MHC class I complexes on nucleated cells [265].

1.5.6.1. Dendritic cell-derived cytokines that drive differentiation and expansion of CD4+ Th cell subtypes

Naïve CD4+ T cell differentiation occurs in secondary lymphoid organs. Th1 cells are induced in response to viral, intracellular bacteria and protozoan infections. Functionally active IL-12p70 is the key Th1 differentiation factor and constitutes a heterodimer of IL-12p40 and IL-12p35. The transcription of p35 and p40 occur independently of one another, with p40 induced by NF-κB activation and p35 driven by type I IFNs and IFN-γ [266]. As outlined in section 1.4, several PRRs promote the endogenous production of type I IFNs in DCs. In contrast, the source of IFN-γ can be manifest and includes existing effector Th1 cells and activated innate lymphoid cells (ILCs), such as NK cells [267]. Signal transducer and activator of transcription (STAT)4 activation downstream of IL-12p70 is critical for Th1 differentiation, expression of the master transcription factor T-bet and production of IFN-γ. IFN-γ produced by Th1 cells has stimulatory effects on
many innate cells, including DCs, macrophages, neutrophils and NK cells and preforms a critical role in optimal CTL activation (Figure 1.11) [267], [268]. In addition to IL-12, Other cytokines have been implicated in Th1 cell polarisation. IL-18 and more recently IL-27 have also been shown to enhance the differentiation or activation of Th1 cells [269], [270]. Although the role of IL-27 in Th1 differentiation is controversial given evidence it promotes IL-10 production [271].

Th2 differentiation is driven by IL-4 and subsequent STAT6 activation. GATA-3 is induced during Th2 cell differentiation and promotes the secretion of IL-5, IL-10, IL-13 and importantly IL-4, revealing an autocrine regulation similar to IFN-γ in Th1 cells. This cytokine signature promotes IgG1 and IgE antibody class switching, eosinophil recruitment, mucus production and mast cell degranulation and is important for protection against extracellular parasites such as helminths and nematodes [267], [268]. Of note, IL-4-induced GATA-3 expression strongly suppresses Th1-associated gene expression and vice versa for T-bet with Th2-associated gene expression during Th1 cell differentiation. In fact, T-bet can directly bind GATA3 to block its transcriptional activity. Interestingly, loss of GATA3 permits Th1 cell differentiation independently of IL-12 and IFN-γ, suggesting that it primarily functions to regulate Th1 vs Th2 cell differentiation [272], [273].

T follicular helper (Tfh) cells are induced by IL-21 and IL-6 driven STAT3 activation. The transcriptional regulator of Tfh cells is BCL-6, an attribute that is shared with germinal center (GC) B cells. BCL-6 induced CXCR5 expression enriches Tfh cells at the edges of B cell zones, follicular regions and GCs, where they specifically interact with cognate antigen-specific B cells and secrete cytokines such as IL-10 and IL-21 that promote the generation of high affinity, class-switched antibodies [268], [274].

Unlike other effector CD4+ T-cell subsets, Tregs can be generated directly within the thymus (tTregs) during thymocyte development. They are characterised by expression of Forkhead box protein P3 (FOXP3) and secrete high levels of IL-10 and membrane bound TGF-β. In addition, Tregs can be induced peripherally (pTregs) from naïve CD4+ T cells under the influence of TGF-β, IL-2 and retinoic acid (RA). Similar to tTregs they are regulated transcriptionally by FOXP3 and function to maintain self-tolerance and limit infection induced immunopathology through an array of mechanisms. These include secretion of inhibitory cytokines, metabolic disruption through depletion of IL-2 and ATP and the upregulation of regulatory molecules such as Lymphocyte-activation gene (LAG)3 and cytotoxic T-lymphocyte-associated protein (CTLA)4 that suppress DC
functions (Figure 1.10). Other Treg subsets are the IL-10 producing Tr1 Tregs and TGF-β producing Th3 Tregs. They are distinct from other Tregs in that differentiation depends on IL-10 and they lack expression of FOXP3 [267], [268].

Th17 cell differentiation is more complicated than Th1 and Th2 subsets and involves a wide range of cytokines such as IL-1β, IL-6, IL-21, IL-23 and TGF-β. STAT3 signalling downstream of IL-6, IL-21 and IL-23 promotes the transcription of ROR-γT and subsequent secretion of IL-17A, IL-17F, IL-25 (IL-17E), IL-21 and IL-22. IL-17 induces potent inflammatory responses, by promoting inflammatory cytokine and chemokine production as well as the recruitment of other immune cells including granulocytes to the site of infection/inflammation. As a consequence of their powerful pro-inflammatory capabilities, Th17 responses are strongly associated with autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, psoriasis and inflammatory bowel disease [267], [268]. Of importance, different Th17-promoting cytokines can induce unique types of TH17 cells: TGF-β promotes an IL-10-producing and less inflammatory subtype, while IL-1β promotes a more pro-inflammatory subtype of Th17 cell [275].

Additional “unconventional” T helper subsets have also been defined. Th9 cells, as the name suggests are IL-9 producers that in 2008 were characterised as a distinct subset from Th2 cells. They additionally secrete the cytokine IL-21 and are involved in the promotion of tissue inflammation and anti-cancer effects [276]. Similarly, Th22 cells exclusively produce IL-22, distinguishing them from Th17 cells. These cells contribute to mucosal defence and in the pathogenesis of a range of inflammatory skin disorders [277].
Figure 1.9 Pathogens and adjuvants direct the induction of Th subsets via distinct effects on innate immune cells.

(a) Th1 cells are induced in response to pathogens and adjuvants that trigger DC maturation and secretion of IL-12 and IL-18. Autocrine type I IFN signalling or NK-derived IFN-γ is required for IL-12 production. IFN-γ has stimulatory effects on many innate cells such as macrophages, neutrophils (not shown) and NK cells and are critical for optimal CTL activation.  
(b) Th2 cells differentiate in response to helminths and licensed vaccine adjuvants alum and MF49. Differentiation normally requires IL-4 produced by DCs and/or basophils. Although in certain cases, Th2 cell induction can be independent of IL-4. Th2 effector cells secrete IL-4 that promotes Th2 differentiation in an autocrine manner and antibody isotype class-switching in B cells to IgG1 and IgE production. Other effector cytokines include IL-13 that drives smooth muscle contraction and mucous production and IL-5 that promotes eosinophil recruitment (not shown).  
(c) Th17 cell differentiation is more complicated than Th1 and Th2 subsets and involves a wide range of cytokines. γδ T cells play a role analogous to NK cells in driving Th1 responses, producing IL-17 and IL-21 in response to DC-derived IL-1 and IL-23. Effector Th17 cells produce IL-17a, a key cytokine that links T cell activation to neutrophil recruitment and activation and TNF-α, a well-established pro-inflammatory cytokine. Much less is known of IL-22 and IL-17F.

Abbreviations; CTL, Cytotoxic T lymphocyte; IC, intracellular; EC, extracellular. Note diagram did not depict the differentiation of Treg cells, Tfh cells, Th22 or Th9 cells.
Figure 1.10 Tregs suppress effector T cells through a variety of mechanisms

(a) IL-10 suppresses Th1 differentiation and inhibits APC functions, TGF-β is a potent anti-proliferative cytokine and IL-35 inhibits T cell functions. (b) CD8 T cells can directly kill effector cells in granzyme-perforin-dependent manner. (c) Tregs can deplete the microenvironment of IL-2 through constitutive expression of CD25, thereby limiting the ability of effector T cells to survive. Concordant expression of CD39 and CD73 ectonucleotidases results in depletion of ADP/ATP cytosolic levels, subsequent pericellular adenosine production and suppressed effector T cell function through activation of the A2AR. (d) Treg cells can modulate the maturation and/or function of DCs required for the activation of effector T cells. Direct interaction of CTLA4 with DCs attenuates the activation of effector T cells and conditions DCs to express IDO, a potent regulatory molecule. The CD4 homologue, LAG3 binds MHCcII with very high affinity and blocks DC maturation through an ERK- SHP1 dependent pathway. Figure taken from [278].

Abbreviations: A2AR, adenosine receptor 2A; cAMP, cyclic AMP; LAG3, Lymphocyte-activation gene; IDO, indoleamine 2,3-dioxygenase; CLTA, cytotoxic T-lymphocyte-associated protein; ERK, Extracellular Receptor Kinase; SHP1, Src Homology Phosphatase 1
1.5.6.2. CD8$^+$ T cells

Priming of CD8$^+$ T cell responses takes place in the lymph nodes and spleen and requires a recently recognized second T cell-priming step, in which a CD4$^+$ Th1 cell and a CD8$^+$ T cell both recognize their respective antigens on the same DC (Figure 1.11). For this to occur, exogenous antigens must bind MHC class I peptides on DCs, a process termed antigen cross presentation. DC-derived type I IFNs are critical for antigen cross presentation and consequently crucial for CD8$^+$ T cell priming and activation [279], [280]. Upon antigen-specific interaction with a DC, CD4$^+$ T cells upregulate expression of CD40L, which then binds to CD40 on the DC. CD40-CD40L binding relays a signal back to the DC to increase its antigen presentation ability and enhance the production of IL-12, IL-15 and/or type I IFNs and expression of CD80, CD86 and CD70 [281]–[283]. IL-12, IL-15 and type I IFNs promote the activation of the CTL master transcriptional regulators T-Bet and Eomesodermin (Eomes) that promote differentiation as well as cytolytic effector functions and survival [284]. Thus, it appears these cytokines have a key yet partially redundant role in the quantity and quality of the CTL response. IFN-γ also supports the differentiation of effector CTLs, although it is likely to be produced by NK cells and Th1 cells, not DCs, during T cell priming. In contrast to the redundancy of IL-12, IL-15 and type I IFNs in CTL differentiation, ligation of both CD28 and CD27 on DCs with CD80 or CD86 and CD70 on CD8$^+$ T cells respectively are needed for optimal CTL responses [282]. Indeed, blocking both CD80/CD86 and CD70 ligation abrogates the capacity of a CD40-activated DC to prime a CTL response. It has been suggested that CD70-CD27 binding promotes clonal expansion of primed CD8$^+$ T cells and their optimal differentiation into effector CTL by increasing the secretion of IL-2 and the expression of IL-12Rβ2 and IL-2Rα on activated CD8$^+$ T cells, whereas CD28 co-stimulation promotes clonal expansion by amplifying TCR signalling, promoting cell cycle progression, counteracting apoptosis and altering cell metabolism to favour rapid proliferation [285]–[287].

Shortly after recognition of antigen on DCs and priming by antigen-specific CD4$^+$ T cells in the central lymphoid organs, CD8$^+$ T cells undergo rapid proliferation and upregulate the expression of CXCR3, allowing them to migrate to peripheral tissues. In the periphery, CD8$^+$ T lymphocytes (CTLs) act as assassins, surveying for signs of viral infection or tumour transformation. Upon TCR mediated recognition of peptide-MHC complexes on nucleated cells, CTLs form an immunological synapse and secrete perforin and granzyme to form large transmembrane pores and initiate apoptosis in the target cell respectively. CTL can then detach from dying cells and interact with another
target endowing them with the ability to perform serial killing. Alternatively, CTLs can induce apoptosis through a Fas-FasL intracellular linkage-mediated pathway or through crosslinking of the TNF and TNFR1 type I. Moreover, activated CTLs secrete various cytokines such as IFN-γ to enhance peripheral antigen presentation and mediate antipathogenic effects [288].

Following antigen clearance, a contraction phase ensues whereby the majority of antigen-specific CD4⁺ and CD8⁺ T cells rapidly die via apoptosis, while a small proportion persist and transition into long-lived memory cells (Figure 1.12). This memory population is critical to a rapid and robust secondary response upon reinfection with the same pathogen. The extent of T cell death is thought to determine the magnitude of T cell memory and be regulated by the IL-2 cytokine, molecules of the TNFR family (Fas and TNFα), TCR signal strength, and effector molecules such as perforin and IFN-γ. If this is the case the number of memory T cells formed is determined primarily by the initial T cell burst size. Thus, it is imperative that vaccines induce as large an effector T cell population as possible to generate sufficient numbers of memory T cells that will confer protection upon future encounter with antigens [289]–[291].

Memory T cells can be divided into central memory (Tcm) cells, which circulate between the blood and secondary lymphoid organs, effector memory (Tem) cells, which can migrate from the blood into non-lymphoid organs and resident memory (Tm) cells, that are seeded in non-lymphoid tissues and serve as sentinels at sites of potential reinfection [289]. Thus, Tm cells are a crucial memory subset in the context of vaccination. The majority of studies on Tm cells have focused on CD8⁺, as little is known of molecular mechanisms underlying the generation of memory CD4⁺ T-cells. This is largely due to CD4⁺ T cells being inherently less proliferative and the rapid decline in CD4⁺ memory T cell populations post infection. Furthermore, the extensive number of functionally distinct Th subsets limits the ability to characterise a common CD4⁺ T cell memory precursor [290]. A better understanding of the molecular mechanisms mediating CD4⁺ T cell memory is required to harness the full protective capacity of this population in vaccination settings.
Figure 1.11 Optimal CD8 CTL activation requires CD4\(^+\) T cell help.
CD40L expressing CD4\(^+\) T cells interact with cognate lymph node-resident dendritic cells through the CD40–CD40L pathway. This interaction leads to licensing of the DC, characterised by increased antigen presentation, expression of co-stimulatory molecules and cytokines. DC-derived type I interferons, IL-12 and IL-15 directly support CD8\(^+\) T cells differentiation into effector CTLs. CD70-CD27 signalling drives differentiation, survival and metabolism in CTLs, directly and indirectly via DC-derived IL-12. CD4\(^+\) T cell-derived IL-2 and IL-21 can also support the CTL response. Figure taken from [284].
Abbreviations: CTL, cytotoxic T lymphocytes; TCR, T cell receptor.
The T-cell immune response consists of three primary stages, expansion, contraction and memory. Expansion begins with the proliferation of antigen-specific T cells in the presence of antigen. CD8$^+$ T cells proliferate to a greater extent than CD4$^+$ counterparts. Following antigen clearance, the contraction phase ensues and the number of antigen-specific T cells decreases by apoptosis. After the contraction phase, the number of antigen-specific T cells stabilises, forming memory pools that can be maintained for great lengths of time. Note that, typically, contraction of the CD4$^+$ T-cell response is more pronounced and less stable than that for CD8$^+$ T cells. Figure taken from [289].
1.6. Chitosan:

Chitosan also known as β (1–4)-linked 2-acetamido-2-deoxy-β-d-glucose (N-acetyl glucosamine), is a cationic polysaccharide investigated as a potential adjuvant in human vaccines due to its biocompatible and biodegradable nature, mucoadhesive properties [292], ability to elicit potent Th1 mediated cellular responses [71] and excellent safety profile [293], [294]. It is derived via the chemical deacetylation of chitin, one of the most abundant polymers in nature, second only to cellulose, which is found naturally in the cell wall of fungi, exoskeletons of crustacea and insects [295]. The history of chitosan dates back to 1811, when Professor Henri Braconnot successfully isolated the parent compound chitin from mushrooms. Since then, the exploration of chitosan has taken on many forms; it is extensively studied for a number of biomedical and pharmaceutical applications, including prolonged or controlled release drug delivery systems [296], wound dressings [297], anti-microbial agents [298], blood anticoagulants [299], cartilage and bone tissue engineering scaffolds [300], [301], space filling implants [302] and weight loss supplements [303].

For chitin to be considered chitosan, more than 50 % of the acetyl groups should be removed and this is achieved through its treatment under alkaline conditions or through the use of chitin deacetylases which catalyse the conversion of the N-acetyl groups of the glucosamine residues into amine groups [295]. The percentage of glucosamine units displayed on chitosan is termed its degree of deacetylation (DD), and high DD are responsible for the polymer's cationic properties. The pKa of the amine groups on the glucosamine units is approximately 6.5, meaning that the majority of these will be protonated under biological conditions, giving the chitosan polymer a net positive charge and subsequently mucoadhesive processes. These protonated amine groups interact with cellular membranes, causing structural reorganization of tight junctions and subsequently their opening. Such a feature suggests significant potential as a mucosal adjuvant. Indeed chitosan and its various derivatives have been investigated as mucosal delivery systems and have enhanced the immunogenicity of various antigens following intranasal and oral vaccination [292]. Furthermore, an intranasal Norwalk virus-like particle (VLP) vaccine adjuvanted with chitosan and a TLR4 agonist, MPL was well tolerated and promoted strong humoral immune responses [304].

The Lavelle lab demonstrated the potential of chitosan to serve as adjuvant capable of driving potent Th1 immune responses [11]. Since this discovery, many studies have
sought to investigate the polymers mode of action. It has have shown that chitosan promotes NLRP3 inflammasome activation, mediated by mechanisms including K⁺ efflux and ROS production that accompany NLRP3 activation by all canonical activators [305]. Furthermore, it has been demonstrated that chitosan can activate macrophages, and induce the secretion of cytokines from NK cells [305], [306]. More recently, Carroll et al., demonstrated the in vivo role of the NLRP3 inflammasome in chitosan’s adjuvanticity. Additionally, the study highlighted the role of DCs and type I IFN-dependent maturation in chitosan-mediated immune responses. The induction of type I IFNs was shown to be dependent on signalling through cGAS and STING as well as the induction of mitochondrial reactive oxygen species. Tying this mechanism to adjuvant function, immunization of STING or cGas knock out mice with the candidate TB vaccine antigen, H1 and chitosan dramatically reduced levels of antigen-specific IFN-γ producing Th1 cells and IgG2c antibodies. Comparable results were obtained in a different experimental model employing mucosal immunization with pneumococcal surface protein A [71].

Important questions left unaddressed concern the specific properties of chitosan which lead to the production of mtROS, whether chitosan polymers disrupt other mitochondrial processes, how the polymers mechanistically elicit mitochondrial stress and how mitochondrial stress activates cGAS-STING signaling. Additionally, it is not yet clear how chitosan activates the NLRP3 inflammasome, how NLRP3 activation contributes to cGAS-STING activation or whether NLRP3 inflammasome activation with chitosan polymers is mtROS dependent. Mounting evidence highlights the fundamental role of cGAS–cGAMP–STING pathway in three major cancer therapies, radiation therapy, chemotherapy, and immunotherapy. Given the pivotal role of the cGAS-STING sensing pathway in chitosan’s mode of action and the lack of translational cGAS-STING adjuvants, chitosan presents as a potential therapeutic adjuvant. With these factors in mind, this research aims to accomplish four main objectives:

1. Identify how chitosan triggers mitochondrial stress, if it affects other aspects of mitochondrial biology and establish how they facilitate cGAS-STING activation.
2. Identify the contribution of NLRP3 inflammasome activation to cGAS-STING activation and determine if mtROS is a shared requirement for chitosan-induced NLRP3 activation and cGAS-STING signalling
3. Identify the physicochemical polymer properties responsible for chitosan’s immunostimulatory effects
4. Compare the effects of conventional STING agonists with those of chitosan and determine the potential of chitosan as an anti-cancer vaccine adjuvant
2. Materials and Methods

2.1. Mice

C57BL/6 mice were obtained from Harlan Olac (Bicester, UK) or were bred in-house by the Comparative Medicine Unit (TCD), and were used at age 8-15 weeks. *Ifnar*<sup>-/-</sup> mice were kindly donated by Paul Hertzog (Centre for Innate Immunity and Infectious Diseases MIMR-PHI and Monash University, Clayton, Victoria, Australia) and were bred in-house by the Comparative Medicine Unit (TCD). *Sting*<sup>-/-</sup> mice were generously provided by Dr. Lei Jin (Centre for Immunology and Microbial Disease, Albany Medical Centre, Albany, New York, USA) and were bred in-house at TCD. *Nlrp3*<sup>-/-</sup> mice were provided by the late Prof. Jurg Tscopp (Department of Biochemistry, University of Lausanne, Switzerland) and bred in-house by the Comparative Medicine Unit (TCD). Animals were maintained according to the regulations of the EU and the Health Products Regulatory Authority (HPRA). All procedures were conducted under animal license number AE19136/P079 and were approved by TCD Animal Research Ethics Committee (Ethical Approval Number 091210).
2.2. Materials

2.2.1. In Vivo Reagents

- **Needles**

  Subcutaneous & Intratumoral injections: Tuberculin syringe 27 G½” (Becton Dickinson and Company #3055620)

  Intraperitoneal injections: Injekt-F Tuberculin 0.01-1mL (#9166017V) attached to 27G ¾” needle (BD Microlance 3 #302200)

- PBS (Gibco)

- Tuberculosis antigen, Hybrid 56 (H56) (Statens Serum Institute)

- *Chlamydia trachomatis* major outer membrane protein (MOMP) (Statens Serum Institute)

- CpG ODN 1826 (Oligos etc)

- Cisplatin (Merck-Sigma)

- Chitosan panel (Table )

2.2.2. General cell culture materials

- **Complete (c)RPMI 1640 medium**: Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera) was supplemented with 2 mM L-Glutamine (Gibco), 50 units/mL penicillin (Gibco), 50 µg/mL streptomycin (Gibco) and heat inactivated foetal calf serum (FCS) (Biosera).

- **Complete (c)DMEM culture medium**: Dulbecco’s Modified Eagle Medium (DMEM) (Biosera) was supplemented with 2mM L-Glutamine, 50 units/mL penicillin, 50 µg/mL streptomycin and heat inactivated FCS.

- **Ca²⁺ free DMEM**: DMEM, high glucose, no glutamine, no Ca²⁺ (Bio-Sciences Ltd) was supplemented with 2 mM L- Glutamine, 50 units/mL penicillin & 50 µg/mL streptomycin. (NO FCS)

- **0.88 % NH₄Cl red blood cell lysis solution**: 0.88g NH₄Cl was dissolved in 100ml of endotoxin-free water (H₂O) (Baxter) and filter sterilised with a 0.22 μm syringe-driven filter (Millipore).

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

- **Macrophage-colony stimulating factor (G-CSF)** was obtained from L929 cells transfected with the murine gene for M-CSF.
● Sterile and Endotoxin-free **Phosphate Buffered Saline (PBS)** was purchased from Gibco.

● **Trypsin-EDTA** was purchased from Sigma.

● **Puromycin dihydrochloride from Streptomyces alboniger** was purchased from Sigma.

● **Trypan Blue** was purchased from Sigma.

● Molecular grade **Ethidium Bromide (E1510)** was purchased from Sigma.

### 2.2.3. Cell culture treatments

**Table 7 Adjuvants used in vitro**

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Estimated Molecular weight</th>
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<td>269</td>
<td>86</td>
<td>Novamatrix, Norway</td>
</tr>
<tr>
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<td>100</td>
<td>38</td>
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<td>72</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C83 Heterogenous</td>
<td>&lt;350</td>
<td>83</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C83 homogenous</td>
<td>&lt;350</td>
<td>83</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C90</td>
<td>&lt;350</td>
<td>90</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C100</td>
<td>115</td>
<td>100</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C100</td>
<td>140</td>
<td>100</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C100</td>
<td>250</td>
<td>100</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>C100</td>
<td>350</td>
<td>100</td>
<td>Viscogel AB</td>
</tr>
<tr>
<td>Alum</td>
<td>N/A</td>
<td>N/A</td>
<td>Breentag Biosector</td>
</tr>
</tbody>
</table>

Molecular weights are estimations based on relative viscosity (Brookfield 1% HoAc, 20°C). Estimations are accurate for molecular weights up to 350 kDa.
**Table 8 Inhibitors used in vitro**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Concentration(s) tested specific to targets *</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAPTA-AM</td>
<td>Cell permeant Ca(^{2+}) chelator</td>
<td>1-10 μM Optimal- 6 μM</td>
<td>Biosciences</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>Sarco/Endoplasmic Reticulum Ca(^{2+})-ATPase (SERCA) channels on ER</td>
<td>100 nm-2 μM Optimal 1 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>U73122</td>
<td>Phospholipase C</td>
<td>0.5-15 μM Optimal: 10 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>2-ABP</td>
<td>Inositol triphosphate receptors (InsP(_3)R) on ER</td>
<td>50- 300 μM Optimal:150 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>N-linked glycosylation</td>
<td>0.1-10 μg/mL Optimal: 4 μg/mL</td>
<td>VWR</td>
</tr>
<tr>
<td>Bafilomycin A1</td>
<td>Vacuolar H(^+) ATPase + SERCA</td>
<td>10-400 nM Optimal: 40-100 μM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>Free actin monomers</td>
<td>2 μM</td>
<td>Merck Chemicals</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Electron transport chain complex I</td>
<td>0.5 μM (ELISA) or 5 μM (MitoSOX)</td>
<td>Sigma</td>
</tr>
<tr>
<td>MitoTEMPO</td>
<td>Mitochondrial ROS scavenger</td>
<td>50-500 μM Optimal 500 μM</td>
<td>Thermo</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Translation Elongation</td>
<td>2 μg/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase</td>
<td>50 μM (PCR &amp; ELISA) 100 μM (DNA damage)</td>
<td>Merck</td>
</tr>
<tr>
<td>S3QEL3</td>
<td>Electron transport chain complex III</td>
<td>5-20 μg/mL Optimal 5 μg/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>mTOR</td>
<td>10 μM</td>
<td>Fisher Scientific</td>
</tr>
</tbody>
</table>

*Optimal concentration based on maximal effect and minimal toxicity in BMDCs or BMDMs.
Table 9 Agonists used in vitro

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Receptor/Target</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (LPS from <em>Escherichia coli</em> (E.coli) LPS, Serotype R515)</td>
<td>TLR4</td>
<td>Enzo life Sciences</td>
</tr>
<tr>
<td>CpG ODN 1826</td>
<td>TLR9</td>
<td>Oligos etc.</td>
</tr>
<tr>
<td>Zymosan</td>
<td>TLR2, TLR4 &amp; Dectin-1</td>
<td>Invivogen</td>
</tr>
<tr>
<td>5,6-Dimethylxanthone-4-acetic Acid (DMXAA)</td>
<td>Murine STING</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mammalian (non-canonical) CDN, cyclic [G(2',5')pA(3',5')p]</td>
<td>STING</td>
<td>Sigma</td>
</tr>
<tr>
<td>FCCP</td>
<td>Weak lipophilic acid- Used to depolarise mitochondrial membrane potential</td>
<td>BioScience LifeSciences</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>NADPH oxidase</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.2.4. Confocal microscopy materials.

- **CELLVIEW cell culture dish** with 35 mm Glass bottom and 4 compartments (627870), were purchased from Cruinn.
- **GFP-LC3 iBMM**
- Immortalised bone marrow macrophages expressing GFP-LC3 were a kind gift from Hardy Kornfeld, University of Massachusetts.

Table 10 Confocal Dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Target</th>
<th>Supplier</th>
<th>Working concentration</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotracker Green</td>
<td>All Mitochondria</td>
<td>BioSciences</td>
<td>50 nM</td>
<td>PBS</td>
</tr>
<tr>
<td>Tetramethylrhodamine, methyl ester, perchlorate (TMRM)</td>
<td>Polarised mitochondria</td>
<td>BioSciences</td>
<td>20 nM</td>
<td>cRPMI</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>DNA</td>
<td>Thermo Fisher</td>
<td>5 µg/mL</td>
<td>cRPMI</td>
</tr>
</tbody>
</table>
2.2.5. Fluorescence-activated cell sorting (FACS)/ flow cytometry materials

- **OneComp eBeads** (eBioscience) were used in single stain sample preparations.
- Cells were collected and stained in **Falcon® Round-Bottom Tubes**, Disposable, Polystyrene, Corning® (VWR 60819-138)
- **Fluorescent chitosan** - 35 mL of Anhydrous methanol (CH$_3$OH) containing 25 mg of FITC was mixed with 25 mL of a 1% chitosan in 0.1M acetic acid (C$_2$H$_4$O$_2$) solution for 3 hours at RT in darkness. FITC-labelled chitosan was then precipitated with 0.2M sodium hydroxide (NaOH) and centrifuged at 4500 g for 30 minutes at RT. The supernatant was discarded and the resultant pellet was washed with 70% CH$_3$OH in water. The wash step was repeated twice and the pellet resuspended in 15 mL of 0.1M C$_2$H$_4$O$_2$ solution and stirred overnight. The polymer solution was dialyzed in 2.5L of distilled H2O for 3 days in the dark before freeze-drying.

### Table 11 FACS Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACs buffer</td>
<td>1 x PBS was supplemented with 2 % heat-inactivated filter sterilised FCS</td>
</tr>
<tr>
<td>10 nM EDTA</td>
<td>0.5 mL 0.5 M EDTA in 49.5 mL PBS</td>
</tr>
<tr>
<td>1 &amp; 2 % Paraformaldehyde (PFA)</td>
<td>PFA solution, 4 % in PBS (Santa Cruz Biotechnology) was diluted in PBS</td>
</tr>
</tbody>
</table>

### Table 12 Flow Cytometry Viability Dyes

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV510 cell stain</td>
<td>BD Horizon</td>
<td>1/1000</td>
<td>PBS</td>
</tr>
<tr>
<td>A700</td>
<td>Biolegend</td>
<td>1/1000</td>
<td>PBS</td>
</tr>
<tr>
<td>Zombie Yellow</td>
<td>Biolegend</td>
<td>1/1000</td>
<td>PBS</td>
</tr>
<tr>
<td>Propidium iodide (PI)/ Rnase A staining buffer</td>
<td>Thermo Fisher</td>
<td>Neat</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 13 Flow Cytometry Dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Target</th>
<th>Supplier</th>
<th>[Working]</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellROX Deep Green</td>
<td>Cellular ROS</td>
<td>Biosciences</td>
<td>5 µM</td>
<td>PBS</td>
</tr>
<tr>
<td>MitoSOX</td>
<td>Mitochondrial ROS</td>
<td>BioSciences</td>
<td>1 µM</td>
<td>PBS</td>
</tr>
<tr>
<td>Mitotracker Green</td>
<td>All Mitochondria</td>
<td>BioSciences</td>
<td>100 nM</td>
<td>cRPMI</td>
</tr>
<tr>
<td>Tetramethylrhodamine, methyl ester, perchlorate (TMRM)</td>
<td>Polarised mitochondria</td>
<td>BioSciences</td>
<td>100 nM</td>
<td>cRPMI</td>
</tr>
</tbody>
</table>

### Table 14 Flow Cytometry Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Colour</th>
<th>Supplier</th>
<th>Clone</th>
<th>Volume /Sample (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c</td>
<td>PecY7</td>
<td>BD Pharmingen</td>
<td>HL3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td></td>
<td>HL3</td>
<td>0.1</td>
</tr>
<tr>
<td>CD11b</td>
<td>PeCy7</td>
<td>BD Pharmingen</td>
<td>M1/70</td>
<td>0.15</td>
</tr>
<tr>
<td>F4/80</td>
<td>PerCP5.5</td>
<td>eBiosciences</td>
<td>BM8</td>
<td>0.4</td>
</tr>
<tr>
<td>Gr1</td>
<td>APC-Cy7</td>
<td>BD Pharmingen</td>
<td>RB6-8C5</td>
<td>0.25</td>
</tr>
<tr>
<td>Dectin1</td>
<td>Alexa647</td>
<td>Bio-Rad</td>
<td>2A11</td>
<td>1.5</td>
</tr>
<tr>
<td>CD80</td>
<td>FITC</td>
<td>BD Pharmingen</td>
<td>16-10A1</td>
<td>0.2</td>
</tr>
<tr>
<td>CD86</td>
<td>PE</td>
<td>Invitrogen</td>
<td>GL1</td>
<td>0.1</td>
</tr>
<tr>
<td>CD40</td>
<td>APC</td>
<td>Invitrogen</td>
<td>IC10</td>
<td>0.3</td>
</tr>
<tr>
<td>MHCcII</td>
<td>E450</td>
<td>Invitrogen</td>
<td>M5/114.15.2</td>
<td>0.15</td>
</tr>
<tr>
<td>BrdU</td>
<td>Alexa488</td>
<td>Thermo Fisher</td>
<td>PRB-1</td>
<td>5</td>
</tr>
</tbody>
</table>
2.2.5.1. Reagents for measuring Autophagy by flow cytometry

- **Digitonin** (BioScience LifeSciences) 50 µg/mL Digitonin was used to permeabilise cells and wash away cytosolic LC3 monomers in GFP-LC3 iBMMs.
- **Bafilomycin** (Sigma)
- **BV 510 viability dye** (BD Horizon).

2.2.5.2. Reagents for measuring DNA fragmentation

- **APO-BrdU™ TUNEL Assay Kit** (Thermo Fisher) (Contents: Positive and negative control cells of a fixed human lymphoma cell line. Terminal deoxynucleotidyl transferase (TdT), 5-Bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP), anti-BrdU mAb Alexa Fluor™ 488 conjugate, Propidium iodide (PI)/ Rnase A staining buffer, Reaction buffer, Wash buffer and rinse buffer).
- **A700 viability dye** (Biolegend)
- **Pure ethanol** (Sigma)
- **1 % PFA** (Santa Cruz Biotechnology diluted in PBS) not included in kit.
2.2.6. Enzyme-linked immunosorbent assay (ELISA) materials

General materials

- **IL-6** Kits BD Biosciences (BD 555240).
- **IFN-β** was detected using antibodies from Santa Cruz Biotechnology Inc (Capture antibody #sc-57201) and R&D systems (detection antibody #32401-1).
- **IFN-β Standard** Recombinant Mouse IFN-β Protein (R&D systems, #12410-1).
- **IFN-γ (DY485)** CXCL10 (DY466) and **IL-1β (DY401)** ELISA kits were purchased from R&D systems.
- ELISA substrate was 0-Phenylenediaminedihydrochloride (OPD) (Sigma # P7288).
- **High Binding 96 well plates** (Cruinn #655061).
- Bovine Serum Albumin (BSA) (Fisher).
- **FCS** (Biosera).
- Sodium Chloride (NaCl), Sodium Phosphate Monobasic (Na₂HPO₄), Potassium dihydrogen phosphate (KH₂PO₄), Potassium chloride (KCl), Citric acid (C₆H₈O₇), sodium carbonate (Na₂CO₃), Sodium bicarbonate (NaHCO₃), sulphuric acid (H₂SO₄) and **Tween 20** were purchased from Sigma.

<table>
<thead>
<tr>
<th>Cytokine of Interest</th>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>All R&amp;D</td>
<td>Supplied in Kits</td>
<td>See individual Kit</td>
</tr>
<tr>
<td>All BioLegend</td>
<td>Supplied in Kits</td>
<td>See individual Kit</td>
</tr>
<tr>
<td>IFNβ</td>
<td>Sigma (A0545)</td>
<td>1:2000 of stock</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine of Interest</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>R&amp;D (except IFN-γ)</td>
<td>1 % BSA (w/v) in PBS</td>
</tr>
<tr>
<td>R&amp;D IFN-γ</td>
<td>0.1 % (w/v) BSA and 0.01 % (v/v) Tween in PBS</td>
</tr>
<tr>
<td>IFN-β</td>
<td>10 % (v/v) FBS in PBS</td>
</tr>
<tr>
<td>Biolegend</td>
<td>1% (w/v) BSA in PBS</td>
</tr>
<tr>
<td>Buffer solution</td>
<td>Composition</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>10 x PBS</td>
<td>400g NaCl, 58g Na$_2$HPO$_4$, 10g KH$_2$PO$_4$ and 10g KCl. Made up to a final volume 5L with dH$_2$O and brought to pH 7.2</td>
</tr>
<tr>
<td>1x PBS</td>
<td>10 mL 10x PBS + 90 mL dH$_2$O</td>
</tr>
<tr>
<td>1 % (w/v) BSA in PBS</td>
<td>1g BSA in 100 mL in 1x PBS</td>
</tr>
<tr>
<td>10 % (v/v) FBS in PBS</td>
<td>10 mL of FBS in 90 mL PBS</td>
</tr>
<tr>
<td>0.05% (v/v) Tween-PBS (Wash buffer)</td>
<td>600 mL 10x PBS, 5400 mL dH$_2$O and 3 mL Tween 20.</td>
</tr>
<tr>
<td>Phosphate citrate buffer</td>
<td>10.19g C$_6$H$_8$O$_7$ and 14.6g Na$_2$CO$_3$. Made up to a final volume of 1L with dH$_2$O and brought to pH 5.0</td>
</tr>
<tr>
<td>Sodium Carbonate Buffer</td>
<td>4.2g NaHCO$_3$ and 1.78g of Na$_2$CO$_3$ was dissolved in 1L dH$_2$O and brought to pH 9.5</td>
</tr>
<tr>
<td>Stop solution</td>
<td>1M H$_2$SO$_4$</td>
</tr>
</tbody>
</table>
2.2.7. Real-time PCR materials

RNA Isolation
RNA was isolated using High Pure RNA Isolation Kit (Roche) with accordance to the protocol provided by the supplier. RNA quality was measured by Nanodrop.

cDNA synthesis: Reverse transcription (RT) reagents (Preformed only at RNA bench)
- PCR Tube strip of 8 with Cap Strip (0.2 mL) purchased from Fisherbrand (#14230215)
- Chemical reagents used for reverse transcription are outlined below in Table 12.

Table 18 Reagents used for cDNA synthesis

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>5</td>
<td>- see below**</td>
<td>-</td>
</tr>
<tr>
<td>Random Hexamer</td>
<td>0.5</td>
<td>1 µg/mL</td>
<td>MWG Biotech</td>
</tr>
<tr>
<td>RT 5x Buffer</td>
<td>2</td>
<td>-</td>
<td>Promega</td>
</tr>
<tr>
<td>Deoxyribonucleotide triphosphates (dNTPs)</td>
<td>2</td>
<td>2.5 mM/NTP</td>
<td>Biolabs</td>
</tr>
<tr>
<td>Ribonuclease Inhibitor (RNAse Out)</td>
<td>0.25</td>
<td>40 U/µL</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>-M-MLV Reverse transcriptase</td>
<td>0.25</td>
<td>200 U/ µL</td>
<td>Promega</td>
</tr>
</tbody>
</table>

**RNA sample concentration was determined by Nanodrop. All RNA sample concentrations were adjusted to the same concentration (minimum of 60 ng/µL) using an RNA/H₂O mix.

Table 19 RT PCR settings for Thermocycler

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>42</td>
<td>60 min</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>∞</td>
<td>-</td>
</tr>
</tbody>
</table>
Quantitative PCR (qPCR) Reagents (Preformed only at DNA bench)

- **KAPA SYBR® FAST qPCR Kit** (Kapa Biosystems) containing ROX reference dye was used in combination with nuclease-free water.
- **RNase/DNase Free water** (Elution buffer from High Pure RNA Isolation Kit (Roche) was taken from kits and utilised only for qPCR purposes to avoid RNA contamination)
- **MicroAMP Fast 96-Well Reaction plate** (0.1 mL) purchased from applied biosystems (4346907)
- **Primers** (Biosciences) were directed against mouse genes and designed as intron-spanning to avoid amplification of genomic DNA (Table 20). Dissociation curve analysis was performed after each real-time qPCR to exclude non-specific products. All PCR primer stocks were reconstituted to a concentration of 100 pmol/µL. Stocks were further diluted 1 in 20 in nuclease free water to yield working aliquots (5 pmol/µL).

### Table 20 qPCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (FP) (5'to 3')</th>
<th>Reverse Primer (RP) (5'to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>actb</td>
<td>TCCAGCCTTTCTTGGT</td>
<td>GCCACTGTGTTGGCATAGAGGTC</td>
</tr>
<tr>
<td>rps18</td>
<td>CTTAGAGGGGACAAGTGGCG</td>
<td>GGACATCTAAGGGCATCACA</td>
</tr>
<tr>
<td>ifna</td>
<td>ATGGCTAGGGCTCTGTGCTTTCTT</td>
<td>AGGGCTCTCCAGAGTTCTGCTG</td>
</tr>
<tr>
<td>ifnb</td>
<td>ATGGTGTTGGCCGAGCAGAGAT</td>
<td>CCACGACTCATCCTGAGGGA</td>
</tr>
<tr>
<td>cxcl10</td>
<td>TCTGAGTGGGACTCAAGGGGA</td>
<td>TCGTGCAATGATCTCAACACG</td>
</tr>
<tr>
<td>il6</td>
<td>AGTCCGGAGAGAGAGACTCA</td>
<td>GCCATTGCACAACCTCTTTCT</td>
</tr>
<tr>
<td>tnfa</td>
<td>GATCGGTCCCCAAAAGGGATG</td>
<td>CCACCTTGTTGGTGTGGTAGG</td>
</tr>
<tr>
<td>il12p40</td>
<td>GTGTAACCAGAAAGGAGCG</td>
<td>TCGGACCCTGCAGGGAAC</td>
</tr>
<tr>
<td>xbp1</td>
<td>TACGGGAGAAAACCTCAGCC</td>
<td>CTTACTCCACCTCCCTTGGC</td>
</tr>
<tr>
<td>Chop</td>
<td>CCACCACACCTGAAAGGAG</td>
<td>AGGTGAAAGGAGGACTCA</td>
</tr>
<tr>
<td>atf4</td>
<td>CTAAGCCATGGGCGCTCTTTCA</td>
<td>GTCCGTTACAGCAACACTGC</td>
</tr>
<tr>
<td>bip</td>
<td>TCATCGGACGACCTTTGGG</td>
<td>CAACCACCTTGAATGCAAGA</td>
</tr>
<tr>
<td>non-numt</td>
<td>CTAGAAACCCCCGAACAA</td>
<td>CCAGCTATCACAAGACAGT</td>
</tr>
</tbody>
</table>
### Table 20 continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (FP) (5’ to 3’)</th>
<th>Reverse Primer (RP) (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dloop</td>
<td>AATCTACCATCCTCCGTGAAA CC</td>
<td>TCAGTTTAGCTACCCCCAAGTTT AA</td>
</tr>
<tr>
<td>β2m</td>
<td>ATGGGAAGCCGAACATACTG</td>
<td>CAGTCTCAGTGGGGTGAAT</td>
</tr>
<tr>
<td>tert</td>
<td>CTAGCTCATGTGTCAAGACC CTCTT</td>
<td>GCCAGCACGTTTCTCTCGTT</td>
</tr>
</tbody>
</table>

### Table 21 Reagents per/well for qPCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA sample</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>KAPA</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>FP</td>
<td>0.5</td>
<td>5 pmol/µL</td>
</tr>
<tr>
<td>RP</td>
<td>0.5</td>
<td>5 pmol/µL</td>
</tr>
<tr>
<td>RNase/DNase free water</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 22 Settings for RT qPCR cycle

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>3s</td>
<td>40</td>
</tr>
<tr>
<td>60</td>
<td>30s</td>
<td>40</td>
</tr>
</tbody>
</table>
2.2.8. Western Blot Materials

General Materials

- Acrylamide/Bis-acrylamide, 30% solution (Merck).
- N,N,N',N'-Tetramethylethlenediamine (TEMED) (Merck).
- Ammonium persulfate (APS), Tris Base, Sodium Dodecyl sulphate (SDS), Glycine & methanol purchased from Sigma.
- Isopropanol (Acros organic).
- Polyvinylidene fluoride (PVDF) Transfer membrane, pore size 0.2 µM (Immoobilon #ISEQ00010).
- Lamelli Buffer, Protease inhibitor, phosphatase inhibitor and protein ladder and filter paper (Extra thick #88620) & BSA were purchased from (Thermo Fisher).
- Powerpack- PowerStation 200 (Labnet).
- SDS Cassette (BioRad Mini-PROTEAN 3 cell).
- Western Blot plates (BioRad Mini-PROTEAN #1653308).
- Transfer Machine (Biometra Fastblot analytikjena).

Table 23 Buffer Solutions

<table>
<thead>
<tr>
<th>Buffer solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M TRIS pH 8.8</td>
<td>18.15 g Tris Base in 100 mL dH₂O</td>
</tr>
<tr>
<td>1 M TRIS pH 6.8</td>
<td>12.1 g Tris Base in 100 mL dH₂O</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>10 g ammonium persulfate in 100 mL dH₂O</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>10 g sodium dodecyl sulfate in 100 mL dH₂O</td>
</tr>
<tr>
<td>10x SDS running buffer</td>
<td>144 g Glycine, 30 g Tris Base, 10 g SDS, 1L dH₂O.</td>
</tr>
<tr>
<td>10x TBST</td>
<td>80 g NaCl 2 g KCl, 30 g Tris Base, 10 mL Tween-20, 990 mL dH₂O.</td>
</tr>
<tr>
<td>1x transfer buffer</td>
<td>10.5 g glycine, 2.25 g Tris, 1 g SDS, 200 mL Methanol, 800 mL dH₂O.</td>
</tr>
<tr>
<td>3% (w/v) BSA in TBST</td>
<td>3 g bovine serum albumin (BSA) in 100 mL 1x TBST</td>
</tr>
</tbody>
</table>
## Table 24 Resolving Gel (20 mL – 2 gels)

<table>
<thead>
<tr>
<th>Composition</th>
<th>% Sodium Dodecyl Sulfate (SDS) acrylamide gels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8%</td>
</tr>
<tr>
<td><strong>Protein size range (kDa)</strong></td>
<td>60-100</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.3 mL</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>5.3 mL</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>5 mL</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>200 μL</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>200 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12 μL</td>
</tr>
</tbody>
</table>

## Table 25 Stacking Gel (15 mL – 2 gels)

<table>
<thead>
<tr>
<th>Composition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>5.5 mL</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>1.0 M Tris pH 6.8</td>
<td>1 mL</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>80 μL</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>80 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 μL</td>
</tr>
<tr>
<td>Antibody</td>
<td>Brand</td>
</tr>
<tr>
<td>----------</td>
<td>-------</td>
</tr>
<tr>
<td>Rabbit monoclonal anti-mouse STING antibody</td>
<td>Cell signalling D2P2F</td>
</tr>
<tr>
<td>Rabbit Monoclonal anti-mouse pIRF3 (S396) antibody</td>
<td>Cell signalling 4D4G</td>
</tr>
<tr>
<td>Rabbit Monoclonal anti-mouse pTBK1/NAK (Ser172) antibody</td>
<td>Cell signalling D52C2</td>
</tr>
<tr>
<td>Rabbit Monoclonal anti-mouse IκBα antibody</td>
<td>Cell signalling #9242S</td>
</tr>
<tr>
<td>Rabbit Monoclonal anti-mouse LC3-II antibody</td>
<td>Cell signalling D3U4C</td>
</tr>
<tr>
<td>Rabbit Monoclonal anti-mouse p-Histone H2A.X (S139) antibody</td>
<td>Cell signalling #2577S</td>
</tr>
<tr>
<td>Mouse monoclonal anti-mouse β-actin antibody</td>
<td>Cell signalling 8H10D10</td>
</tr>
</tbody>
</table>
Table 26 continued

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Brand Cat number</th>
<th>Dilution</th>
<th>Incubation time and temperature</th>
<th>Volume per membrane (µL)</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye 800CW Goat anti-rabbit IgG (H+L)</td>
<td>Odyssey #926-32211</td>
<td>1 in 20,000</td>
<td>1 hr at RT</td>
<td>3 mL</td>
<td>3 % (w/v) BSA in TBST</td>
</tr>
<tr>
<td>IRDye 680CW Goat anti-mouse IgG (H+L)</td>
<td>Odyssey #926-68070</td>
<td>1 in 10,000</td>
<td>1 hr at RT</td>
<td>3 mL</td>
<td>3 % (w/v) BSA in TBST</td>
</tr>
</tbody>
</table>

2.2.9. Lactate dehydrogenase (LDH) assay materials

Pierce LDH Cytotoxicity Assay Kit (contents: substrate mix, assay buffer, 10x lysis buffer and stop solution), 1% (w/v) BSA in PBS, ultrapure water, cRPMI and RPMI without FCS.
2.3. Methods

2.3.1. Mice

2.3.1.1. Prophylactic Vaccination Model

Two clinically relevant antigens were used, the *Mycobacterium tuberculosis* vaccine antigen, H56, a fusion protein composed of Ag85B-ESAT6-Rv2660c and the *Chlamydia trachomatis* antigen MOMP [307], [308]. Mice were vaccinated by the subcutaneous (s.c) route on day 0 with PBS, H56 (5 µg/mouse), or H56 with adjuvants, MOMP (5 µg/mouse) or MOMP with adjuvants. On day 14, mice were boosted with the same treatments. On day 21, mice were sacrificed and spleens and inguinal lymph nodes were collected to allow for the measurement of antigen-specific responses. Cells were restimulated ex-vivo with H56 (2 µg/mL and 10 µg/mL) or MOMP (5 µg/mL) for 72 hours and antigen-specific IFN-γ was determined by ELISA.

2.3.1.2. Therapeutic Tumour Model

B16-OVA Cells were cultured in vitro utilising T175 cell culture flasks. Upon reaching logarithmic phase of growth (≤50% confluency) cells were harvested using Trypsin-EDTA. Cells were washed, counted and resuspended to appropriate concentration in ice-cold HBSS. 12-week-old C57BL/6J mice were injected s.c with $3.5\times10^5$ B16-F10 cells on the right flank. Tumour growth was measured daily and tumour volume was determined as $(\text{Length} \times (\text{Width})^2)/2$. Upon tumour volume reaching $75\text{mm}^3$, mice were randomly allocated to experimental groups and injected intratumorally (i.t). Three days later, mice were injected in an identical manner. Mice were culled at a humane endpoint determined as reaching 15 mm in diameter.
2.3.2. Cell culture

Cells were cultured at 37 °C in an atmosphere maintained at 95 % humidity and 5 % CO₂.

2.3.2.1. Cell viability and counting

Cell viability was determined by using a trypan blue exclusion method. 10 μL of cells was mixed with 90 μL Trypan Blue solution (Sigma). 10 μL of this solution was injected into a hemocytometer chamber (Hycor Biomedical Inc). Cells were viewed under a light microscope, assessed by dye exclusion and counted inside the three diagonal squares. Cells on the bottom and right edges were excluded from counting. The number of cells/mL was determined using the following formula.

\[
\text{Cells/mL} = \text{Average cell count} \times \text{dilution factor} \times 10^4
\]

2.3.2.2. Culture of Bone Marrow Derived Dendritic cells (BMDCs):

On day 1, C57BL/6 mice were sacrificed. The femurs and tibiae were removed, cleaned of muscle tissue with a sterile scissors and separated. Bones were washed in petri dishes containing 70 % ethanol (EtOH) for 5 seconds and then in cRPMI. A 10 mL syringe was filled with cRPMI and attached to a 27G needle. The tips of the femur and tibia bone were minimally cut and kept in a sterile petri dish with fresh cRPMI. The bone marrow was flushed out into a petri dish by inserting the 27G needle into the crevice of the bone and injecting cRPMI. Cell clumps were disrupted by repeat gentle pipetting with a 19G needle. The cells were transferred to 50 mL falcon tubes and centrifuged (1200 rpm, RT, 5 min). The supernatant was removed and 1 mL of 0.88 % ammonium chloride (NH₄Cl) red blood cell lysis solution was added. After two minutes, the reaction was stopped with 25 mL of cRPMI. After centrifugation (1200 rpm, RT, 5 min), supernatants were discarded and the cell pellet was resuspended in 10 mL cRPMI. Cell numbers were counted using the trypan blue dye exclusion (Section 2.3.2.1). Cell suspension volume was adjusted with cRPMI containing 20 ng/mL GM-CSF to give a final concentration of 4.25 x 10^5 cells/ml. Cells were transferred to T175 flasks (12.75x10^6 cells/flask). From day 1 on, extreme care was taken when handling T175 flasks to avoid DC activation. On day 3, 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 spun down (1200 rpm, RT, 5 min). The supernatant was discarded and the cell pellet was resuspended in 10 mL cRPMI. Cell suspensions were adjusted to a final concentration of 6.25x10⁵ 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 for cytokine analysis or in 12 well flat-bottom plates at a volume of 950 μL/well for FACs, qPCR or WB analysis. The plates were incubated for at least 2 hours at 37 °C with 5 % CO₂ before stimulation.

2.3.2.3. Culture of mtDNA depleted BMDCs (EtBr BMDCs)

BMDCs were grown as normal until day day 6, when media was removed and replaced with 30 mL fresh cRPMI containing 20 ng/mL GM-CSF and 150 ng/mL EtBr. On Day 7, 30 mL of cRPMI containing 20 ng/mL GM-CSF and 150 ng/mL EtBr 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 spun down (1200 rpm, RT, 5 min). Cells were resuspended and DNA from 5x10⁶ cells was isolated using Roche DNeasy Blood and tissue kits. Relative total mtDNA amounts were then quantified by qPCR with primers specific for the mitochondrial D-loop region (dloop) or a region of mtDNA that is not inserted into nuclear DNA (non-numt) and primers specific for nuclear DNA telomerase reverse transcriptase (tert) and β2 microglobulin (β2m) (Table 20).

2.3.2.4. Culture of Bone Marrow Derived Macrophages (BMDMs)

BMDMs were collected in an identical manner to that of BMDCs unless stated otherwise. Bone marrow was flushed from femurs and tibiae using cDMEM medium. After determining the cell count, the volume was adjusted to give 1x10⁶ cells/mL in cDMEM with 20 % L929-conditioned medium containing M-CSF. The cell suspension was transferred to large petri dishes. On day 3, 20 mL of cDMEM containing 20 % L929 medium was added to large petri dishes. On day 6, the petri dish culture media was removed using a pipette. The cells were washed in PBS without Ca²⁺/Mg²⁺. The PBS was removed and 10 mL trypsin EDTA was added for 20 minutes at 4 °C. Cells were collected, transferred into 50 mL falcon tubes and centrifuged (1200 rpm, RT, 5 min). The supernatants were discarded and cells were resuspended in cDMEM supplemented with 10 % L929 medium at 1x10⁶ cells/mL. Cells were plated in flat bottom 96 well plates at a volume of 200 μL/well for cytokine analysis. The plates were incubated for at least 2 hours at 37 °C with 5 % CO₂ before stimulation.
2.3.2.5. Culture of L929 macrophage colony-stimulating factor (M-CSF)-expressing cell line

The murine gene encoding M-CSF was cloned into a mammalian expression vector and transfected into the murine aneuploid fibrosarcoma cell line, L929. These cells were seeded at 0.5 x 10^6 cells/mL in cDMEM medium in T175 flasks (40 mL/flask) and cultured for 7 days. After 7 days, the supernatants were collected, filter-sterilised and stored at -20 °C for later use. The cells were washed with sterile PBS, and then left to incubate in 10 mL of trypsin-EDTA for 10 minutes at 37 °C. The cells were washed from the T175 flasks with 30 mLs of media, transferred into 50 mL falcon tubes and spun down (1200 rpm, RT, 5 min). Cells were resuspended in cDMEM to a concentration of 0.5 x 10^6 cells/mL and the culture process repeated again. BMDMs were grown in 20 % filter-sterilised L929 supernatant in cDMEM (Section 2.3.2.4) and characterised for expression of F4/80, CD11b, Gr1 and CD11c to ensure growth of a pure macrophage populations (Figure 2.1).

![Figure 2.1 Gating strategy for macrophage characterisation](image)

2.3.2.6. Culture of immortalised bone marrow-derived macrophages (iBMMs)

Immortalised bone marrow-derived macrophages (iBMDMs) were a gift from Professor Katherine Fitzgerald (University of Massachusetts Medical School, USA). iBMDMs were cultured in cDMEM medium. Every fourth day cells were scraped, washed with cDMEM medium and passaged at a 1:10 dilution. For experiments, iBMMs were plated at 1x10^6 cells/ml in 12 well flat-bottom tissue culture plates. Cells were left to adhere for two hours prior to stimulation.
2.3.2.7. Culture of GFP-LC3 immortalised bone marrow-derived macrophages

Immortalised bone marrow macrophages expressing GFP-LC3 were a kind gift from Hardy Kornfeld, University of Massachusetts. iBMM GFP-LC3 cells were cultured in cDMEM medium containing 10 µg/mL puromycin to maintain positive selection. iBMM GFP-LC3 cells were passaged and plated in the same manner as iBMMs (above).

2.3.2.8. Culture of B16 and B16-OVA melanoma cell line

The B16F10 and B16F10-OVA murine melanoma cell line was a Kind Gift from Prof Kingston Mills, Trinity College Dublin (previously purchased from American Type Culture Collection (ATCC) (Manassas, VA)). Cells were cultured in cDMEM and Upon reaching 60-80% confluency, cells were harvested using trypsin EDTA. Cells were washed, counted, and resuspended to 0.5 x10⁶ cell/mL and plated in 12 well flat-bottom tissue culture plates. Cells were left to adhere overnight and stimulated the next morning.

2.3.3. ELISA

R&D and Biolegend ELISA’s were performed according to the kit manufacturer’s instructions. IFN-β was carried out as follows: High binding plates were coated with 50 µl/well capture antibody (1:1000 dilution) in Sodium Carbonate buffer overnight at 4 °C. The next day, plates were washed three tunes with Wash Buffer. Plates were then blocked for 2 hours at 37 °C with 100 µL/well of 10% FCS in PBS. Plates were washed as before. A seven-point standard curve using 2-fold serial dilutions in 10 % FCS in PBS was carried out. Standards and samples were added to wells and left overnight at 4 °C or for 2 hours at RT. Plates were washed as before and loaded with 50 µL/well detection antibody (1 in 2000 dilution) in 10 % FCS in PBS and left for 1.5 hours at RT. Plates were washed three times before addition of 50 µL/well streptavidin-HRP antibody (1 in 2000 dilution) in 10 % FCS in PBS for 1 hour at RT. Plates were washed and developed with 50 µL/well OPD. The reaction was stopped with 25 µL/well Stop solution. Plates were analysed immediately.
2.3.4. Flow Cytometry

2.3.4.1. Measuring cell surface marker expression and viability in BMDCs and BMDMs

After stimulation, plates were centrifuged (1200 rpm, RT, 5 min) and supernatants were discarded. Cells were re-suspended in 10 nM EDTA in PBS. The EDTA/PBS solution containing cells was transferred to labelled FACs tubes and washed in 2 mL PBS. FACs tubes were centrifuged (1200 rpm, RT, 5 min), supernatants removed and cells resuspended in PBS containing fixable viability stain 510 (200 µL: 1 in 1000 dilution). The cells were left for 15 minutes in the dark at RT. After 15 minutes, cells were washed with 2 mL PBS, centrifuged (1200 rpm, RT, 5 min) and resuspended in 100 µL FACs buffer containing Fc block (100µL: 1 in 200 dilution). The tubes were left for 10 minutes on ice, after which the master mix was added to appropriate tubes. The tubes were left for 30 minutes in the dark at RT. After 30 minutes, cells were washed in 1 mL PBS, centrifuged (1200 rpm, RT, 5 min), resuspended in 200 µL FACs buffer and acquired immediately.

2.3.4.2. Flow cytometric analysis of viability in B16F10 cells:

After 20 hours of stimulation, cells were removed from wells using ice cold PBS-EDTA, transferred to labelled FACS tubes and washed with 2 mL of PBS. Cells were stained with BV510 viability dye (1 in 1000 dilution) for 15 minutes at RT. Cells were then washed in PBS, and stained for cell surface markers for 30 minutes at RT. Cells were then washed in PBS, resuspended in 200µl of FACs buffer, with samples acquired via BD FACSCanto and the data analysed using Flowjo™ software (Treestar, Oregon).

2.3.4.3. Flow cytometric analysis of mitochondrial depolarisation

After 3, 6 and 24 hours of treatment with chitosan, plates were centrifuged and supernatants were discarded. Cells were removed from wells using PBS-EDTA, transferred to labelled FACs tubes and washed with 2 mL PBS. Once washed and spun down (1200 rpm, RT, 5 min), cells were stained with fixable viability stain 510 (200 µL: 1 in 1000 Dilution) in PBS at 37°C for 15 minutes. After 15 minutes, cells were washed in PBS and re-suspended in 500 µL pre-warmed cRPMI containing TMRM (100 nM) and mitotracker green (100 nM). Cells were placed back into the incubator for 30 minutes at 37 °C. Following this, cells were washed twice with PBS and re-suspended in 200 µL FACs buffer. FCCP (5 µM) was added to cells 10 minutes before acquisition [225].
2.3.4.4. **Flow cytometric analysis of mitochondrial superoxide and cellular reactive oxygen species**

After treatment, plates were centrifuged 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 and spun down (1200 rpm, RT, 5 min), cells were co-stained with fixable viability stain 510 (1 in 1000 Dilution), MitoSOX Red (1 in 5000 dilution) and CellROX (1 in 500) in 500 µL PBS for 15 minutes at RT. Cells were then washed twice in PBS, re-suspended in 200 µL FACS buffer and acquired immediately [225].

2.3.4.5. **Flow cytometric analysis FITC-labelled Protasan uptake**

After treatment with FITC-labelled protasan, plates were centrifuged 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 and spun down (1200 rpm, RT, 5 min), cells were stained with BV510 (1 in 1000 Dilution) in 500 µL PBS for 15 minutes at RT. Cells were then washed twice in PBS, re-suspended in 150 µL FACS buffer and acquired immediately. Trypan blue was used to quench external FITC fluorescence and distinguish between surface-bound and internalised chitosan-FITC. The total FITC-fluorescense was analysed in 15,000 single, live events before and after addition of 100 µL trypan blue. To demonstrate the efficacy of trypan blue quenching, BMDCs were treated with 1 mg/mL of FITC-labelled protasan at the FACS machine and acquired with and without tryphan blue. A 100 % drop in FITC-fluorescense after trypan blue addition signified complete external FITC quenching [71].

2.3.4.6. **Flow cytometric analysis of DNA damage.**

The Apo-TUNNEL DNA damage kit was used to measure DNA fragmentation. After stimulation, cells were removed from wells using ice-cold PBS, transferred to labelled FACs tubes and washed with 2 mL of PBS. Cells were stained with A700 viability dye (400 µL; 1 in 1000 dilution) for 15 minutes at room temperature. After the elapsed time, cells were washed and fixed in 1 % PFA for 15 minutes. Cells were washed as before and then permeabilised overnight in pure ethanol at -20°C. The next morning, cells were washed and stained with the DNA labelling solution containing reaction buffer (10 µL/sample), TdT enzyme (0.75 µL/sample), BrdUTP (8 µL/sample) and dH2O (31.25 µL/sample) for 1 hour at 37°C. Note, FACS tubes were shaken every 15 minutes to keep cells in suspension. After 1 hour, cells were washed with Rinse buffer (provided in kit).
and stained with 100 µL of the antibody staining solution (10 µL Alexa 488 anti-BrdU Ab and 90 µL rinse buffer) for 30 minutes at room temperature. Without washing, 200 µL PI/RNase A staining buffer was added to each FACS tube already containing the antibody staining solution. Cells were analysed 30 minutes-2 hours after the addition of the PI/RNase A buffer on FACSCanto and the data analysed using Flowjo™ software (Treestar, Oregon) [309].

2.3.4.7. Measuring Autophagy with Green Fluorescent Protein-Light Chain 3 (GFP-LC3) cells

After stimulation, cells were spun down at (1500 rpm, RT, 5 min). Media was replaced with ice-cold PBS for ten minutes to allow cells to detach. Once detached, cells were transferred to appropriate FACs tubes and stained with A700 Live Dead (500 µL: 1/1000 dilution) for 15 minutes at RT. After 15 minutes cells were washed with 2 mL 0.5 % FCS in PBS and spun down at 1200 RPM for 5 minutes. The supernatant was poured off and the resulting pellet was re-suspended in 100 µL of 0.5 % FCS in PBS followed by 100 µL of 120 µg/mL Digitonin (final concentration 60 µg/mL). After two minutes, cells were washed twice with 2 mL 0.5 % FCS in PBS and spun at 150 g for 5 minutes. To prevent loss of cells, supernatants were aspirated rather than poured off. Cells were then re-suspended in 200 µL 0.5 % FCS in PBS and acquired immediately [310].
2.3.5. Confocal microscopy

2.3.5.1. Mitochondrial division

BMDCs were seeded overnight in 4 well glass bottom cell culture dishes at 1.5 x10^6 cells/well (500 µL/well). The next day, media was aspirated off to remove non-adherent DCs. Cells were then resuspended in fresh cRPMI containing treatments. Cells were stained in the cRPMI containing treatments with TMRM (20 nM) and Mitotracker Green FM (50 nM) for 30 minutes at 37 °C. After 30 minutes, cells were washed once with 750 µL pre-warmed PBS and resuspended in cRPMI containing 5 nM TMRM and 10 µg/mL Hoescht 33342 [225].

2.3.5.2. Mitochondrial depolarisation

BMDCs were seeded overnight in 4 well glass bottom cell culture dishes at 1.5 x10^6 cells/well. The next morning, cRPMI was removed and replaced with fresh cRPMI. TMRM (20 nM) and Mitotracker Green FM (50 nM) were added directly to wells containing media and left for 30 minutes at 37 °C. After 30 minutes, cells were washed once with 1 mL pre-warmed PBS and resuspended in cRPMI containing 5 nM TMRM and 10 µg/mL Hoescht 33342 [225], [311]
2.3.6. Western Blot

2.3.6.1. Sample preparation

BMDCs were seeded at 1.5 x10^6 cells/mL in 12 well plates and left to rest for a minimum of two hours before stimulation. Cells were lysed in 150 µL Laemmli Buffer containing 1 % Protease inhibitor (1/100 dilution) and 1 % phosphatase inhibitor (1/100 dilution) and left on ice for 5 minutes. After 5 minutes, the Laemmli-cell mixture was transferred to Eppendorf tubes and heated for 5 minutes at 95 °C in a heating block.

2.3.6.2. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

SDS polyacrylamide gels were prepared (Table 24 & 25). Isopropanol was used to removed bubbles from the lower gel and was poured out prior to addition of the stacking gel. Gels were placed in SDS electrophoresis stands with plates facing inwards. The SDS stand containing the two gels was then placed in the SDS Cassettes (BioRad Mini-
PROTEAN 3 cell) and the centre column was filled to the top with SDS running buffer. The central column was checked for leaks. Combs were removed from the two gels to reveal lanes for protein loading. The samples (20 µL) and diluted protein ladder (7 µL protein ladder + 13 µL laemmli buffer) were added into lanes. Any remaining empty lanes were filled with 20 µL laemmli buffer to ensure that proteins travelled at an equal rate across the gel. Once fully loaded, the outer part of the SDS cassette was filled with running buffer to above the silver electrode line. The machines were then attached to PowerStation 200 powerpack and ran at 80 Volts for 30 minutes (or until the lamelli buffer has passed through the stacking gel). The voltage was then increased to 100 V and left to run until the smallest protein band (10 kDa) was 5mm away from the bottom of the plate.

2.3.6.3. Protein Transfer: Semi-Dry Method.

The two gels were removed from the SDS cassette. The upper plate was removed carefully exposing the gel. The gel was quickly submerged in transfer buffer. Two pieces of 1.5 mm filter paper were cut (each slightly bigger in size than the gel) and soaked in transfer buffer. PVDF was cut to the same size of the gels and then activated in methanol for 2 minutes, followed by two 2-minute washes in water and transfer buffer. One piece of soaked filter paper was placed on the transfer machine. The activated PVDF membrane was then placed on top of the filter paper. The gel was carefully removed from the transfer buffer and placed on top of the PVDF membrane. The second soaked filter paper was then placed on top of the gel. A roller was used to remove any air
bubbles, taking care not to move the gel off the PVDF membrane. The gel was transferred using the Biometra Fastblot Transfer Machine and PowerStation 200 powerpack with the number of mAmperes dependent on the size of the protein of interest. For example, a 40 kDa protein was transferred using 50 mAmperes per gel for 1 hr 30 minutes. If two gels were transferred at the same time, transfer was carried out at 100 mAMPs for 1 hr 30 minutes. Following transfer, PVDF membranes were placed in 50 mL tubes with the side of the membrane in contact with the gel facing into the centre of the tube.

2.3.6.4. Blocking

Membranes were blocked in 50 mL tubes, under rotation (to ensure equal blocking), in 10 mLs of 3 % BSA in TBST for 1 hour at RT or overnight at 4 °C.

2.3.6.5. Capture antibody

Blocking buffer was removed from the 50 mL tubes and replaced with 3 mL Primary antibody solution (Table 26). Tubes were placed on rotators overnight at 4 °C.

2.3.6.6. Secondary antibody

The primary antibody mixture was poured out and the membrane was washed three times in TBST for 5 minutes each. Gels were placed in secondary antibody solutions (Table 26) for 1 hour at RT on rotators. Gels were then washed three times in TBST for 5 minutes each. When the protein of interest was not phosphorylated, membranes were additionally washed twice in PBS for 5 minutes. Blots were analysed on an Odyssey LI-COR machine with the intensity set at 5 for the 700 channel and 6 for the 800 channel and resolution set to 169 µm. Images were analysed using Image studio Lite softwar
2.3.7. Lactate dehydrogenase (LDH) assay

One vial of substrate mix (lyophilizate) was dissolved in 11.4 mL of ultrapure water (Baxter). 0.6 mL assay buffer was thawed and added to 11.4 mL substrate mix. The solution was mixed well by inversion and protected from light until use. A complete medium control without cells was included to determine LDH background activity resulting from the FCS used in media supplementation. Serum-free media control without cells was used to determine the amount of LDH activity in the sera. 45 minutes before the full 24 hr stimulation, 20 µL of ultrapure, sterile water was added to triplicate wells containing cells (200 µL) as a spontaneous LDH activity control. 20 µL of 10x Lysis buffer was added to another set of triplicate wells as a maximum LDH activity positive control. Plates were put back in the incubator for 45 minutes. After the incubation period, plates were spun at 1200 rpm for 5 minutes. Supernatants were collected and diluted 1:2 with PBS. 50 µL supernatant/ PBS was transferred to a fresh 96 well plate, followed by 50 µL of the reaction mixture. After a 30-minute RT incubation, reactions were stopped by adding 50 µL stop Solution. LDH activity was determined by measuring absorbance at 492 nm. To calculate cytotoxicity, the following formula was used:

\[
\% \text{ Cytotoxicity} = \frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100
\]

2.3.8. Statistical Analysis

All statistical analysis was carried out on GraphPad Prism, version 6f (GraphPad software). The means for 2 groups were compared by unpaired student t tests. The means for 3 or more groups were compared by one-way analysis of variance (ANOVA). For tests involving more than two variables, groups were compared by two-way ANOVA.
3. Endoplasmic Reticulum Ca\(^{2+}\) regulates chitosan-induced mitochondrial stress and mtDNA-dependent cGAS-STING activation.

3.1. Introduction, Aims and Hypothesis

A major obstacle in the development of vaccines against diseases such as TB, HIV, Malaria, Leishmaniasis and cancer is a lack of adjuvants that can promote potent cellular immunity. Licensed human adjuvants such as alum, MF59, AS03, virosomes and AS04 find wide clinical application and promote humoral immunity and Th2 biased responses [5], [6]. However, there is limited knowledge of their mechanism of action or understanding as to why they fail to elicit potent Th1 responses. The cationic polysaccharide, chitosan is a 1-4-β-linked polymer comprised of N-Ac-glucosamine and glucosamine units that presents as an attractive alternative to alum- It is biocompatible, flexible in terms of formulation and DD, efficacious when administered mucosally and superior to alum in promoting Th1 responses. Moreover, in contrast to the ill understood licensed adjuvants, there is solid evidence implicating two major PRR pathways, the cGAS-STING pathway and NLRP3 inflammasome in chitosan-induced Th1 immunity [71].

The maturation of DCs is a key determinant in the immunostimulatory effects of adjuvants. Adjuvants can activate DCs either directly by engaging with PRRs or indirectly through the delivery of danger signals to the cell which bind to PRRs located both externally and internally in the cells [30]. Indeed, DAMPs are postulated to underlie the efficacy of live attenuated vaccines, alum-adjuvanted vaccines, and DNA vaccines. PRR activation leads to enhanced expression of MHC and costimulatory molecules on the DC surface in addition to the secretion of cytokines which direct T cell polarization and differentiation [32]. Type I IFNs have important roles in the induction of adaptive immunity, they promote the generation of cytotoxic T cell responses as well as a Th1 biased CD4\(^{+}\) T cell phenotype. Furthermore, type I IFNs promote the activation and functional maturation of DCs, increasing their migration and facilitating antigen presentation to CD4\(^{+}\) T cells as well as cross priming of CD8\(^{+}\) T cells [279], [280].

The cGAS–STING signalling axis has emerged as a crucial regulator of type I IFN responses to both exogenous and endogenous DNA. cGAS recognises dsDNA
independently of its sequence, bestowing the ability to sense DNA from both endogenous and microbial origins. Upon DNA binding, cGAS catalyses the synthesis of the CDN cGAMP, which serves as a second messenger that binds to and activates STING [312]. STING traffics from the ER to perinuclear sites, where it engages in TBK1 and IRF3 dependent type I IFN gene expression [313]. The cGAS-STING pathway plays a vital part in orchestrating chitosan-induced Th1 immune responses [71]. However, the DNA entity responsible for cGAS activation is not known. Although most endogenous ligands for cGAS are presumed to originate from nuclear DNA, it is becoming increasingly clear that mtDNA also serves as a cell-intrinsic cGAS ligand in certain contexts [314].

Mitochondria are signalling organelles with diverse roles in innate immunity. When mitochondria are damaged, the dysfunctional mitochondria increase generation of mtROS in cells [315]. These dysfunctional mitochondria are prone to enhance immune responses, by triggering spontaneous MAVs oligomerisation or by promoting the translocation of mitochondrial DAMPs into the cytosol [170], [226], [227], [316], [317]. The translocation of matrix DAMPS, such as mtDNA occurs through a process involving MOMP. The mechanism of MOMP is poorly understood, with conflicting reports suggesting the involvement of BAX-BAK pores or mitochondrial permeability transition pores (MPTPs) [221], [318]–[320].

Mitochondria undergo processes of fission and fusion to regulate function and quality in response to changing environments [321]. Mitochondrial fission is regulated by the GTPase Drp1 that drives division at specific points along mitochondria. These points are pre-marked by close contacts with the ER that facilitate mitochondrial Ca$^{2+}$ uptake leading to IMM constriction and cristae remodelling [213]–[215], [322]. IMM constriction permits Drp1 to oligomerise into rings encircling the OMM and pinch the mitochondrion apart [216]. Fission has no direct role in MOMP-induced mtDNA release- Drp-1 deficient cells have no defect in MOMP or mtDNA release [221]. However, in T cells it promotes mtROS production, by disrupting ETC structures and promoting electron leaks that then form ROS [220], [321]. It is unknown whether fission promotes mtROS production in innate immune cells.
The hypothesis underlying this work is that ER Ca$^{2+}$ triggers chitosan-induced mitochondrial dysfunction that permits mtDNA egress and cGAS-STING activation. Accordingly, this chapter hopes to accomplish the following aims:

- Identify the DNA entity responsible for chitosan-induced type I IFN responses.
- Investigate if, in addition to mitochondrial stress, chitosan alters mitochondrial dynamics, mass and membrane potential.
- Investigate the mechanism of chitosan-induced mitochondrial dysfunction, with particular focus on the ER.

**Figure 3.1 Overview of the proposed role of Ca$^{2+}$ in chitosan-induced immune signalling**

(a) Chitosan is hypothesised to trigger Ca$^{2+}$-dependent mitochondrial fission and mitochondrial stress resulting in the egress of mtDNA. mtDNA binds to cGAS generating cGAMP, which then binds STING at the ER and triggers relocation to the ERGIC where type I IFN signalling can occur.

Abbreviations: MOMP; mitochondrial outer membrane permeabilization; Mito, mitochondria; ER, endoplasmic reticulum; ER-Golgi intermediate compartment.
3.2. Results

3.2.1. Chitosan-induced DC maturation is dependent on STING-mediated type I IFN

Chitosan was previously demonstrated to drive the induction of type I IFNs through the cGAS-STING pathway, resulting in interferon-α/β receptor (IFNAR)-dependent DC maturation and subsequent Th1-mediated immune responses [71]. To corroborate these findings, BMDCs were stimulated for 24 hours with indicated concentrations of chitosan and assessed for secretion of IFN-β and the interferon stimulatory gene (ISG), CXCL10 by ELISA. The TLR9 agonist CpG and TLR4 agonist LPS, were used as positive controls for IFN-β and CXCL10 production. Both controls induced high levels of IFN-β and CXCL10 secretion. As previously shown, low concentrations (0.38 – 3 µg/mL) of chitosan induced a dose-dependent increase in IFN-β and CXCL10 secretion. Above 3 µg/mL, increasing chitosan concentration did not correlate with increased IFN-β or CXCL10 production (Figure 3.2 a-b).

To elucidate the kinetics of chitosan-induced type I IFN responses, BMDCs were stimulated with 5 µg/mL of chitosan for 2, 4, 8, 12, 16, 20 and 24 hours and ifna, Ifnb and cxcl10 expression was assessed by qPCR. As a positive control, cells were stimulated with the murine STING agonist DMXAA for 4 hours or LPS for 3 hours. Primers were designed to amplify the ifnb gene, a large subset of the 13 murine ifna subtypes described [323] and the cxcl10 gene. Induction of type I IFN genes was calculated with respect to actb (β-actin) and mRNA expression was validated with respect to a second reference gene, rps18 (18S rRNA) that displays invariant expression across different cells, tissues and experimental conditions [324]. An initial ifna and ifnb mRNA expression peak was observed 8 hours after treatment with chitosan (Figure 3.3 a-b). Despite being an ISG, CXCL10 also exhibited an initial peak 8 hours after treatment (Figure 3.3 c). For all three genes, expression reduced at 12 hours, before reaching a second higher peak at 16 hours. Ifna, ifnb and cxcl10 expression continued to be observed up to 24 hours after stimulation (Figure 3.3 a-c).

As mentioned, chitosan engages the cGAS-STING pathway to mediate type I IFN production. Here, BMDCs from WT and Tmem173−/−mice were stimulated with chitosan and monitored for type I IFN responses. CpG and LPS were used as negative controls for STING-dependent IFN responses. As expected, the induction of ifna and Ifnb in
response to chitosan was completely abolished in $Tmem173^{-/-}$ BMDCs (Figure 3.4 a-b). This impairment was additionally observed at the IFN-β protein level when BMDCs from WT and $Tmem173^{-/-}$ mice were stimulated with chitosan for 24 hours. BMDCs generated from $Tmem173^{-/-}$ mice retained the capacity to induce $ifna$ and $ifnb$ transcription and IFN-β translation in response to LPS (Figure 3.4 a-c). While BMDCs from $Tmem173^{-/-}$ mice were still capable of secreting IFN-β in response to CpG, transcription of the $ifnb$ gene was significantly impaired (Figure 3.4 b-c). CpG drove low levels of $ifna$ expression in BMDCs from WT and $Tmem173^{-/-}$ mice (Figure 3.4 a).

Type I IFNs exert a multitude of effects on both the innate and adaptive immune systems. In particular, these cytokines are implicated in the phenotypic and functional maturation of DCs in response to dsRNA and viral infection. To investigate the extent of DC maturation with chitosan, BMDCs were treated for 24 hours with indicated concentrations of chitosan or CpG and LPS separately as positive controls. Single, live CD11c$^+$ cells were then monitored for the percentage and expression levels of MHCcII, CD40, CD86 and CD80.

CpG and LPS treatment significantly increased the percentage of CD86$^+$, CD40$^+$ and MHCcII$^+$ cells and the expression of CD40, CD86 and CD80 on DCs compared to the media controls (Figure 3.5 b,d,e,h,I & g). Chitosan treatment significantly increased the percentage of MHCcII$^+$, CD40$^+$ and CD86$^+$ to levels comparable to CpG and LPS. While the chitosan-induced percentage increase in CD40$^+$ cells was dose dependent, 2,4 and 8 µg/mL of chitosan enhanced the percentage of CD86$^+$ and MHCcII$^+$ cells to similar extents (Figure 3.5 b,d,f). Of note, despite all concentrations of chitosan increasing the percentage of CD40 and CD86 expressing cells, only high concentrations of chitosan significantly enhanced CD40 and CD86 expression in terms of the mean fluorescence intensity (Figure 3.5 e,g). Similarly, despite increasing the percentage of MHCcII$^+$ cells, neither chitosan, CpG or LPS significantly enhanced basal MHCcII expression (Figure 3.5 c). There was no significant increase in the percentage of CD80$^+$ cells in response to chitosan, CpG or LPS, as the majority of media-stimulated cells were already CD80$^+$ (Figure 3.5 h). However, chitosan did increase CD80 expression, albeit to a much lesser extent than CpG and LPS treatments (Figure 3.5 i). The chitosan-induced increase in CD80 mean fluorescence intensity (MFI) was dose-dependent. Chitosan-induced CD40 expression was similar to that of CD80, being dose-dependent and lower than that mediated by CpG and LPS (Figure 3.5 e). Chitosan increased CD86 expression in a
dose-dependent manner and in contrast to CD40 and CD80, was comparable to both positive controls at the highest concentrations tested (Figure 3.5 g).

Cell death can influence the priming and differentiation of T cells. For instance, alum-induced cytotoxicity results in the release of host DNA, which can act as a DAMP, and has been proposed to contribute to the adjuvant activity of alum [12]. Chitosan-induced cytotoxicity was monitored by flow cytometry using BV510 LIVE/DEAD viability stain which works by binding cellular amines. The dye permeates compromised membranes of dying cells and binds free amines on the interior and exterior of the cell, yielding brightly stained cells. In contrast, only cell surface amines of live cells react with BV510 resulting in weakly fluorescent cells. Single, live CD11c⁺ cells were analysed for AQUA LIVE/DEAD fluorescence. Chitosan induced significant cell death in a dose-dependent manner, with the highest concentration driving on average 60 % toxicity. In contrast, CpG and LPS were not toxic to DCs (Figure 3.6 a-b). BMDCs were additionally monitored for lactate dehydrogenase (LDH) activity in the supernatant after 24-hour treatment with 2, 4 and 8 µg/mL chitosan. LDH is a cytosolic enzyme that is released upon damage to the plasma membrane. It is an oxidoreductase enzyme that catalyses the interconversion of pyruvate and lactate, and simultaneously reduces NAD to NADH, which is specifically detected by colorimetric (450 nm) assay. Chitosan drove LDH accumulation in a dose-dependent manner, with the highest concentration resulting in 60 % cytotoxicity (Figure 3.6 c).
Figure 3.2 Chitosan drives the secretion of IFN-β and CXCL10 in BMDCs.

BMDCs were stimulated with media, 0.38, 0.75, 1.5, 3 or 6 µg/mL of protasan, CpG (4 µg/mL) or LPS (10 ng/mL) for 24 hours. ELISAs were used to determine levels of IFN-β and CXCL10 in the supernatants. Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by one-way ANOVA. *p<0.05, **p<0.01, ****p<0.001, *****p<0.0001.
Figure 3.3 Time course analysis of chitosan-induced *ifna*, *ifnb* and *cxcl10* mRNA in BMDCs.

BMDCs were stimulated with protasan (5 µg/mL) for indicated times, DMXAA (10 µg/mL) for 4 hours or LPS (10 ng/mL) for 3 hours. mRNA levels were calculated by qPCR for *ifna*, *ifnb* and *cxcl10* with respect to *actb* and *rps18*. Data shows technical triplicate mRNA levels with respect to *actb* and is representative of three independent experiments. Data was analysed by one-way ANOVA. ****p<0.0001.
Figure 3.4 Chitosan-induced IFN responses are dependent on STING.

(a-b) BMDCs from WT and \textit{Tmem173}\textsuperscript{-/-} mice were treated for 24 hours with protasan (8 µg/mL) or for 3 hours with CpG (4 µg/mL) or LPS (10 ng/mL). mRNA levels were calculated by qPCR for \textit{ifna} and \textit{ifnb} with respect to \textit{actb} and \textit{rps18}. Data shows technical triplicate mRNA levels with respect to \textit{actb} and is representative of three independent experiments. (c) BMDCs from WT and \textit{Tmem173}\textsuperscript{-/-} mice were stimulated with protasan (6 µg/mL), CpG (4 µg/mL) or LPS (10 ng/mL) for 24 hours. Data represents technical triplicates of IFN-β in the supernatant of treated cells and is representative of four independent experiments. Statistical significance was determined using two-tailed unpaired student’s \textit{t} tests with the Holm-sidak method for multiple comparisons. ****\(p<0.0001\).
Figure 3.5 Chitosan induces DC maturation

BMDCs were treated with indicated concentrations of protasan, CpG or LPS for 24 hours. After this period, cells were collected and stained for flow cytometry. BMDCs were gated as single, live, CD11c+ cells and then analysed for percentages of (b) MHCcII+, (d) CD40+, (f) CD86+ and (h) CD80+ cells and expression of (c) MHCcII, (e) CD40, (g) CD86 and (i) CD80. (b-i) Data are expressed as mean ± SD for biological quadruplets and was analysed by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
**Figure 3.6 Chitosan is toxic to BMDCs in a dose-dependent manner.**

(a-b) BMDCs were treated with protasan, CpG or LPS for 24 hours. After this period, cells were collected and stained for flow cytometry. (a) % cell death in single, CD11c+ cells (see Figure 3.5a for gating) (b) Data are expressed as mean ± SD for biological quadruplets and was analysed by one-way ANOVA. (c) The supernatants of BMDCs stimulated with indicated concentrations of protasan for 24 hours were monitored for cytotoxicity by LDH assay. Data are expressed as mean ±SD of technical triplicates and was analysed by one-way ANOVA. n=1. **p<0.01, ****p<0.0001.
3.2.2. Chitosan-induced mitochondrial stress triggers the egress of mtDNA and activation of the cGAS-STING pathway

Chitosan-induced mitochondrial stress is critical for cGAS-STING activation and occurs three hours after treatment [71]. A time course experiment was set up to visualise the induction, onset, and longevity of chitosan-induced mitochondrial stress. BMDCs were treated with chitosan (5 µg/mL) for 1, 2, 3, 6, 9, 12 and 24 hours or Rotenone, a complex I inhibitor for 6 hours. The degree of stress was quantified by flow cytometry using a mitochondrial-specific ROS indicator, MitoSOX, that selectively detects superoxide in mitochondria and BV510 LIVE/DEAD to allow mtROS detection specifically in live cells (Figure 3.7 a). As expected, Rotenone drove mitochondrial stress, visualised by an enhancement in the percentage of mtROS+ cells and MitoSOX fluorescence. Chitosan drove mitochondrial stress as early as one hour after treatment, the signal peaked 3 hours after treatment and began to level off after 6 hours. Of note, mitochondrial stress was still apparent after 24 hours of treatment with 9.34 % of cells remaining mtROS+ compared to 2.32 % in the medium control (Figure 3.7 b-c).

From this point on, chitosan-induced mitochondrial stress was always quantified 1-2 hours after treatment. DC maturation was analysed to address whether chitosan-induced mitochondrial stress is dose-dependent in line with downstream type I IFN production. BMDCs, were treated with chitosan (2.5, 5 or 7.5 µg/mL) for 1.5 hours or rotenone for 6 hours as a positive control. Chitosan-induced mtROS production was dose-dependent with 5 and 7.5 µg/mL being more potent than rotenone in terms of percentage of mtROS+ cells and MitoSOX fluorescence. Treatment with chitosan at concentrations of 5 µg/mL and 7 µg/mL increased MitoSOX MFI from 248 to 576 and 876 respectively compared to 331 with rotenone. Likewise, these treatments increased the percentage of mtROS+ cells from 3.73 % in the media control to 35.0 % and 55.6 % compared to 22.2 % after rotenone treatment (Figure 3.8 b-c).

Next, to ascertain whether chitosan-induced stress is exclusively localised to the mitochondria cells were co-stained with MitoSOX and CellROX Deep Red (CellROX) (Figure 3.9 a). CellROX localises in the cytoplasm and predominately detects hydroxyl radicals and to a lesser extent superoxide anion. In the absence of ROS, the probe emits no signal, but upon detection it emits a red fluorescence. BMDCs were treated with chitosan (5 µg/mL) for 1.5 hours, Rotenone for 6 hours or phorbol myristate acetate (PMA) for 9 hours to target NADPH oxidase (NOX) and exclusively stimulate NOX-
dependent respiratory burst [325]. In contrast to the low levels of mitochondrial stress in media-treated DCs (Figure 3.9 b-c), basal cellular stress levels were high with 92.5 % of single live cells gated as CellROX+ (Figure 3.9 e). When a population is already highly saturated in a process or protein, it is more informative to monitor deviations between samples by analysing the MFI of individual events. For this reason, CellROX fluorescence was used to monitor alterations in cellular stress levels. While chitosan treatment drove significant mitochondrial stress (Figure 3.9 b-c), there was no substantial difference in CellROX fluorescence (Figure 3.9e). Rotenone treatment enhanced the percentage of mtROS+ cells and MitoSOX fluorescence (Figure 3.9 b-c), but reduced CellROX fluorescence, albeit not to a significant degree (Figure 3.9 e). As expected, PMA had no effect on mitochondrial stress (Figure 3.9 b-c) and enhanced CellROX fluorescence above the media control (Figure 3.9 e).

The Lavelle Lab have demonstrated the essential role of mitochondrial stress in chitosan-induced cGAS-STING activation. Pre-treatment with the mitochondrial-specific antioxidant MitoTEMPO abolished chitosan-induced IFN-β and CXCL10 production [71]. As complex I and III of the electron transport chain are the main sources of ROS emanating from the mitochondria, it was proposed that inhibition of these complexes would reduce chitosan-induced type I IFN responses. The complex I inhibitor Rotenone is unusual in that it only lowers complex I-derived ROS if reverse electron transport is occurring and otherwise induces ROS production [220]. Rotenone significantly reduced chitosan-induced IFN-β production while LPS and zymosan-induced IFN-β production remained intact (Figure 3.10 a). As rotenone additionally blocks complex I dependent oxygen consumption, it cannot be assumed that the reduced IFN-β response is independent of this process. In contrast, the complex III inhibitor S3QEL3 selectively suppresses superoxide production at site IIIQo, without altering ETC activity. This provides the means to implicate complex 3 electron leakage in chitosan-induced responses without otherwise altering DC metabolism. Similar to Rotenone, S3QEL3 significantly reduced chitosan induced IFN-β production while leaving LPS and zymosan induced IFN-β production unchanged (Figure 3.10 b).

As mtROS is responsible for chitosan-induced cGAS-STING activation, levels of mitochondrial stress should be comparable in BMDCs from wild-type (WT) and Tmem173−/− mice. To verify this, BMDCs from WT and sting−/− mice were stimulated with chitosan (2,4 or 8 μg/mL) for 1.5 hours or alum for 2 hours. BMDCs from WT and Tmem173−/− mice displayed similar levels of mtROS production in terms of percentage of mtROS+ cells and MitoSOX fluorescence. Alum appeared to be a relatively weak inducer
of mitochondrial stress, particularly with respect to the percentage of mtROS* cells (Figure 3.11 b-c).

Chitosan-induced mitochondrial stress stimulates the release of host DNA, which then, acting as a DAMP, activates the cGAS-STING pathway [71]. Carroll et al. hypothesised that mitochondrial stress leads to the opening of the MPTPs and the subsequent release of mtDNA that serves as a trigger for cGAS-activation. To confirm this theory, an ethidium bromide (EtBr) culture method aimed at depleting mtDNA in BMDCs was set up (Figure 3.12 a). The DNeasy Blood and tissue kit was used to isolate total DNA in normal BMDCs and EtBr-cultured BMDCs. Relative total mtDNA amounts were then quantified by qPCR with primers specific for the mitochondrial D-loop region (dloop) or a region of mtDNA that is not inserted into nuclear DNA (non-numt) and primers specific for nuclear DNA telomerase reverse transcriptase (tert) and β2 microglobulin (β2m). Low dose EtBr treatment significantly decreased the levels of dloop relative to tert and to a lesser extent non-numt relative to β2m, signifying a near complete depletion of mtDNA (Figure 3.12 d-e). There was a moderate reduction in viability of cells cultured in EtBr compared to control BMDCs (Figure 3.12 b-c). For this reason, ELISA quantification of protein secretion was precluded to assess the role of mtDNA in chitosan-induced type I IFN responses. Instead, qPCR was carried out to allow normalisation of gene expression against actb and rps18 and across the treatment groups.

Control and mtDNA-depleted BMDCs were stimulated with chitosan for 24 hours, etoposide (a topoisomerase II inhibitor that damages DNA) for 24 hours or DMXAA for 3 hours. mtDNA depletion significantly reduced chitosan-induced ifnb transcription, particularly at high concentrations. In contrast, etoposide- and DMXAA-induced ifnb transcription were unaffected (Figure 3.13).
Figure 3.7 Chitosan-induces mitochondrial stress in BMDCs within 1 hour of treatment

BMDCs were treated with media, protasan (5 µg/mL) for 1, 2, 3, 6, 9, 12 or 24 hours or Rotenone (5 µM) for 6 hours. Single, live cells were analysed for MitoSOX fluorescence. Data are representative of two independent experiments. FMO= Fluorescence minus one, MFI= Mean fluorescence intensity.
Figure 3.8 Chitosan drives mitochondrial Reactive Oxygen Species (mtROS) production in BMDCs.

BMDCs were treated with media, indicated concentrations of protasan for 1.5 hours or Rotenone (5 µM) for 6 hours. Single, live cells were analysed for MitoSOX fluorescence. Data are representative of six independent experiments. FMO= Fluorescence minus one, MFI= Mean fluorescence intensity.
Figure 3.9 Chitosan does not alter basal Cellular levels of ROS.

BMDCs were treated with media, protasan (5 µg/mL) for 1.5 hours, Rotenone (5 µM) for 6 hours or phorbol 12-myristate 13-acetate (PMA) (5 µM) for 9 hours. After this period, cells were collected and stained for flow cytometry. (a) Single, live cells were analysed for MitoSOX and CellROX fluorescence. (b) FSC-A vs MitoSOX, (c) Unit area vs MitoSOX, (d) FSC-A vs CellROX, (e) Unit area vs CellROX plots. Data expressed as mean ± SD of experimental duplicates (n=2). Statistical significance was measured by one-way ANOVA. **p<0.01. FMO= Fluorescence minus one, MFI= Mean fluorescence intensity.
Figure 3.10 Chitosan-induced IFN-β secretion is dependent on Complex I and III activity.

BMDCs were pre-treated with the (a) complex 1 inhibitor rotenone (500 nM) for 3 hours or (b) the complex III inhibitor S3QEL3 (10 µM) for 45 minutes. Cells were then stimulated with indicated concentrations of protasan, LPS (10 ng/mL) or Zymosan (10 µg/mL). After 24 hours, supernatants were collected and the levels of IFN-β were determined by ELISA. Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by two-way ANOVA. *p<0.05, ***p<0.001, ****p<0.0001.
Figure 3.11 Chitosan-induced mtROS production is not dependent on STING.

WT and Tmem173^-/- BMDCs were treated indicated concentrations of protasan for 1.5 hours or Alum (10 µg/mL) for 2 hours. (a) Single, live cells were analysed for MitoSOX fluorescence. n=1 b) FSC-A vs MitoSOX, (c) Count vs MitoSOX. n=1.
Figure 3.12 Low dose ethidium bromide treatment depletes mtDNA in BMDCs.
(a) Experimental schematic used to deplete mitochondrial DNA (mtDNA) in BMDCs (see materials and methods for more details). (b) Day 11 flow cytometric analysis of EtBr toxicity in single cells (c) Toxicity data combined from two independent experiments was analysed by a two-tailed unpaired student’s t test. *p<0.05. (d) on day 10, relative total mtDNA amounts were quantified by qPCR with primers specific for the mitochondrial D-loop region (dloop) or a region of mtDNA that is not inserted into nuclear DNA (non-numt) and primers specific for nuclear DNA (tert, B2m). Statistical analysis on technical triplicates was performed by two-tailed unpaired student’s t tests. Data are representative of four independent experiments. ***p<0.001; ****p<0.0001.
Figure 3.13 Chitosan-induced ifnb mRNA transcription requires mtDNA.

WT bone marrow precursors were cultured into BMDCs as usual (Ctrl BMDCs) or in low dose ethidium bromide (EtBr DCs) as explained in figure 10 to deplete mtDNA. Cells were then treated with indicated concentrations of protasan for 24 hours, DMXAA (10 µg/mL) for 3 hours or etoposide (50 µM) for 24 hours. ifnb mRNA levels were calculated by qPCR with respect to actb and rps18. Data are expressed as mean ± SD for technical triplicates with respect to actb and are representative of 3 independent experiments. Statistical analysis was performed by two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. **p<0.01; ***p<0.001.
3.2.3. The NLRP3 inflammasome is not required for chitosan-induced type I IFN responses in BMDCs.

cGAS and STING-deficient mice fail to induce type I IFNs \textit{in vitro} or a Th1 response \textit{in vivo} in response to chitosan. These striking phenotypes demonstrate the importance of this pathway in chitosan’s mode of action. In addition, chitosan-induced Th1 responses are also highly dependent on the inflammasome component NLRP3 through an unresolved mechanism [71]. The NLRP3 specific inhibitor MCC950 was used to investigate the contribution of NLRP3 in chitosan-induced type I IFN responses. The adjuvant alum was used as a positive control for IL-1β release given its ability to exclusively activate the NLRP3 inflammasome [73]. As MCC950 significantly suppressed chitosan- and alum-induced IL-1β release it was used to monitor the effect of NLRP3 on mitochondrial stress and subsequent type I IFN responses (Figure 3.14 a). BMDCs were pre-treated with MCC950 and then stimulated for 1.5 or 24 hours with chitosan to quantify mtROS production and Type I IFN secretion respectively. BMDCs were treated with rotenone for 6 hours as a positive control for mtROS production and CpG for 24 hours as a positive control for IFN-β or CXCL10 production. Inhibition of the NLRP3 inflammasome did not reduce chitosan or rotenone-induced mitochondrial stress, evident in the similar percentages of mtROS⁺ cells and MitoSOX fluorescence in cells treated with and without the inhibitor (Figure 3.15 b-c). Similarly, inhibition of the NLRP3 inflammasome did not reduce chitosan or CpG-induced IFN-β or CXCL10 production (Figure 3.14 b-c).
Figure 3.14 Inhibition of the NLRP3 inflammasome does not compromise chitosan induced IFN-β and CXCL10 secretion in BMDCs.

(a) BMDCs were pre-treated with the NLRP3 inflammasome inhibitor MCC950 (200 nM) for 45 minutes and then stimulated with indicated concentrations of CpG and Protasan or CpG and Alum. (b-c) BMDCs were pre-treated with MCC950 (200 nM) for 45 minutes and then stimulated with indicated concentrations of protasan or CpG (4 µg/mL). After 24 hours, supernatants were collected and concentrations of (a) IL-1β (b) IFN-β and (c) CXCL10 were determined by ELISA. Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by (a) Two-way ANOVA or (b-c) two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. ****p<0.0001.
Figure 3.15 The NLRP3 inflammasome does not drive chitosan-induced mtROS production.

WT BMDCs were pre-treated for 1 hour with the NLRP3 inflammasome inhibitor MCC950 (200 nM). Cells were then treated with media, indicated concentrations of protasan for 1.5 hours or Rotenone (5 µM) for 6 hours. After this period, cells were collected and stained for flow cytometry. (a) Single, live cells were analysed for MitoSOX. (b) FSC-A vs MitoSOX, (c) Count vs MitoSOX. n=1. FMO= Fluorescence minus one, MFI= Mean fluorescence intensity.
Figure 3.16 Overview of chitosan-induced immune signalling in dendritic cells

Chitosan induces mitochondrial stress through an unknown mechanism resulting in the egress of mtDNA. mtDNA in turn binds to cGAS generating cGAMP, which then stimulates the STING pathway for type I IFN synthesis. Dendritic cells undergo IFNAR-dependent maturation which is required for Th1 polarisation. Chitosan also requires NLRP3 for Th1 cell polarisation. NLRP3 does not promote Th1 polarisation by stimulating chitosan-induced mitochondrial stress. The role of mtROS and/or mtDNA in chitosan-induced NLRP3 inflammasome activation is unknown.
3.2.4. Chitosan drives mitochondrial fission in BMDCs

To explore the mechanism by which chitosan drives mitochondrial stress, chitosan’s affects on other aspects of mitochondrial dynamics such as size, shape, mass and membrane potential were investigated. To address the effect of chitosan on mitochondrial size and shape, BMDCs were stimulated for three hours with chitosan and then stained with tetramethylrhodamine methyl ester (TMRM). TMRM is a lipophilic and cationic dye that equilibrates across membranes in a Nernstian fashion, and therefore will accumulate across mitochondrial membranes and into the matrix in inverse proportion to the membrane potential. Untreated cells displayed long elongated mitochondria, whereas chitosan-treated mitochondria were small and punctate, characteristic of mitochondrial fission (Figure 3.17).

Next, the ability of chitosan to alter mitochondrial mass in BMDCs was addressed using MitoTracker Green. As this reagent labels mitochondria in a largely membrane potential-independent manner, its intensity purely relates to the mitochondrial mass of the cell. BMDCs were treated with chitosan for 3 hours or FCCP for 10 minutes. Chitosan moderately reduced mitochondrial mass compared to media-treated cells, albeit not to a significant degree. The moderate reduction was dose-dependent, became more apparent after 6 hours of treatment and recovered to normal if not higher mitochondrial mass after 24 hours. In contrast, FCCP significantly increased mitochondrial mass compared to media treated or chitosan-treated BMDCs (Figure 3.18).

A non-quenching TMRM concentration was used to quantify alterations in membrane potential by FACS. There are several drawbacks, limitations, and considerations that must be addressed when using membrane potential dyes. Firstly, TMRM and similar derivatives, solely measure the charge gradient across the inner mitochondrial membrane and cannot specifically measure the mitochondrial proton gradient. As membrane potential does not always mirror mitochondrial pH, these probes cannot be used to make direct inferences regarding the mitochondrial proton gradient and respiratory status. Secondly, all membrane potential probes have varying degrees of toxicity to mitochondria and cells, principally due to phototoxicity from singlet oxygen generation and inhibition of the ETC. TMRM is the least toxic and the non-quenching concentrations employed in this experiment do not inhibit the ETC. Thirdly, for acute stimulants, like the lipophilic acid FCCP that directly dissipates the mitochondrial membrane potential, TMRM should be loaded prior to stimulation. In contrast, for slower...
acting stimulants (>10 minutes) TMRM should be loaded after treatment. This will provide a static comparison of pre-existing relative mitochondrial polarization between control and experimental treatments. Fourthly, MitoTracker green should be used to ensure alterations in TMRM signals are not due to alterations in mitochondrial mass [311]. Finally, during the experiment it was noted that dead cells fail to take up TMRM dye (Figure 3.19 a). As chitosan is a toxic compound (Figure 3.6), it became clear that viability dyes should be used to ensure that TMRM intensity is only quantified in the mitochondria of single live cells.

BMDCs were treated with chitosan (1.5, 3 or 6 µg/mL) for 3 hours and then stained with TMRM, MitoTracker Green and BV510 viability stain. As a positive control, cells were pre-loaded with dyes and then treated with the ionophore uncoupler FCCP (5 µM) for 5 minutes. In contrast to chitosan’s robust effects on mtROS production and mitochondrial division in BMDCs, none of the concentrations of chitosan tested had an effect on TMRM intensity in live cells. In fact, TMRM intensity was comparable between chitosan treated and untreated-cells in terms of percentages of polarised live cells and TMRM MFI in live cells. As expected, FCCP dramatically reduced TMRM intensity; the percentage of polarised live cells and TMRM MFI in live cells dramatically decreased to 2.88 % and 471 respectively compared to 95.8 % and 13793 in media-treated cells (Figure 3.19 b). When TMRM intensity was analysed without the use of a live dead stain, untreated cells appeared to have a large percentage of depolarised mitochondria. This percentage further increased upon chitosan treatment (Figure 3.19 c). As mentioned above, this fall in polarised mitochondria is due to cell death, as when the live dead stain in included, the depolarised populations largely disappeared (Figure 3.19 a).

To corroborate these results, membrane potential was visualised in BMDCs by Confocal microscopy. BMDCs were grown overnight in live imaging wells. The next morning, non-adherent cells were removed and cells were treated with media or chitosan. One well was left untreated for later addition of FCCP. After 2.5 hours, TMRM, MitoTracker Green and Hoescht stain, a viability stain which targets nuclear DNA, were added to the three wells. After 30 minutes, media-treated and chitosan-treated cells were visualised. FCCP was added to the third well and analysed roughly 30 minutes later. Both FCCP and chitosan treatments caused cell death, evident from the reduced number of stained nuclei compared to the untreated wells. The red TMRM signal was clearly maintained in the live cells of medium-treated cells. This resulted in an orange signal surrounding blue nuclei when TMRM, MitoTracker Green and Hoescht were overlaid. The TMRM signal was also preserved in the mitochondria of live cells upon stimulation with chitosan.
However, the TMRM signal disappeared in the mitochondria of dead cells, visualised as green specks not surrounded by blue nuclei. As expected, FCCP completely dissipated the membrane potential, preventing TMRM accumulation. As a result, the FCCP treated cells displayed green-only mitochondria (Figure 3.20 a).

Figure 3.17. Chitosan induces mitochondrial fission in BMDCs.
BMDCs were treated with media or protasan (4 µg/mL) for 3 hours. Cells were subsequently washed and mitochondria were stained with TMRM (red) and nuclei stained with Hoechst 33342 (blue). Cells were analysed by confocal microscopy. N=3.
Figure 3.18 Chitosan treatment moderately reduces Mitochondrial Mass.

BMDCs were treated with protasan (1.5, 3, 6 and 12 µg/mL) for 3 hours or FCCP (5 µM) for 10 minutes. Single, live cells were analysed for MitoTracker Green fluorescence. Data are expressed as mean ± SD of biological quadruplets normalised to media controls. Statistical analysis was performed by one-way ANOVA. ****p<0.0001. BMDCs were treated with protasan (1.5, 3, 6 and 12 µg/mL) for 3 hours, 6 or 24 hours. Single, live cells were analysed for MitoTracker Green fluorescence. n=1. MG= Mitotracker Green. MFI= Mean fluorescence intensity.
Figure 3.19 Chitosan treatment does not affect the membrane potential of live cells
(a) Gating strategy for cells stained with TMRM, MitoTracker Green, and BV510 viability stain. (b-c) BMDCs were treated with media, protasan (4 µg/mL) or FCCP (5 µM) as a positive control for 5 minutes. (b) TMRM fluorescence in single, MitoTracker Green+ cells (Mitochondria of all cells). (c) TMRM fluorescence in single, MitoTracker Green+, Live cells (mitochondria of live cells). Data are representative of four independent experiments. MFI= Mean fluorescence intensity. FMO= Fluorescence minus one
Figure 3.20. Chitosan does not drive BMDC depolarisation

BMDCs were treated with media or protasan (4 µg/mL) for 3 hours. Cells were subsequently washed and stained with Mitotracker Green (green), TMRM (red) and Hoechst 33342 (blue). Cells were analysed by confocal microscopy. n=2.
3.2.5. Chitosan increases DC size and granularity.

During the course of the MitoSOX and DC maturation experiments it became clear that chitosan treatment impacted on cell size and granularity. To quantify this effect, BMDCs were treated with chitosan, CpG or LPS for 24 hours and then live cells were analysed for size (FSC-A) and granularity (SSC-A) (Figure 3.21 a). While all treatments increased cell size compared to the media control, only chitosan treatment increased cell granularity. Chitosan-induced increases in cell size and granularity were dose-dependent, while the highest concentration of chitosan increasing cell size to levels equivalent to LPS and CpG, and increasing cell granularity significantly above LPS and CpG (Figure 3.21 b-c).

In FACS, doublet exclusion gates are used to exclude any event (a cell passing through the laser beam) that appears to consist of 2 independent particles. Given the dramatic effect of chitosan on cell size and granularity, it is possible that doublet exclusion gating is incorrectly identifying portions of the chitosan-treated cell population as two cells rather than one larger and more granular cell. If this were the case, doublet exclusion could affect the evaluation of mitochondrial stress and DC maturation. To determine if doublet exclusion gating affected chitosan-induced mitochondrial stress, BMDCs were treated with a range of concentrations of chitosan and then analysed for MitoSOX intensity with and without doublet exclusion (Figure 3.22 a). Importantly, while doublet exclusion did moderately reduce the percentage of mtROS* cells and MitoSOX MFI with all concentrations of chitosan tested, it was not significant (Figure 3.22 b). As such, doublet exclusion was consistently included in all experiments.
Figure 3.21 Chitosan-treatment increases cell size and granularity.
BMDCs were treated with indicated concentrations of protasan, CpG or LPS for 24 hours. (a) Gating strategy. (b) Representative histograms of FSC-A of live cells and median FSC-A ± SD of live cells from four independent experiments (c) Representative histograms of SSC-A of live cells and median SSC-A ± SD of live cells from four independent experiments. (d). (e) Statistical analysis was performed by one way ANOVA. **$p<0.01$; ****$p<0.0001$. FMO, Fluorescence minus one; FSC-A, Forward scatter area; SSC-A, Side scatter area.
Figure 3.22 Doublet exclusion moderately reduces chitosan-induced mitochondrial stress signals

BMDCs were treated with indicated concentrations of protasan. (a) Gating strategy for MitoSOX analysis in live cells with or without doublet exclusion gating. (b) MitoSOX MFI ± SD from four independent experiments. (c) % mtROS⁺ ± SD from four independent experiments. Statistical analysis was performed by two-tailed unpaired student t tests with the Holmes-Sidak method for multiple comparisons. MFI, Mean fluorescence intensity.
3.2.6. Chitosan requires Ca\textsuperscript{2+} to drive mitochondrial fission, mitochondrial stress, type I IFN production and DC maturation.

Increased intra-mitochondrial Ca\textsuperscript{2+} is a prerequisite for mitochondrial division in human osteosarcoma U2OS cells [213] and rat neurons [326]. In both studies it was shown that blockade of intra-mitochondrial Ca\textsuperscript{2+} entry inhibits efficient mitochondrial division by preventing IMM constriction [213], [326]. To elucidate the role of Ca\textsuperscript{2+} in chitosan-mediated mitochondrial division, BMDCs were pre-treated for one hour with BAPTA-AM (6 µM), a cell permeable ester that is rapidly hydrolysed to the Ca\textsuperscript{2+} chelator, BAPTA, in the cytosol, followed by a 3-hour stimulation with chitosan (4 µg/mL). BAPTA-AM blocked chitosan-induced mitochondrial fission. While chitosan treated cells displayed small and punctate mitochondria, cells pre-treated with BAPTA-AM displayed elongated mitochondria similar to the media control (Figure 3.23 a).

Intra-mitochondrial Ca\textsuperscript{2+} influx has been implicated in many mitochondrial processes, not just mitochondrial division. In particular, elevated IMM Ca\textsuperscript{2+} levels have been shown to drive mitochondrial ROS production and initiate formation of the MPTP to allow transport of mitochondrial matrix compounds [327]. Given that chitosan requires mtDNA to activate the cGAS-STING pathway, the confirmed role of mitochondrial stress in this process, and the ability of a MPTP inhibitor to block chitosan-induced IFN responses [328], it seemed highly plausible that Ca\textsuperscript{2+} could play a role in chitosan-induced mtROS production and subsequent type I IFN responses. To investigate the role of Ca\textsuperscript{2+} in chitosan-induced mtROS production, cells were pre-treated with BAPTA-AM (4 µM) and then stimulated with chitosan (2.5, 5 and 7 µg/mL) for 1.5 hours. Rotenone was used as a positive control. Ca\textsuperscript{2+} chelation dramatically reduced the percentage of mtROS\textsuperscript{+} and MitoSOX fluorescence upon chitosan treatment at all concentrations tested (Figure 3.24 a-b). This reduction was found to be significant when data from 3 independent experiments treated with 7 µg/mL of chitosan were pooled together (Figure 3.24 c). Importantly, pre-incubation of DCs with BAPTA-AM had no effect on Rotenone induced mitochondrial stress, with both control and BAPTA-AM treated DCs displaying similar percentages of MtROS\textsuperscript{+} cells and MitoSOX MFI (Figure 3.24 a-c).

Next it was investigated whether Ca\textsuperscript{2+} chelation blocks chitosan-induced IFN-β production. BMDCs were pre-treated for 1 hour with a range of concentrations of BAPTA-AM (4-10 µM), followed by three concentrations of chitosan (1.5, 3 and 6 µg/mL). Due to the toxicity of BAPTA-AM, the IFN-β ELISA results were not considered reliable for
concentrations greater than 4 µM (Figure 3.25 b). 4 µM BAPTA-AM supressed chitosan-induced IFN-β expression at all concentrations tested. However, CpG-induced IFN-β secretion was also compromised in the presence of 4 µM BAPTA-AM (Figure 3.25 a).

To further implicate Ca$^{2+}$ in chitosan-induced responses, the ability of Ca$^{2+}$ chelation to block chitosan-induced DC maturation was determined. BMDCs were pre-treated for 40 minutes with BAPTA-AM followed by 24 hours with media, indicated concentrations of chitosan or LPS. As previously demonstrated, chitosan treatment increased CD80 fluorescence and the percentage of CD86$^+$ and MHCcII$^+$ cells in a dose dependent manner (Figure 3.26 b-d). Ca$^{2+}$ chelation reduced chitosan-induced CD80 expression to near basal levels at all concentrations tested, while LPS-induced CD80 expression was not affected (Figure 3.26 b). Similarly, the percentage increase in CD86 and MHCcII expression with chitosan was reduced by Ca$^{2+}$ chelation. Importantly, BAPTA-AM treatment did not affect the ability of LPS to increase the percentage of CD86$^+$ and MHCcII$^+$ cells (Figure 3.26 c-d).
Figure 3.23. Ca\textsuperscript{2+} is needed for chitosan-induced mitochondrial division.
BMDCs were pre-treated for 40 minutes with 6 µM BAPTA-AM followed by a 3-hour stimulation with protasan (4 µg/mL). Cells were subsequently washed and mitochondria were stained with MitoTracker Green (green) and nuclei stained with Hoechst 33342 (blue). Cells were analysed by confocal microscopy. n=1
Figure 3.24 Chitosan requires Ca\textsuperscript{2+} to induce mtROS.

BMDCs were pre-treated for 1 hour with BAPTA-AM (4 μM) followed by 1.5 hours 2.5, 5 and 7 μg/mL of protasan. As a positive control, cells were treated with Rotenone (5 μM) for 6 hours. Single, live cells were analysed for MitoSOX fluorescence. (a) Count vs MitoSOX and (b) Unit area vs MitoSOX plots. (c) Data are expressed as mean ± SEM of experimental triplicates and was analysed by two-tailed unpaired student \textit{t} tests. Protasan: 7 μg/mL. ***\textit{p}<0.001. MFI= Mean fluorescence intensity.
Figure 3.25 Ca\(^{2+}\) is needed for chitosan-induced IFN-β secretion.

BMDCs were pre-treated for 1 hour with indicated concentrations of BAPTA-AM. Cells were then stimulated with media, indicated concentrations of protasan or CpG (4 µg/mL) for 24 hours. (a) Levels of IFN-β in the supernatants of stimulated cells. (b) Cytotoxicity induced by BAPTA-AM (range 4-10 µM) determined by LDH assay. Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by two-way ANOVA. ****p<0.0001.
Figure 3.26 Chitosan-induced CD80, CD86 and MHCII upregulation on BMDCs requires Ca^{2+}.

BMDCs were pre-treated for 1 hour with BAPTA-AM (6 µM) followed by 24 hours with protasan (2, 4, or 8 µg/mL) or LPS (10 ng/mL). (a) Single, live, CD11c^{+} cells were analysed for CD80, CD86 and MHCII expression. (b) CD80 MFI (c) % of CD86^{+} cells and (d) % of MHCII^{+} cells. n=2
3.2.7. Chitosan drives IP₃R-Ca²⁺ release from the endoplasmic reticulum.

There are two major sources of Ca²⁺ for a cell- the extracellular medium, a virtually unlimited reservoir of Ca²⁺ with concentrations up to 1 mM and intracellular pools such as the ER that allow the rapid release of Ca²⁺ through store resident channels [329]. To determine the source of Ca²⁺ needed for chitosan-induced responses, a series of experiments were set up to block Ca²⁺ uptake from the extracellular medium or the release of Ca²⁺ from the ER. As Ca²⁺ free RPMI was not available, the experiment was performed in another APC previously shown to respond to chitosan (Dr. Liz Carroll, PhD and [330]). BMDMs were stimulated in Ca²⁺-free DMEM followed by a 24-hour stimulation with chitosan. FBS media supplementation is critical for cell survival but is also a source of Ca²⁺. To avoid toxicity and keep extracellular Ca²⁺ levels low, FBS supplementation was reduced from 10 to 2 %. CpG was used as a positive control for IFN-β production, while LPS was used as a positive control for CXCL10 production. Chitosan failed to induce IFN-β secretion by cells in both complete and Ca²⁺ free medium (Figure 3.27 a), but did induce secretion of the ISG CXCL10 in the presence or absence of extracellular Ca²⁺ (Figure 3.27 b). Both CpG and LPS were similarly unaffected by the absence of extracellular Ca²⁺ (Figure 3.27 a-b). These data indicated that intracellular Ca²⁺ is required for chitosan-induced IFN-β.

To test this theory, BMDCs were pre-treated with 2-Aminoethoxydiphenyl borate (2-APB), an inhibitor of the IP₃R responsible for the release of Ca²⁺ from the ER (Figure 3.28). As CpG induced responses were inhibited by BAPTA-AM treatment (Figure 3.25 b), two additional controls, LPS and zymosan, were included. 2-ABP treatment dramatically reduced chitosan-induced IFN-β secretion at all concentrations tested (Figure 3.29 b and c). LPS and zymosan-induced IFN-β secretion remained intact (Figure 3.29 c), while CpG-induced IFN-β was significantly reduced (Figure 3.29 b).

To further explore the role of ER Ca²⁺ in chitosan-induced IFN-β production, the generation of IP₃ was blocked. Phospholipase C (PLC) hydrolyses phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P₂) into the soluble ligand inositol-1,4,5-triphosphate (IP₃) and the lipid diacylglycerol (DAG) (Figure 3.28). BMDCs were pre-treated for 40 minutes with the PLC inhibitor, U73122. Consistent with blocking IP₃R-dependent ER Ca²⁺ release, blocking this enzyme dramatically reduced IFN-β secretion upon stimulation with all three concentrations of chitosan, while LPS and zymosan-induced IFN-β production was not significantly changed (Figure 3.30 a). This pattern was also seen at
the mRNA level: U73122 treatment impaired the capacity of high concentrations of chitosan to induce *ifnb* mRNA transcription (Figure 3.30 b) but had no effect on the induction of *ifnb* mRNA by LPS and zymosan (Figure 3.30 c).

**Figure 3.27 Chitosan-induced CXCL10 secretion does not require extracellular Ca²⁺.**

BMDMs were stimulated in DMEM (media) or Ca²⁺ free DMEM with PBS, indicated concentrations of protasan, CpG (4 µg/mL) or LPS (10 ng/mL). Concentrations of IFN-β and CXCL10 in supernatants were determined by ELISA 24 hours later. Results are expressed as the mean ± SD for technical triplicate samples and represent data from two independent experiments. Statistical analysis was performed by two-tailed unpaired student *t* tests.
The ER is the largest reservoir of Ca\textsuperscript{2+} in the cell. ER Ca\textsuperscript{2+} importing mechanisms are mainly driven by SERCA proteins. Thapsigargin inhibits SERCA and causes depletion of the ER Ca\textsuperscript{2+} pool. ER- Ca\textsuperscript{2+} release is mediated by channels on its membrane that are gated by IP\textsubscript{3}R. Phospholipase C hydrolyses PIP\textsubscript{2} into membrane bound DAG and water soluble IP\textsubscript{3}. IP\textsubscript{3} migrates to the ER where it binds and opens IP\textsubscript{3}R-gated channels allowing Ca\textsuperscript{2+} release into the cytosol. U73122 blocks phospholipase C activity. 2-ABP prevents IP\textsubscript{3}R-gated channel opening and subsequent Ca\textsuperscript{2+} release.

Abbreviations: SERCA, sarco/endoplasmic-reticulum Ca\textsuperscript{2+} ATPase; IP\textsubscript{3}, Inositol 1,4,5-triphosphate; IP\textsubscript{3}R, IP\textsubscript{3}-receptor; PIP\textsubscript{2}, Phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol.
Figure 3.29 Chitosan-induced IFN-β secretion requires endoplasmic reticulum Ca²⁺ stores.

BMDCs were pre-treated for 40 minutes with indicated concentrations of the IP₃R inhibitor 2-ABP. Cells were then stimulated with media, indicated concentrations of protasan, CpG (4 µg/mL), LPS (10 ng/mL) or Zymosan (10 µg/mL) for 24 hours. (a) Cytotoxicity induced by 2-ABP (range 100-250 µM) determined by LDH assay. (b) Concentrations of IFN-β in the supernatants from independent experiments with different controls, one with CpG and the second with LPS and Zymosan. Results are expressed as the mean ± SD for technical triplicate samples and represent data from three independent experiments. (b-c) Statistical analysis was performed two-tailed unpaired student t tests. ***p<0.001, ****p<0.0001.
Figure 3.30 Phospholipase Cγ activity is required for the Chitosan-induced type I IFN response.

(a) BMDCs were pre-treated for 40 minutes with indicated concentrations of the PLC inhibitor U73122 (10 µM). Cells were then treated with medium, indicated concentrations of protasan, LPS (10 ng/mL) or Zymosan (10 µg/mL) for 24 hours. Results are expressed as the mean concentrations of IFN-β in the supernatant ± SD for technical triplicates and are representative of three independent experiments. Statistical analysis was performed by two-way ANOVA. ****p<0.0001.

(b) BMDCs were pre-treated for 40 minutes with indicated concentrations of the PLC inhibitor U73122 (10 µM). Cells were then stimulated with indicated concentrations of protasan for 24 hours or LPS (10 ng/mL) or Zymosan (10 µg/mL) for 4 hours. ifnb mRNA levels were calculated by qPCR with respect to actb. Results represent mRNA levels in technical triplicates, n=1. Statistical analysis was performed by two-way ANOVA. *p<0.05; ****p<0.0001.
3.2.8. Chitosan requires intact ER Ca\textsuperscript{2+} levels to drive mitochondrial ROS production and IFN-\(\beta\) secretion.

To further implicate ER Ca\textsuperscript{2+} stores in chitosan-induced mtROS and IFN-\(\beta\) production, BMDCs were pre-treated with the SERCA inhibitor thapsigargin (tpg). Ca\textsuperscript{2+} is constantly seeping out of the ER into the cytoplasm and SERCAs tirelessly pumps it back into the ER. When these pumps are blocked, ER Ca\textsuperscript{2+} stores become depleted [331] (Figure 3.28). BMDCs were pre-treated for 1 hour with 1 µM tpg, followed by 1.5 hrs with chitosan (1.5, 3 or 6 µg/mL) or 6 hours with 5 µM Rotenone. Tpg treatment reduced MitoSOX fluorescence and the percentage of mtROS\textsuperscript{+} cells at high concentrations of chitosan, albeit not significantly. The effect on lower concentrations of chitosan was more modest. Tpg had no effect on rotenone-induced MitoSOX fluorescence (Figure 3.31 a-c).

To analyse the effect of tpg treatment on chitosan-induced IFN-\(\beta\), BMDCs were pre-treated for 1 hour with tpg, followed by 24 hours with chitosan, CpG, LPS or zymosan. To rule out toxicity-dependent cytokine reductions, the effect of tpg on viability was assessed. Tpg treatments induced little cell death in BMDCs, despite potential impairment of ER function. This was demonstrated via LDH assay and live dead viability staining (Figure 3.32 a-b). Similar to the results obtained in the BAPTA-AM experiments, tpg treatment dramatically reduced chitosan- induced IFN-\(\beta\) production (Figure 3.32 d). In contrast, when cells were stimulated with CpG, LPS or zymosan, IFN-\(\beta\) was synergistically induced (Figure 3.32 d). These results were mimicked at the mRNA level (Figure 3.32 c). Tpg could not be used to implicate ER Ca\textsuperscript{2+} in chitosan-induced DC maturation as treatment alone increased CD80 and CD86 and the percentage of CD86\textsuperscript{+} cells, while reducing MFI and percentage of MHCcII\textsuperscript{+} cells (Figure 3.33 a-f).

It is speculated that the induction of type I IFNs by low concentrations of chitosan requires Dectin-1 engagement (Dr. Liz Carroll, PhD Thesis). Tpg-mediated ER Ca\textsuperscript{2+} disruptions cause protein folding dysfunction, and the accumulated misfolded proteins subsequently induce ER stress, ultimately leading to a halt on translation [167]. To ensure tpg-mediated suppression of IFN-\(\beta\) production was not due to reduced receptor expression, Dectin-1 expression levels were measured in tpg-treated CD11c\textsuperscript{+} DCs. Dectin-1 expression was preserved in tpg-treated BMDCs (Figure 3.34 b).
Figure 3.31 Chitosan requires ER Ca\(^{2+}\) to induce mtROS.

BMDCs were pre-treated for 40 minutes with Tpg (1 µM) followed by 1.5 hours with indicated concentrations of protasan. As a positive control cells were treated with Rotenone (5 µM) for 6 hours. Single, live cells were analysed for MitoSOX fluorescence. (a) Count vs MitoSOX and (b) Unit area vs MitoSOX plots are representative of three independent experiments. (c) Data combined from three independent experiments were analysed by two-tailed unpaired student t tests. MFI= Mean fluorescence intensity.
Figure 3.32 Chitosan-induced IFN-β secretion requires endoplasmic reticulum Ca²⁺ stores.

(a) Cytotoxicity induced by 24-hour tpg treatment (range 125-1000 nM) determined by LDH assay. Results are expressed as mean ± SD for technical triplicates and were analysed by one-way ANOVA. n=1 (b) Cytotoxicity induced by 24-hour tpg treatment assessed by flow cytometry. Results are expressed as mean ± SD for biological duplicates and were analysed by two tailed unpaired t test. **p<0.01 (c) BMDCs were pre-treated for 40 minutes with tpg and then stimulated with indicated concentrations of protasan for 24 hours or CpG (4 µg/mL), LPS (10 ng/mL) or Zymosan (10 µg/mL) for 4 hours. ifnb mRNA levels were calculated by qPCR with respect to actb. Results represent mRNA levels in technical triplicates and represent data from three independent experiments. (d) BMDCs were pre-treated for 40 minutes with tpg. Cells were then stimulated with media, indicated concentrations of protasan, CpG (4 µg/mL), LPS (10 ng/mL) or Zymosan (10 µg/mL). After 24 hours, the levels of IFN-β in the supernatants were measured by ELISA. Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by two-tailed unpaired student t tests *p<0.05; ***p<0.001, ****p<0.0001.
Figure 3.33 Thapsigargin treatment alters basal expression of CD80, CD86 and MHCII

BMDCs were treated for 24 hours with media or tpg (1 µM) (a) Single, live, CD11c+ cells were analysed for (a-b) CD80, (c-d) CD86 and (e-f) MHCII expression. n=2. Values on histograms correspond to mean fluorescence intensity.
Figure 3.34 Thapsigargin does not alter dectin-1 cell surface expression.
(a) Gating strategy used to define CD11c+ BMDC population. Cells were gated to include single and live cells. F4/80− CD11b+ CD11c+ positive cells were gated on for dectin-1 analysis. (b) BMDCs were left untreated (Grey shaded) or treated with 1 µM tpg for 2 hours. N=1.
3.2.9. Chitosan induces Ca\textsuperscript{2+}-dependent ER stress.

The data so far suggests that chitosan drives ER-mitochondria Ca\textsuperscript{2+} transfer resulting in mitochondrial stress and mtDNA egress. Unfortunately, ER matrix and intramitochondrial Ca\textsuperscript{2+} levels cannot be accurately measured in BMDCs as these cells are exquisitely sensitive to transfection techniques that permit organelle-specific Ca\textsuperscript{2+} measurements [332]. As ER Ca\textsuperscript{2+} perturbation induces ER stress, the ER stress response was used as an indirect method of determining ER Ca\textsuperscript{2+} efflux. BMDCs were treated with chitosan for 2, 4 or 6 hours or tpg for 3 hours as it is known to induce Ca\textsuperscript{2+}-dependent ER stress. ER stress is monitored by measuring activation of the UPR. The downstream outputs of the UPR master regulators were monitored by qRT-PCR.

Chitosan induced a classic ER stress-UPR response, as measured by the expression of the transcription factor \textit{atf4} (Figure 3.35 b), its downstream pro-apoptotic target \textit{chop} (Figure 3.35 c), the ER chaperone \textit{bip} (Figure 3.35 d), and the transcription factor \textit{xbp1} (Figure 3.35 e). This response was comparable in magnitude to the positive control tpg for \textit{bip} and \textit{atf4} (Figure 3.35 a-c). Tpg repeatedly failed to upregulate \textit{xbp1} expression, so an additional control, tunicamycin (tun) was used for this gene (Figure 3.35 d). Interestingly, chitosan drove an oscillating ER stress expression pattern; whereby 2 and 6-hour stimulations drove high ER stress, while 4-hour stimulations displayed little to no ER stress (Figure 3.35 a-d).
Figure 3.35 Chitosan induces ER stress.

(a) In response to ER stress, the UPR is activated. The UPR is initiated by three ER transmembrane proteins: IRE1α, PERK and ATF6. During unstressed conditions, the ER chaperone, BiP binds to the luminal domains of these master regulators, keeping them inactive. ATF6 transits to the Golgi where it is cleaved by S1 and S2 proteases, generating an active transcription factor. This processed form of ATF6 translocates to the nucleus to activate UPR that can be monitored by qRT-PCR. PERK oligomerizes, autophosphorylates, and directly phosphorylates the α subunit of EIF2. The transcription factor ATF4 mRNA requires eIF2α phosphorylation for translation. ATF4 drives UPR gene expression that can be monitored by qRT-PCR. IRE1α dimerizes and autophosphorylates to become active. Activated IRE1α splices XBP-1. Spliced XBP-1 mRNA encodes a transcription factor that upregulates UPR target genes that can be monitored by qRT-PCR.

(b-e) BMDCs were treated with protasan (8 µg/mL) for 2, 4 or 6 hours. As positive controls, cells were treated with 1 µM tpg for 3 hours or 4 µg/mL tun for 5 hours. Figures a-d show the expression of mRNA encoding (a) chop, (b) bip, (c) atf4 or (d) xbp1. mRNA levels were calculated by qPCR with respect to actb and results are presented relative to those of untreated cells. Data are expressed as the mean ± SD of samples in technical triplicate and are representative of five independent experiments. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparisons test. UT vs treated. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Abbreviations: IRE1α, Inositol Requiring 1α; PERK, PKR-like ER kinase; ATF, Activating Transcription Factor; BiP, Binding-immunoglobulin protein; S1, Site 1; EIF2, eukaryotic translation initiation factor 2; CHOP, CCAAT-enhancer-binding protein homologous protein; XBP-1, X-box binding protein 1.
Figure 3.36 Ca\(^{2+}\) regulates chitosan-induced mitochondrial fission, stress and DNA release

Chitosan drives IP\(_3\)-dependent Ca\(^{2+}\) release from the ER. ER Ca\(^{2+}\) depletion results in ER stress and activation of the UPR response. Ca\(^{2+}\) flows into the mitochondria, dictating sites for IMM constriction and subsequent fission and reduction in mitochondrial mass. In addition, Ca\(^{2+}\) influx promotes mitochondrial stress and opening of the MPTP, driving MOMP and eventual rupture of the inner mitochondrial membrane (IMM). mtDNA is then released into the cytosol where it engages with cGAS generating cGAMP. cGAMP binds to STING at the ER and triggers relocation to the ERGIC where type I IFN signalling occurs.

Abbreviations: ER, Endoplasmic Reticulum; ERGIC, ER-golgi intermediate compartment; Mito, mitochondria; IP\(_3\), inositol-1,4,5-triphosphate; IP\(_3\)R, IP\(_3\) receptor; IMM, inner mitochondrial membrane; MPTP, mitochondrial permeability transition pore; MOMP, mitochondrial outer membrane permeabilization.
3.3. Discussion

This work implicates ER Ca\(^{2+}\) in chitosan-induced IFN responses by promoting mitochondrial stress that triggers mitochondrial DNA egress and subsequent cGAS-STING activation. Depletion of ER Ca\(^{2+}\) stores limited chitosan-induced mtROS and abrogated \(i\text{fnb}\) transcription and translation. Chelation of cytosolic Ca\(^{2+}\) with BAPTA-AM, which would block Ca\(^{2+}\) transfer from the ER into mitochondria, reduced mtROS and blocked IFN-\(\beta\) production and the upregulation of co-stimulatory molecules on DCs. Blockage of IP\(_3\)-R-gated Ca\(^{2+}\) channels or the ligand responsible for opening these channels reduced chitosan-induced IFN-\(\beta\) responses. Finally, chitosan was shown to induce ER stress two hours after treatment to levels comparable to the positive control, thapsigargin. Taken together this data places ER to mitochondrial Ca\(^{2+}\) transfer at the heart of chitosan-induced mtROS production.

This work identifies mtDNA as the link between chitosan-induced mtROS production and cGAS-STING activation. mtDNA depleted DCs failed to elicit type I IFN responses upon chitosan treatment. Oxidized DNA can enhance STING-dependent type I IFN induction due to its increased resistance to cytosolic TREX1-mediated degradation [119]. Given the rapid (within 1 hr) and sustained (up to 24 hours) mitochondrial oxidative stress induced by chitosan, it is likely that mtDNA becomes oxidised to enhance cGAS-STING signalling. This work contributes to the large body of literature implicating mitochondrial dysfunction and matrix DAMPs in PRR activation settings.

How mtDNA is released from the matrix under conditions of cellular stress is controversial. During apoptosis, BAX and BAK can promote the formation of pores within the OMM causing MOMP. Over time, these pores can expand through an unresolved mechanism, forming macropores that allow the release of mtDNA. Alternatively, the IMM may control MOMP through the MPTP. The MPTP is a multiprotein complex that forms in response to high mitochondrial Ca\(^{2+}\) levels and spans both mitochondrial membranes. Opening of the pore permits the efflux of matrix molecules that are much smaller than a mtDNA nucleoid. However, it is possible that sustained opening of the pore can lead to swelling of the mitochondria and subsequent rupture of the inner membrane, which would permit the efflux of mtDNA into the cytoplasm. Previous work demonstrated that scavenging of mtROS or inhibition of cyclophilin D, a core component of mitochondrial MPTP pore abrogates the capacity of chitosan to promote type I IFN production [328]. Building on these findings, this work suggests that chitosan promotes an MPTP-
dependent type I interferon response through increased mitochondrial Ca\(^{2+}\) levels and oxidative stress.

Mitochondrial division is known to support mtROS production in lymphocytes; T cells undergoing fission generate fragmented mitochondria with increased ROS production [333] that is essential for T effector cell activation [218]. Specifically, fission disrupts ETC supercomplex formation causing inefficient electron transfer along the respiratory chain and increasing electron leak that can generate mtROS [334], [335]. In a similar timeframe to the induction of mtROS, chitosan drove mitochondrial fission, characterised by a population of small, round and fragmented mitochondria with lower overall mass. This would suggest that the relationship between mitochondrial fission and ROS production is not limited to lymphocytes. In support of this, stimulation of macrophages with live Escherichia coli was shown to decrease supercomplex formation in a ROS-dependent manner, resulting in impaired bacterial clearance [336]. Unfortunately, the authors did not directly visualise mitochondrial fission and further research is needed to delineate the relationship between mitochondrial fission/fusion and metabolic reprogramming in DCs and macrophages.

This work demonstrates for the first time a role for Ca\(^{2+}\) in mitochondrial fission in an innate immune cell. Chitosan-mediated formation of small and fragmented mitochondria was blocked by cytosolic Ca\(^{2+}\) chelation with BAPTA-AM. Experiments carried out in human osteosarcoma U20S cells [213] and rat neurons [326] would suggest this is due to a block on ER-mitochondrial Ca\(^{2+}\) transfer that is critical for IMM constriction and subsequent Drp-1-mediated fission. Increased mitochondrial intra-luminal Ca\(^{2+}\) levels additionally promote loose cristae and activate matrix dehydrogenases that ramp up ETC activity [213], [322], [326], [337]. Since fission can redirect the output of the ETC from ATP to ROS, this work proposes a mechanism whereby Ca\(^{2+}\) promotes chitosan-induced mtROS by increasing ETC activity in mitochondria that are skewed toward electron leakage. Indeed, inhibition of complexes I and III (the main sources of ROS in the ETC) decrease chitosan-induced IFN-\(\beta\) production. Future studies should demonstrate the direct effect of ETC inhibitors on chitosan-induced ROS and quantify the reduced oxygen consumption rates when chitosan-induced intra-mitochondrial Ca\(^{2+}\) influxes are blocked.

Chitosan was not shown to induce mitochondrial depolarisation in BMDCs. This was unexpected given that mitochondrial Ca\(^{2+}\) uptake consumes membrane potential [338]. However, membrane potential was analysed at fixed time points, and as Ca\(^{2+}\) influx events can be promptly followed by MPTP formation and CytC release [339], it is
possible that depolarisation events were discounted as death. Alternatively, although Ca\(^{2+}\) influx reduced membrane potential, Ca\(^{2+}\) export occurs via antiporters in an exchange process for either sodium or hydrogen. Thus, simultaneous functioning of the mitochondrial Ca\(^{2+}\) uniporter (MCU) and the antiporters can maintain mitochondrial membrane potential and optimal Ca\(^{2+}\) concentrations in the mitochondria [340]. Live imaging of chitosan-treated DCs will provide concrete evidence of whether chitosan induces membrane depolarisation.

ER stress was monitored after chitosan stimulation as an indirect method of monitoring a Ca\(^{2+}\) efflux event. Changes in intra-luminal ER Ca\(^{2+}\) cause ER stress as protein chaperone functions and protein folding processes require high levels of calcium. However, several studies have reported ER stress to be a downstream consequence of STING activation. It appears that while inactive STING maintains ER Ca\(^{2+}\) homeostasis, activated STING stimulates the release of ER Ca\(^{2+}\), subsequently causing ER stress [138], [179], [341]. Chitosan-mediated expression of UPR target genes displayed two peaks, one after 2 hours of treatment and another 6 hours after treatment. Chitosan-induced mtROS production is detectable after 1 hour of treatment, whereas type I IFNs appear after 9 hours. Taken together this data suggests the first wave of UPR gene transcription is the result of chitosan-mediated ER Ca\(^{2+}\) release, and not a consequence of STING activation. This must be verified by monitoring ER stress induction and more importantly ER Ca\(^{2+}\) release in settings where chitosan-induced STING activation cannot occur, such as in mtDNA depleted DCs and DCs from cgas\(^{-/-}\) mice. Critically, ER stress plays a negative role in DC cross presentation [206]–[208]. For instance, hyperactivation of IRE1α in CD8α\(^{+}\) DCs drives RIDD-dependent defects in cross presentation to OT-1 T cells due to the degradation of components of the cross-presentation machinery, such as tapasin [205]. Several ER stress inhibitors are US Food and Drug Administration–approved drugs with an excellent safety profile; thus, future work should investigate whether alleviating ER stress can amplify STING-dependent CD8\(^{+}\) T cell responses.

Chitosan-induced Th1 immune responses, in addition to cGAS-STING signalling, are dependent on NLRP3. While it remains unclear how chitosan activates NLRP3 and how NLRP3 promotes chitosan-induced Th1 responses, this work has ruled out the notion that NLRP3 contributes by supporting mitochondrial stress and thus cGAS-STING signalling. DCs pre-treated with the NLRP3 specific inhibitor MCC950 and stimulated with chitosan had no impairment in chitosan-induced mtROS production or type I IFN responses. Future work should investigate the role of mtROS in chitosan-induced NLRP3 inflammasome activation, as it may be required for both cGAS-STING and NLRP3
activation and the point in which these pathways separate. Although controversial, mtDNA that is oxidised by mtROS is speculated to directly bind and activate NLRP3 in murine BMDMs [57], [342]. As such, it should be explored whether ox-mtDNA serves as a DAMP for NLRP3 inflammasome activation after chitosan treatment.

Another avenue that should be explored is the role of lysosomal rupture in chitosan-induced NLRP3 activation. Previous work has demonstrated that chitosan polymers with similar features to the protasan formulations used in this study, drive lysosomal rupture [343]. cGAS-STING signalling can induce a cell death programme dependent on lysosomal rupture that initiates K⁺ efflux upstream of NLRP3 [344]. With this in mind, it is possible that chitosan polymers drive STING-dependent lysosomal rupture that triggers K⁺ efflux to activate NLRP3. It’s also conceivable that ER stress is responsible for chitosan-induced NLRP3 inflammasome activation. In murine macrophages, ER stress can trigger mitochondrial damage and the release of contents that activate the NLRP3 inflammasome [197], [345].

The work presented in this chapter enhances our knowledge of the mechanism by which the vaccine adjuvant chitosan activates DCs to elicit Th1 mediated cellular immunity. Treatment of DCs with chitosan triggers the release of Ca²⁺ from the ER, resulting in ER stress and more importantly, mitochondrial dysfunction that promotes the release of mtDNA and activation of the cGAS-STING pathway. Future work must address the role of chitosan-induced mitochondrial dysfunction in NLRP3 activation.
4. Chitin-derived polymer deacetylation regulates mitochondrial reactive oxygen species dependent cGAS-STING and NLRP3 inflammasome activation

4.1. Introduction, Aims and Hypothesis

The cationic polysaccharide, chitosan is a 1-4-β-linked polymer comprised of N-Ac-glucosamine and glucosamine units that is under investigation as a vaccine adjuvant and biomaterial (Figure 4.1). The polymer is generated by the chemical deacetylation of chitin, one of the most abundant polymers in nature, second only to cellulose, which is found naturally in the cell wall of fungi, exoskeletons of crustacea and insects [295]. Deacetylation of chitin through the use of chitin deacetylases or treatment in alkaline conditions, leads to the conversion of acetyl groups to amine groups, which are distributed along a glucosamine backbone. The pKa of the amine groups on the glucosamine units is approximately 6.5, meaning that the majority of these will be protonated under biological conditions, giving the chitosan polymer a net positive charge and subsequently mucoadhesive properties [346].

![GlcN and GlcNAc](image)

**Figure 4.1** Chemical structure of glucosamine (GlcN) in its protonated form and N-acetylglucosamine (GlcNAc).

Much of the literature to date has focused on chitosan preparations with DD in the region of 70–95%. Such preparations generally have the remaining acetyl groups arranged in blocks, or clusters that are distributed heterogeneously along the chitosan chain.
Alternatively, chitosan polymers can be produced with much lower DD—typically ranging from 35-70%. These polymers display even dispersal of acetyl groups along the chitosan chain, commonly referred to as a homogenous distribution [346].

Although several groups have addressed the effects of the DD on the immunostimulatory properties of chitosan, some of these studies have been inconclusive due to issues such as endotoxin contamination and inconsistencies between individual batches and formulations from different sources [347], [348]. Indeed, it is hugely difficult to obtain chitosan formulations that are standardised with respect to DD or molecular weight. This study compared a series of highly characterized and purified polymers, each with a different DD or molecular weight, prepared in the same laboratory. In doing so, it identified the mechanistic basis underlying the differential immunomodulatory effects of chitin-derived polymers and identified an in vitro process that can be used to choose the optimal chitosan formulation for different biomedical applications from vaccine adjuvants to drug delivery systems and scaffolds.

Adjuvants can be incorporated into vaccines to enhance the magnitude and type of innate and adaptive immune response induced [267], [349]. Their success depends on the maturation of DCs, a key determinant in the immunostimulatory effects of vaccines. Adjuvants can activate DCs either directly by engaging with PRRs or indirectly through the delivery of danger signals to the cell which bind to PRRs located both externally and internally in the cells. This activation leads to enhanced expression of MHC and co-stimulatory molecules on the DC surface in addition to the secretion of cytokines which direct T cell polarization and differentiation [33]. The Lavelle lab previously demonstrated that that the chitosan salt, protasan CL213 is superior to the most widely used vaccine adjuvant alum, in promoting Th1 immune responses [11], [71]. Protasan triggers indirect activation of the cGAS-STING pathway through the induction of mitochondrial stress and subsequent release of mtDNA into the cytosol. This results in the production of type I IFNs, leading to IFNAR-dependent DC maturation and Th1 immune responses. In addition, through an unknown mechanism, NLPR3, in conjunction with cGAS-STING signaling, is pivotal to chitosan’s adjuvanticity [71]. This study aimed to identify the precise characteristics of chitin-derived polymers that dictate the extent of DC activation and NLRP3-dependent IL-1β release and the mechanistic basis by which polymer physicochemical properties regulate immune activation.
The hypothesis underlying this work is that the ability of chitin-derived adjuvants to promote activation of the NLRP3 inflammasome, the cGAS-STING pathway and antigen-specific cellular immunity is principally dictated by the degree of polymer deacetylation and the distribution of acetyl groups. With this in mind, the specific aims of this chapter include

- Determining the effect of altering chitosan deacetylation and molecular weight on cGAS-STING activation.
- Determining the effect of altering chitosan deacetylation and molecular weight on IL-1β release.
- Determine the contribution of mtROS on chitosan-induced IL-1β release.
- Identify the optimal chitosan formulation for promotion of antigen-specific cellular immunity.
4.2. Results

4.2.1. High levels of deacetylation are required for chitosan-induced mitochondrial stress

The Lavelle group reported in 2016 that commercially available 86 % deacetylated chitosan, referred to as protasan, activates the cGAS-STING pathway resulting in IFNAR-dependent DC maturation and Th1-immune responses [71]. While this finding gave great impetus for its translational use as an adjuvant to promote cellular immunity, it was at odds with a phase I/IIa clinical trial containing a novel adjuvant called Viscogel and Haemophilus influenzae type b glycoconjugate vaccine (Act-Hib). Viscogel was a chitosan-based formulation containing water and 50 % deacetylated chitosan, and while it was safe and well tolerated, it had a modest effect on cellular or humoral immune responses in vaccinated subjects compared to antigen alone (NCT01578070) [293], [294]. It was speculated that this was a result of the adjuvant’s low DD and subsequent inability to activate the cGAS-STING pathway. To prove this, a panel of chitosan polymers with 38 %, 49 %, 72 %, 90 %, and 100 % deacetylation were generated, herein referred to as C38, C49, C72, C90 and C100 (Figure 4.2 b).

Mitochondrial stress was measured given its role in chitosan-induced cGAS-STING activation. As in section 3.2, the mitochondrial-specific ROS indicator MitoSOX was used to selectively detect superoxide in the mitochondria of live cells after stimulation with C38, C49, C72, C90, C100, protasan or rotenone (Figure 4.2 a). As expected, rotenone drove mitochondrial stress, visualised as an increase in MitoSOX fluorescence and the percentage of mtROS+ cells. C38, C49 and C72 failed to enhance MitoSOX fluorescence or percentage of mtROS+ cells compared to media treatment alone. In contrast, protasan, C90 and C100 drove robust mitochondrial ROS (Figure 4.2 c-d). C100 was the strongest mitochondrial stress inducer, particularly when cells were treated with low concentrations of polymers (Figure 4.3 a-b).

C100 differs from the other chitosan polymers in that chains of positively charged amines are not disrupted by acetyl groups at random intervals. It was speculated that chains of amines are responsible for chitosan-induced mitochondrial ROS. To prove this, two chitosan polymers were generated with identical degrees of deacetylation but alternative distribution of acetyl groups. While both polymers had 83 % of acetyl groups removed,
the remaining 17% of acetyl groups were either clustered together to leave long chains of amines free (referred to as heterogenous distribution) or distributed evenly over the polymer (homogenous distribution) (Figure 4.4 a). As expected, homogenous chitosan polymers failed to drive mitochondrial stress, while heterogenous chitosan significantly enhanced MitoSOX fluorescence and the percentage of mtROS+ cells compared to media alone (Figure 4.4 b-d).
Figure 4.2 Highly deacetylated chitosan polymers enhance production of mitochondrial reactive oxygen species in BMDCs.

BMDCs were treated with chitin-derived polymers (5 µg/mL) for 1.5 hours or rotenone (5 µM) for 6 hours. (a) Chemical structure and chitin and chitosan polymers (b) Gating strategy (b) FSC-W vs MitoSOX (c) Unit area vs MitoSOX. Values correspond to mean fluorescence intensity. Data are representative of $n=4$. 
Figure 4.3 The degree of polymer deacetylation dictates the extent of mitochondrial stress in BMDCs.

(a-b) Analysis of mtROS production in single, live BMDCs treated with indicated concentrations of chitin-derived polymers (2, 4 or 8 µg/mL) for 1.5 hours or rotenone (5 µM) for 6 hours. (a) FSC-W vs MitoSOX (b) Unit area vs MitoSOX. Values correspond to mean fluorescence intensity. Data are representative of $n=4$. 

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Figure 4.4 Chains of deacetylated amines are responsible for chitosan-induced mitochondrial stress

(a) Deacetylation of chitin can be homogenous or heterogenous. Polymers with a homogenous pattern of deacetylation have uniform distribution of acetyl and amine groups along the polyglucosamine backbone. Heterogenous chitosan polymers have clusters of acetyl groups separated by long chains of positively charged amine groups along the polyglucosamine backbone. (b-d) Analysis of mtROS production in single, live BMDCs treated with 8 µg/mL 83 % (C83) homogenously or heterogeneously deacetylated chitosan polymers for 1.5 hours or 5 µM rotenone for 6 hours. (a) FSC-W
vs MitoSOX (b) Unit area vs MitoSOX. Values correspond to mean fluorescence intensity. (c) Data expressed as mean ± SD of biological duplicates were analysed by one-way ANOVA. *p<0.05 **p<0.01. MFI= Mean fluorescence intensity

4.2.2. The degree of polymer deacetylation and the acetylation pattern dictates the extent of STING-dependent type I IFN production and IFNAR-dependent DC maturation.

Protasan-induced mitochondrial stress activates the cGAS-STING pathway, resulting in type I IFN production and IFNAR-dependent DC maturation [71]. It was speculated that polymers which failed to drive mitochondrial ROS would not produce type I IFNs or cause DC maturation, and that C90 and C100 would be the strongest inducers of type I IFN secretion and DC maturation. The TLR9 agonist, CpG and TLR4 agonist, LPS were used as positive controls for IFN-β and CXCL10 expression and secretion. As expected, both drove significant IFN-β and CXCL10 production. C38, C49 and C72 treated cells failed to produce IFN-β or CXCL10 compared to media controls. C90 treatment drove higher levels of IFN-β and CXCL10 secretion compared to protasan treatment. The potency of C90 over protasan was particularly evident when comparing protein secretion in response to low concentrations of polymers. In contrast to C90, C100-induced type I IFN responses were weak, with little or no increase in IFN-β or CXCL10 respectively compared to media controls (Figure 4.5 a-b). As with protasan, C90 and C100-induced secretion of IFN-β or CXCL10 were STING dependent (Figure 4.6 a-b). Despite driving lower levels of IFN-β protein secretion than protasan and C90, C100-induced similar expression levels of ifna and ifnb mRNA and this process was entirely STING dependent (Figure 4.6 c-d). In contrast, LPS and CpG induced secretion of IFN-β and CXCL10 were STING independent, as was LPS-induced upregulation of ifna and ifnb mRNA (Figure 4.6 a-d).

In line with the mitochondrial stress data (Figure 4.4 a-c), a heterogenous chitosan polymer (83% deacetylation) drove high levels of IFN-β and CXCL10 secretion while 83% deacetylated chitosan with a homogenous distribution of acetyl groups did not (Figure 4.7 a-b), further supporting the notion that long chains of positively charged amine groups, not interrupted by acetylated sugar moieties, are critical for mitochondrial stress and subsequent cGAS-STING activation.
Next, BMDCs were treated for 24 hours with chitin-derived polymers, LPS or CpG and monitored for DC maturation. Two additional controls, the synthetic STING agonist DMXAA and mammalian STING agonist, 2’3’-cGAMP were used. While DMXAA is cell permeable, 2’3’-cGAMP is large, electronegative and thus extremely impermeable to the cell membrane. In order to access the cytosol to activate STING it must be transfected into cells or used at very high concentrations. Given the sensitivity of BMDCs to transfection, saturating concentrations of 2’3’-cGAMP were used. Single, live CD11c<sup>+</sup>, MHC<sub>II</sub><sup>hi</sup> cells were analysed for the expression of CD80 and CD86 (Figure 4.8 a). As expected, LPS, CpG, DMXAA and 2’3’ cGAMP enhanced the expression of CD80 and CD86 compared to media controls (Figure 4.8 b-c). In line with the mitochondria stress and type I IFN data, C38, C49 and C72 treated cells failed to enhance CD80 or CD86 expression compared to media controls. In fact, CD80 and CD86 expression appeared to decrease as the degree of acetylation increased. Notably, C38 treatment reduced the MFI of CD80 from 9165 to 4700 and CD86 from 2628 to 482 (Figure 4.8 b-c). In contrast, C90, C100 and protasan treatment enhanced expression of CD80 and CD86. Despite driving lower levels of IFN-β protein secretion than protasan and C90, C100 promoted the highest expression of CD80 and CD86 (Figure 4.8 b-c). While DMXAA and 2’3’-cGAMP drove much higher levels of CD86 expression compared to the three chitin-derived STING agonists, CD80 expression was comparable in response to all five STING agonists (Figure 4.8 b).

To determine whether C90 and C100-induced DC maturation was IFNAR-dependent, BMDCs from WT and Ifnar1<sup>−/−</sup> mice were stimulated for 24 hours with 2, 4 or 8 µg/mL of chitin-derived polymers and then analysed for the expression of CD80 and CD86 on single, live, CD11c<sup>+</sup>, MHC<sub>II</sub><sup>hi</sup>cells. BMDCs were additionally stimulated with LPS, CpG as negative controls and DMXAA and 2’3’-cGAMP for 24 hours. To our knowledge the role of IFNAR in DMXAA and 2’3cGAMP-induced DC maturation has not been explored. As expected, LPS and CpG induced CD80 and CD86 expression was IFNAR-independent. As before, C100-induced expression of CD80 and CD86 was higher than that seen with C90 and protasan. Its potency over both compounds was most obvious when BMDCs were treated with the low concentration of the three polymers. Importantly, all three polymers failed to enhance CD80 and CD86 expression in Ifnar1<sup>−/−</sup> BMDCs. DMXAA and 2’3cGAMP induced upregulation of CD80 and CD86 was not reduced in Ifnar1<sup>−/−</sup> BMDCs (Figure 4.9 a-b). In fact, 2’3’-cGAMP induced expression of CD86 rose from 4751 in WT treated DCs to 9008 in Ifnar1<sup>−/−</sup> treated BMDCs (Figure 4.9 b). Similarly, 2’3 cGAMP induced expression of CD80 rose from 8315 in WT treated DCs to 11666.
Ifnar1<sup>−/−</sup> treated BMDCs (Figure 4.9 a). DMXAA induced expression of CD80 and CD86 was comparable between WT and Ifnar1<sup>−/−</sup> treated BMDCs (Figure 4.9 a-b).

**Figure 4.5 The degree of chitin polymer deacetylation dictates the extent of IFN-β and CXCL10 secretion in BMDCs**

ELISA analysis of (a) IFN-β and (b) CXCL10 secretion in the supernatants of BMDCs stimulated for 24 hours with 0.8, 1.5, 3, 6 or 8 ug/mL of chitin-derived polymers, CpG (4 µg/mL) or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by multiple one-way ANOVAs. Media Vs Treatment. *p<0.05, ***p <0.001, ****p< 0.0001.
**Figure 4.6 Chitosan-induced type I IFN responses are dependent on STING.**

ELISA analysis of (a) IFN-β and (b) CXCL10 secretion in the supernatants of WT and \( Tmem173^{-/-} \) BMDCs stimulated for 24 hours with indicated concentrations of chitin-derived polymers, CpG (4 µg/mL) or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. (c-d) BMDCs from WT and \( Tmem173^{-/-} \) mice were treated with chitin-derived polymers (8 µg/mL) for 24 hours or LPS (10 ng/mL) for 2 hours. mRNA levels were calculated by qPCR for (a) \( ifna \) and (b) \( ifnb \) with respect to \( actb \) and \( rps18 \). Data shows mean mRNA expression ± SD for technical triplicate calculated with respect to \( actb \) and is representative of three independent experiments. Statistical significance was determined by Two-tailed unpaired student’s \( t \) tests with the Holm-sidak method for multiple comparisons. *\( p < 0.05 \), **\( p < 0.001 \), ****\( p < 0.0001 \).
Figure 4.7 Chains of deacetylated amines are responsible for chitosan-induced IFN-β and CXCL10 secretion in BMDCs

ELISA analysis of (a) IFN-β and (b) CXCL10 secretion in the supernatants of BMDCs stimulated for 24 hours with indicated concentrations of homogenous or heterogenous C83 or CpG (4 µg/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was determined by Two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. Homogenous C83 vs Heterogenous C83. ***p < 0.001.
Figure 4.8 Deacetylation dictates the extent of chitosan-induced CD80 and CD86 upregulation

BMDCs from WT mice were treated with C90, C100 or protasan (8 µg/mL), CpG (4 µg/mL), LPS (10 ng/mL), DMXAA (10 µg/mL) or 2’3’-cGAMP (1 µM) for 24 hours. After this period, cells were collected and stained for flow cytometry. (a) BMDCs were gated as single, live, CD11c+, MHCIIhi cells and then analysed for expression of (b) CD80 and (c) CD86. Black line: media. Shaded histogram: treatment. Values correspond to Mean fluorescence intensity. n=5.
Figure 4.9 C90, C100 and protasan-induced upregulation of CD80 and CD86 is IFNAR-dependent.

BMDCs from WT and Ifnar1−/− mice were treated with C90, C100 or protasan (2, 4 or 8 µg/mL), CpG (4 µg/mL), LPS (10 ng/mL), DMXAA (10 µg/mL) or 2′3′-cGAMP (1 µM) for
24 hours. Single, live, CD11c<sup>+</sup>, MHC<sub>II<sup>Hi</sup> cells were analysed for expression of (a) CD80 and (b) CD86. Black line: media. Shaded histogram: treatment. Values correspond to Mean fluorescence intensity. n=3

4.2.3. The immunostimulatory effects of C100 apply across a range of molecular weights

The data thus far highlights the importance of chitosan deacetylation for mitochondrial stress and subsequent activation of the cGAS-STING pathway. It was suggested that molecular weight could be an additional defining parameter in the immunogenicity of chitin-derived polymers, as increasing the molecular weight would increase the length of positively charged, deacetylated chitosan chains (Figure 4.10 a). BMDCs were treated with C100 polymers of four molecular weights, 115 kDa, 140 kDa, 250 kDa and 350 kDa and analysed for mitochondrial stress, type I IFN secretion and DC maturation. Of note, all previous experiments utilised 115 kDa C100 polymers. All four C100 polymers were more effective than C90, protasan and rotenone at driving mitochondrial ROS, evident by the larger increase in percentage of mtROS<sup>+</sup> cells and MitoSOX fluorescence. There was no substantial difference in mitochondrial stress between the four C100 polymers of different molecular weight (Figure 4.10 b-d-c).

In line with previous data, C100 was a poor inducer of IFN-β and CXCL10 secretion regardless of polymer chain length. Despite a lack of IFN-β secretion, all C100 polymers outperformed protasan and C90 in terms of upregulation of CD80 and CD86 expression. C100-induced CD80 and CD86 expression was moderately affected by polymer chain length, as low molecular weight C100 polymers were less effective at enhancing CD80 and CD86 expression compared to high molecular weight C100 polymers. As before, CpG, LPS, DMXAA and 2’3’-cGAMP enhanced expression of CD80 and CD86 (Figure 4.12 a-b).
a

Chitin

Acetyl group

PG backbone

Protonated amine group

↓

100 % Deacetylation

C100

Low Mw

PG chain length = Mw

High Mw

b

<table>
<thead>
<tr>
<th>Medium</th>
<th>Rotenone</th>
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<tr>
<td>mtROS+ cells 5.24</td>
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<tr>
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<td>C100 140 kDA</td>
</tr>
<tr>
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</tr>
<tr>
<td>mtROS+ cells 48.5</td>
<td>mtROS+ cells 52.1</td>
</tr>
</tbody>
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C90

mtROS+ cells 28.6

Protasan

mtROS+ cells 18.6
Figure 4.10 C100 induces mitochondrial ROS across a broad range of molecular weights.

(a) BMDCs were treated with chitin-derived polymers (8 µg/mL) for 1.5 hours or Rotenone (5 µM) for 6 hours. Single live cells were analysed for % of mtROS⁺ cells and MitoSOX fluorescence. (a) Molecular weight determines the length of polyglucosamine chains (b) FSC-A vs MitoSOX, (c-d) Unit area vs MitoSOX. Values correspond to mean fluorescence intensity. n=3.
Figure 4.11 C100 polymers are poor inducers of IFN-β and CXCL10 secretion irrespective of molecular weight

ELISA analysis of (a) IFN-β and (b) CXCL10 secretion in the supernatants of WT BMDCs stimulated for 24 hours with chitin derived polymers (1.5, 3 or 6 µg/mL), CpG (4 µg/mL) or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments.
Figure 4.12 C100-induced CD80 and CD86 expression in DCs is affected by polymer molecular weight

BMDCs were treated with 115, 140, 250 or 350 kDa C100 polymers (2, 4 or 8 µg/mL), CpG (4 µg/mL), LPS (10 ng/mL) DMXAA (10 µg/mL) or 2’3’-cGAMP (1 µM) for 24 hours. After this period, cells were collected and stained for flow cytometry. BMDCs were gated as single, live, CD11c+, MHCIIhi cells and then analysed for expression of (a) CD80 and (b) CD86. Figures show representative histograms and Mean fluorescence intensity values for CD80 and CD86. n=3. Black line=Media control. Shaded histograms = treatments.
4.2.4. Incubation with C100 drives rapid BMDC cell death

As demonstrated in section 3.2.1, protasan drives a two phase-type I IFN response, with the first wave occurring 8 hours after stimulation and the second more extensive wave occurring >12 hours after stimulation. Given that C100 drove the strongest IFNAR-dependent DC maturation, yet was the weakest driver of IFN-β secretion, it was speculated that premature cell death after C100, but not C90 and protasan treatment, prevented the second wave of type I IFN and CXCL10 secretion after IFNAR ligation. To investigate whether C100 was more toxic to BMDCs than C90 and protasan, BMDCs were treated for 2 hours with indicated concentrations of the chitin-derived polymers and then collected and stained with the viability exclusion dye BV510. The ability of chitosan polymers to drive cell death depended on the extent of deacetylation. Polymers with a DD of 70% and lower drove little or no death, while highly deacetylated polymers were the most toxic, with C100 driving the most cell death in BMDCs, followed by C90 and then protasan (Figure 4.13 a). The higher toxicity of C100 over other chitosan polymers was more apparent after 24 hours treatment, with all three concentrations of C100 tested driving at least 20% more death than less deacetylated polymers. Of note, as with LPS and CpG, 24-hour treatment of BMDCs with DMXAA and 2’3cGAMP induced little or no cell death (Figure 4.14 a). In line with the inability to drive IFN-β and CXCL10 secretion, C100 polymers irrespective of molecular weight were highly toxic to BMDCs driving more death than protasan or C90 (Figure 4.15 a).
**Figure 4.13 C100 drives BMDC death after 2 hours.**

BMDCs were treated for 2 hours with indicated concentrations of chitin-derived polymers or for 6 hours with rotenone (5 µM). Cells were collected and analysed for death using a live/dead stain (BV510). Gated on single cells. n=3.
Figure 4.14 Prolonged C100 treatment is highly toxic to BMDCs

BMDCs were treated for 24 hours with indicated concentrations of chitin-derived polymers, CpG (4 µg/mL), LPS (10 ng/mL), DMXAA (10 µg/mL) and 2’3’-cGAMP (1 µM). Cells were collected and analysed for death using a live/dead stain (BV510). Gated on single cells. n=3.
Figure 4.15 C100 polymers are toxic to BMDCs across a broad range of molecular weights

BMDCs were treated with C100 polymers of various molecular weight, C90 or protasan for 2 hours or rotenone (5 µM) for 6 hours. Cells were collected and analysed for death using a live/dead stain (BV510). Gated on single cells. $n=3$. 
4.2.5. **C100 toxicity prevents type I IFN feedback signalling**

Next, it was necessary to address if C90 and C100, like protasan, trigger a two-phase type I IFN response. BMDCs were treated for 30 minutes, 1, 2, 4, 8, 12, 16, 20 or 24 hours with C90, C100, protasan, DMXAA, 2’3’-cGAMP and etoposide and then lysed and analysed for mRNA expression of *ifna* and *ifnb*. Etoposide is a topoisomerase inhibitor that activates STING via the induction of DNA damage [350]. Protasan displayed the expected two-phase type I IFN response, with an initial increase in *ifna* and *ifnb* mRNA expression emerging after 8 hours of treatment, and a second more extensive wave peaking after 16 hours of treatment. The kinetic profile of type I IFN expression was nearly identical between C90 and protasan treated BMDCs. However, the C90-induced *ifna* and *ifnb* mRNA waves were higher than that seen with protasan and C90-induced *ifnb* continued to rise 24 hours later, unlike protasan which appeared to have plateaued. In contrast, C100-induced *ifna* and *ifnb* mRNA were delayed in comparison to protasan and C90 with the first peak appearing 12 hours after treatment and the second wave emerging 20 hours after treatment and continuing to rise 24 hours later (Figure 4.16 a-b). A limitation of many STING agonists is the transient nature of their activated signalling. Indeed, DMXAA-induced *ifna* mRNA expression arose one hour after stimulation, with a second wave appearing 4 hours later and the signal disappearing within 8 hours (Figure 4.16 a). Likewise, DMXAA-induced *ifnb* mRNA expression appeared rapidly, within one hour of treatment, peaked within two hours of treatment and began to decline by 4 hours, with mean fold mRNA expression falling from -40,000 to 10,0000 (Figure 4.16 b). 2’3’-cGAMP-induced *ifna* and *ifnb* mRNA expression occurred in two phases, the first peaking after 2 hours of treatment, and the second peaking after 8 hours of treatment. Of note, the 2’3’-cGAMP-induced *ifna* and *ifnb* mRNA expression was slower to arise, peak and dissipate than with DMXAA. 2’3’-cGAMP-induced *ifna* and *ifnb* mRNA expression, albeit low, was still detectable 20 hours after stimulation. In contrast to both DMXAA and 2’3’-cGAMP, Etoposide-induced *ifna* and *ifnb* mRNA expression was low and slow, with significant expression only arising 16 hours and 20 hours after stimulation respectively (Figure 4.16 a-b). As with C90 and C100 treatment, etoposide-induced *ifnb* mRNA expression was highest 24 hours later (Figure 4.16 b).

To further explore the role of feedback signalling on chitosan-induced type I IFN responses, BMDCs were stimulated for 12, 24, 36 or 48 hours with protasan, C90, C100, DMXAA, cGAMP or Etoposide and then monitored for the accumulation of IFN-β and CXCL10 over a time course. Consistent with the mRNA data, none of the three chitosan
formulations or etoposide drove IFN-β or CXCL10 secretion within 12 hours of treatment (Figure 4.17 a-b). In contrast, DMXAA and cGAMP stimulated DCs reached peak IFN-β secretion after 12 hours of stimulation, with levels remaining largely steady over the following 36 hours (Figure 4.17 a). DMXAA-induced CXCL10 production reached its peak after 24 hours, with comparable levels detected in the supernatants of 36- and 48-hour stimulations. 2’3’-cGAMP-induced secretion of CXCL10 was slower than DMXAA, requiring 36 hours of stimulation for peak CXCL10 secretion (Figure 4.17 b). While 2’3’-cGAMP and DMXAA induced CXCL10 secretion preceded IFN-β production, protasan and C90 induced IFN-β and CXCL10 production was concurrent and both cytokines displayed a similar kinetic profile. For instance, protasan-induced IFN-β and CXCL10 secretion appeared after 24 hours and slightly increased to its highest levels after 36 hours of treatment. C90-induced IFN-β and CXCL10 secretion required a 24-hour incubation, with levels of IFN-β and CXCL10 remaining consistent regardless of additional incubation times (Figure 4.17 a-b). Despite having slower kinetics, protasan and C90 drove similar levels of CXCL10 secretion to DMXAA and 2’3’-cGAMP (Figure 4.17 b). C100 drove minimal IFN-β and no CXCL10 secretion across the range of incubation times tested. Despite driving less IFN-β than C100, etoposide drove the secretion of CXCL10, with similar levels observed after 24-, 36- and 48-hours of stimulation (Figure 4.17 a-b).

To confirm the contribution of feedback signalling to chitosan-induced type I IFN responses, BMDCs from WT and *Ifnar1*−/− mice were stimulated for 24, 48 and 72 hours with protasan, C90, C100, DMXAA, cGAMP or Etoposide. IFN-β secretion was markedly reduced in *Ifnar1*−/− DCs treated for 24 hours with C90, Protasan, 2’3’-cGAMP, and etoposide compared to WTs. As for C100, *Ifnar1*−/− DCs failed to produce any detectable IFN-β. Of note, the low levels of IFN-β produced by *Ifnar1*−/− BDMCs stimulated with C90 or protasan was equivalent to levels produced by C100 stimulated WTs (Figure 4.18a). 48-hour incubations bore similar results; C90, C100 and protasan treated *Ifnar1*−/− BMDCs showed significantly reduced production of IFN-β compared to WTs. Again, the residual levels of IFN-β in *Ifnar1*−/− BMDCs stimulated with C90 and protasan was equivalent to that produced by C100-stimulated WTs. In contrast to the 24-hour treatments, 48-hour treatment with etoposide and 2’3’-cGAMP masked the requirement of the IFNAR receptor for optimal IFN-β secretion (Figure 4.18 b). A similar observation was observed after 72-hour treatment with C90, protasan, etoposide and 2’3’-cGAMP, with WTs producing similar levels to *Ifnar1*−/− DCs. C100-induced IFN-β secretion remained IFNAR-dependent even after 72-hour incubation. As with 24- and 48-hour treatments, 72-hour C100 treatment drove equal levels of IFN-β secretion to *Ifnar1*−/− DCs treated with C90.
and protasan (Figure 4.18 c). C90 and protasan induced CXCL10 production was IFNAR-dependant at all timepoints investigated. BMDCs from both WT and Ifnar1⁻/⁻ mice did not secrete CXCL10 in response to stimulation with C100 at all timepoints tested (Figure 4.18 d-f). Etoposide induced CXCL10 production appeared after 72 hours of treatment and was IFNAR-dependent (Figure 4.18 f). DMXAA and 2’3’-cGAMP induced CXCL10 secretion was partially dependent on IFNAR, with IFNAR deficiency significantly reducing but not eliminating CXCL10 production at all timepoints tested (Figure 4.18 d-f).
a

**C90**

- Etoposide
  - ****
  - **

- DMXAA
  - ***
  - ****

**C100**

- Protasen
  - ****
  - ****
  - ****
  - ****

- cGAMP
  - ****
  - ****
  - ****

*Time (Hrs)*
Figure 4.16 Highly deactylated polymers drive a two phase *ifna* and *ifnb* mRNA expression profile

BMDCs from WT mice were treated with chitin-derived polymers (5 µg/mL), Etoposide (50 µM), DMXAA (10 µg/mL) or 2’3’-cGAMP (1 µM) for 30 minutes, 1, 2, 4, 8, 12, 16, 20 or 24 hours. mRNA levels were measured by qPCR for (a) *ifna* and (b) *ifnb* with respect to *actb* and *rps18*. Data are expressed as mean ± SD for technical triplicate mRNA levels with respect to *actb* and is representative of three independent experiments. Statistical significance was determined by one-way ANOVA. **p<0.01, ***p<0.001, ****p<0.0001.
Figure 4.17 Time course analysis of IFN-β and CXCL10 secretion in response to STING agonists.

BMDCs from WT mice were treated for 12, 24, 36 or 48 hours with chitin-derived polymers (6 µg/mL), 2’3’-cGAMP (1 µM), DMXAA (10 µg/mL) or etoposide (50 µM). Supernatants were collected and levels of IFN-β and CXCL10 were determined by ELISA. Results are expressed as the mean ± SD for technical triplicates and represent data from two independent experiments.
Figure 4.18 C90, C100 and protasan-induced IFN-β is partially dependent on IFNAR.

ELISA analysis of (a-c) IFN-β and (d-f) CXCL10 secretion in the supernatants of WT and Ifnar1−/− BMDCs stimulated for 24, 48 or 72 hours with chitin-derived polymers (6 µg/mL), Etoposide (50 µM), DMXAA (10 µg/mL) or 2’3’-cGAMP (1 µM). Results are expressed as the mean ± SD for technical triplicates and represent data from two independent experiments. Statistical significance was determined by two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 4.19 Viability is required for chitosan-induced positive IFN feedback signalling

C90, C100 and protasan drive STING-dependent IFN-β secretion. (1) IFN-β binds the IFNAR receptor driving ISGF3-dependent gene transcription. DCs subsequently express increased levels of CD80 and CD86 on their surface and secrete IFN-β and CXCL10. (2) Newly secreted IFN-β binds the IFNAR receptor initiating a positive feedback loop. Viability is required for positive feedback signalling. Unlike protasan and C90, C100 treatment is highly toxic to DCs preventing the IFN feedback amplification loop in vitro.
4.2.6. Toxic chitin-derived polymers elicit autophagy to limit cell death and sustain IFN-I responses

Fong et al, previously demonstrated that chitosan entry into the endocytic pathway causes lysosomal rupture [343]. The DD dictated the extent of lysosomal rupture, as primary amines have a pK\(_a\) value of 6.5, and became protonated in acidifying lysosomal lumens, driving the accumulation of chloride ions and water, causing lysosomal swelling and subsequent rupture. It was hypothesised that lysosomal rupture was responsible for chitosan-induced cell death, as C100 are the most toxic of the chitosan panel and have the highest number of amine groups. To investigate, BMDCs were pre-treated with bafilomycin A (BafA), an inhibitor of endosomal acidification, stimulated with chitosan for 2 or 24 hours and then monitored for cell viability. BafA treatment alone was not toxic to BMDCs, had no effect on zymosan-induced cell death and slightly reduced the toxicity of rotenone treatment. Lysosomal rupture was not the cause of chitosan-induced cell death, as BafA pre-treatment increased the toxicity of high concentrations of C90 and C100 after two hours of treatment (Figure 4.20 a). This unexpected protective role of endosomal acidification on C100 and C90 viability was mirrored 24 hours later. Surprisingly in this instance, C90 induced more cell death than C100. Long term BafA treatment slightly reduced basal viability but had no effect on cycloheximide-induced cell death (Figure 4.20 b). Short and long term BafA treatments had no effect on the protasan-induced viability (Figure 4.20 a-b).

BafA blocks endosomal acidification downstream of endocytosis and closely-related autophagy [351]. It was postulated that BafA increases the toxicity of highly deacetylated chitosan polymers by blocking autophagy and allowing the accumulation of damaged organelles. To investigate, BMDMs were pre-treated for 40 minutes with BafA and then stimulated for 2 hours with C38, C49, C72, C90, C100, protasan or rapamycin as a positive control. Note, BMDCs were not used to monitor autophagic flux as high basal levels result in very little or no induction of autophagy on stimulation [352]. As expected, BafA blocked autophagic flux, evident in the increased expression of LC3-II in the media control. Rapamycin caused an increase in LC3-II levels, that was marginally increased by blocking LC3 degradation with BafA. In contrast, C38, C49 and C72 polymers drove no net increase in LC3-II accumulation and BafA addition only elevated LC3-II to the extent of the BafA alone control. C90, C100 and protasan increased autophagic flux was so rapid that LC3-II accumulation could only be visualised in the presence of BafA (Figure 4.21 a).
These results were mirrored in iBMM expressing GFP-LC3. To quantify the membrane associated fraction of LC3-II, the membrane unbound, cytosolic LC3-I form of the protein was removed from cells by digitonin treatment (Figure 4.22 a). As before, BafA was crucial for detecting protasan and C100-induced LC3-II accumulation. While bafilomycin A treatment alone caused a small increase in GFP-LC3 fluorescence, reflecting basal levels of autophagy, a strong increase in fluorescence was observed when cells were co-cultured with C90 or C100 (Figure 4.22 b). As seen in BMDCs, BafA treatment rescued iBMMs from chitosan-induced cell death. When iBMMs were treated with BafA, C90 or C100 alone, they exhibited little or no cell death. However, when treated in combination with BafA and C90, or BafA and C100, there was a dramatic drop in cell viability (Figure 4.22 c).

Altogether this data suggests that autophagy regulates chitosan-induced type I IFN responses by limiting cell death that allows sustained signalling. To verify autophagy’s role in promoting chitosan-induced type I IFNs, BMDCs were grown from the bone marrow of ATG16L partial knock down (PKD) mice [13]. ATG16L is required for correct localisation of the ATG12-ATG5 conjugate to the isolation membrane (Figure 1.2). CpG was used as a positive control given the role of LAP in CpG-induced type I IFN responses [174], [353]. LPS was used as a negative control. As hypothesised, there was a modest reduction in chitosan-induced IFN-β production in ATG16L−/− PKD BMDCs. Furthermore, CpG-induced IFN-β was dramatically reduced and there was no significant change in LPS-induced IFN-β production (Figure 4.23 a).
Figure 4.20 VATPase activity is protective against chitosan-induced cell death

(a) BMDCs were pre-treated for 40 minutes with BafA (100 nM) followed by 1.5 hours with indicated concentrations of protasan. As a positive control, cells were treated with Rotenone (5 µM) for 6 hours or zymosan (10 µg/mL) for 2 hours. Viability of single cells. n=1. (b) BMDCs were pre-treated for 40 minutes with BafA (100 nM) followed by 24 hours with chitin-derived polymers (4 µg/mL) or cycloheximide (2 µg/mL). Data are expressed as mean viability of single cells ± SD of biological duplicates. Statistical analysis was determined by Two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. *p<0.05, **p<0.01.
Figure 4.21 Highly deacetylated chitin-derived polymers drive rapid LC3 lipidation and degradation

BMDMs were pre-treated for 40 minutes with BafA (40 nM) and then stimulated for 2 hours with chitin-derived polymers (20 µg/mL) or 5 hours with rapamycin (10 µM). Cells were then lysed and blotted for endogenous levels of LC3. β-actin was employed as a loading control. n=3
a

Cells

Single cells

Live cells

GFP-LC3+ cells

+ Digitonin

Cells

Single cells

Live cells

GFP-LC3+ cells

FMO L/D

Unstained iBMM

b

Media C9C

Media C100

Ctrl

GFP-LC3

Baf

GFP-LC3

GFP-LC3
Figure 4.22 C100-induced autophagy is protective against cell death in iBMMs
GFP-LC3 iBMM were pre-treated with BafA (100 nM) for 40 minutes and then stimulated for 5 hours with C90 or C100 (15 µg/mL) (a) Single, live cells were analysed for GFP-LC3 expression before and after 60 µg/mL digitonin treatment. (b) Histogram analysis of membrane-bound GFP-LC3 after digitonin treatment (c) Representative viability plots for GFP-iBMMs. n=2.
Figure 4.23 Autophagy regulates chitosan-induced IFN-β production.

WT and ATG16L partial knock BMDCs were stimulated for 24 hours with protasan (4 µg/mL), CpG (4 µg/mL), or LPS (10 ng/mL). The levels of IFN-β protein in the supernatant of cells was determined by ELISA. Results are expressed as the mean ± SD for technical triplicates. Statistical analysis was performed using two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. ****p <0.0001. n=3.
Figure 4.24 Highly deacetylated chitin-derivatives drive autophagy to promote cell survival and IFNAR signalling

Highly deacetylated chitin derivatives drive mitochondrial stress and subsequent STING-dependent type I IFN signalling. In addition, these polymers activate autophagy through an unknown mechanism. Autophagy promotes cell survival, likely through the removal of damaged organelles, allowing positive IFNAR feedback signalling.
4.2.7. High levels of deacetylation and clusters of deacetylated amines are required for NLRP3 inflammasome activation.

Given that the NLRP3 inflammasome, alongside the cGAS-STING pathway is critical for protasan-induced cellular immunity, we sought to determine whether C90 and C100 also drive IL-1β secretion. As a positive control, cells were treated with alum and LPS. As expected, alum and LPS co-treatment drove high amounts of IL-1β release. C90, C100 and protasan were robust inducers of IL-1β release, with 115 kDa C100 outperforming C90 at all concentrations tested, and C90 similarly outperforming protasan. In fact, all four C100 polymers outperformed protasan, C90 and alum. While high concentrations of 115 and 140 kDa Mw C100 polymers were stronger at driving IL-1β release, the opposite was true for low concentrations with 1.5 µg/mL of 115 kDa C100 driving less IL-1β release than 140, 250 and 350 kDa C100 polymers (Figure 4.25 a).

Next, it was assessed whether chitosan-induced IL-1β release in DCs is sensitive to patterns of deacetylation. In line with the IFN-β and CXCL10 secretion data, mitochondrial stress inducing heterogenous C83 polymers drove IL-1β release, while homogenous polymers that do not drive mitochondrial stress failed to enhance IL-1β secretion (Figure 4.26 a).
Figure 4.25 C100-induced inflammasome activation by BMDCs occurs across a broad range of molecular weights

BMDCs were stimulated with media or LPS (10 ng/mL) alone or together with chitin-derived polymers (0.8, 1.5, 3 & 6 µg/mL) or Alum (10 ng/mL). Levels of IL-1β were measured in supernatants 24 hours later. Results are expressed as the mean ± SD for technical triplicates and are representative of three independent experiments.
Figure 4.26 Chitin-derived polymer-induced inflammasome activation by BMDCs is dependent on the distribution of deacetylation.

BMDCs were stimulated with media or LPS (10 ng/mL) alone or in combination with homogenous or heterogenous C83 polymers (1.5, 3 or 6 µg/mL) or alum (10 µg/mL). IL-1β concentrations were measured in supernatants 24 hours later. Results are expressed as the mean ± SD for technical triplicates and are representative of three independent experiments. Statistical analysis was performed by two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. **p<0.01, ****p<0.0001.
4.2.8. C90, C100 and protasan-induced inflammasome activation is dependent on mitochondrial stress

It seems plausible that NLRP3, like cGAS-activation, is dependent on mitochondrial stress given that the optimal polymers for activation of both pathways are the strongest inducers of mtROS. BMDCs were pre-treated with saturating concentrations of MitoTEMPO and co-stimulated with LPS and chitin-derived polymers or LPS and alum. Of note, this concentration of MitoTEMPO was not toxic to BMDCs (data not shown). As before, C100 was the strongest inducer of IL-1β release at all concentrations tested, followed by C90 and then protasan. MitoTEMPO completely inhibited protasan-induced IL-1β secretion at all concentrations tested and C90-induced IL-1β release at low concentrations. MitoTEMPO inhibition of IL-1β release was only partial with increasing C90 concentrations. A similar outline held true for C100-treated DCs with low concentrations more susceptible to ROS scavenging than higher concentrations. Of note, MitoTEMPO had little effect on alum-induced IL-1β release (Figure 4.27 a).

Two scenarios were postulated- (1) that C90 and C100 polymers can additionally drive mitochondrial-independent stress that contributes to NLRP3 activation or (2), that residual IL-1β release is due to un-scavenged mtROS after MitoTEMPO treatment. To investigate, BMDCs were stimulated for 2 hours with C90, C100 and Protasan, 6 hours with Rotenone or 9 hours with PMA and analysed for mitochondrial and cellular ROS levels. As demonstrated previously, C100, C90, protasan and rotenone increased MitoSOX fluorescence, PMA enhanced CellROX fluorescence and protasan had no effect on CellROX fluorescence (Figure 3.9 a-b). Similar to protasan, C90 and C100 treatment failed to enhance CellROX MFI compared to the media control (Figure 4.28 b).

To quantify the degree of mitochondrial scavenging by MitoTEMPO, BMDCs were pre-treated for 45 minutes with MitoTEMPO and then stimulated for 1.5 hours with 2,4 or 8 µg/mL of chitin-derived polymers. As demonstrated previously, protasan, C90 and C100-induced mitochondrial stress was dose-dependent, with C100 being the optimal inducer of mtROS at all concentrations tested. While MitoTEMPO alone increased MitoSOX fluorescence from an MFI of 461 to 560, it reduced protasan-induced mitochondrial stress at all concentrations tested, with the highest concentration displaying a drop in MFI from 2129 to 778. C90-induced mitochondrial stress was less sensitive to MitoTEMPO treatment: in cells treated with 8 µg/mL C90, the MFI was reduced from
1355 to 1180 and in cells treated with 4 µg/mL C90 the MitoSOX MFI was reduced from 842 to 689. In line with this, the MitoSOX MFI in cells incubated with C100 was untouched by MitoTEMPO treatment, with all concentrations sustaining robust mitochondrial stress (Figure 4.29 a).

Figure 4.27 Protasan, C90 and C100-induced IL-1β release in BMDCs requires mitochondrial stress
(a) BMDCs were pre-treated for 40 minutes with MitoTEMPO (500 µM* saturating concentration) and then stimulated with media or LPS (10 ng/mL) alone or together with chitin-derived polymers (0.8-6.25 µg/mL) or Alum (10 ng/mL). IL-1β concentrations were measured in supernatants 24 hours later. Results are expressed as the mean ± SD for technical triplicates and are representative of three independent experiments. Statistical
analysis was performed by two-tailed unpaired student’s $t$ tests with the Holm-Sidak method for multiple comparisons. ***$p<0.001$, ****$p<0.0001$.

**Figure 4.28 C90 and C100 do not alter Cellular levels of ROS.**

BMDCs were treated with media, chitin-derived polymers (5 µg/mL) for 1.5 hours, Rotenone (5 µM) for 6 hours or phorbol 12-myristate 13-acetate (PMA) (5 µM) for 9 hours. Single, live cells were analysed for MitoSOX and CellROX fluorescence (see Figure 3.9 for gating). Data are expressed as the mean ±SD of experimental duplicates ($n=2$). (a) MitoSOX MFI (b) CellROX MFI. Statistical analysis was determined by one-way ANOVA. *$p<0.05$, **$p<0.01$. 
Figure 4.29 MitoTEMPO does not completely scavenge chitin-derived polymer-induced mtROS

BMDCs were pre-treated for 40 minutes with MitoTEMPO (500 µM* saturating concentration) and then stimulated with chitin-derived polymers (2, 4 or 8 µg/mL) for 1.5 hours. Single, live cells were analysed for MitoSOX fluorescence. Values on graph correspond to mean fluorescence intensity. n=2.
Figure 4.30 Chitosan-induced mitochondrial stress is required for activation of the NLRP3 inflammasome and the cGAS-STING DNA sensing pathway. Protasan induces Ca\(^{2+}\)-dependent mitochondrial stress. Mitochondrial stress subsequently activates the NLRP3 inflammasome to allow the release of active IL-1\(\beta\). The contribution of IL-1\(\beta\) in Th1 polarisation is unknown. Mitochondrial stress additionally triggers the egress of mtDNA, which engages the cGAS-STING pathway resulting in IFNAR-dependent DC maturation and subsequent Th1-mediated immune responses.
4.2.9. **C100 is the optimal polymer formulation for driving cellular immunity.**

Note: The following section has been taken from Turley and Moran, *et al.*, Chitin-derived polymer deacetylation regulates reactive oxygen species dependent cGAS-STING and NLRP3 inflammasome activation (manuscript ready for submission, see appendix). The *in vivo* vaccination data was performed by Dr Hannah Moran and Dr Craig McEntee.

The previous section has demonstrated the necessity of clustered amine groups on chitosan polymers for inducing mitochondrial ROS and subsequent type I IFN-dependent DC maturation and NLRP3 inflammasome activation *in vitro*. It was sought to translate these findings to an *in vivo* setting to validate the use of mitochondrial stress as a readout for the adjuvanticity of chitosan polymers and to confirm C100 as the optimal chitosan formulation. The clinically relevant TB antigen, hybrid 56 (H56), was used to assess the capacity of the adjuvants to promote antigen-specific Th1 cell type responses. C57BL/6 mice were immunized subcutaneously (s.c.) on day 0 with PBS, antigen alone, or antigen and C38, C49, C72, C90, C100 or protasan. On day 14, mice were boosted in the same manner and on day 21 mice were sacrificed and spleens and lymph nodes collected to allow for the measurement of antigen-specific IFN-γ. Re-stimulation with H56 at two different concentrations revealed a higher antigen-specific IFN-γ response in splenocytes and lymph nodes from mice vaccinated with H56 and C100 compared to those immunized with antigen alone. Importantly, C100 was the only chitosan polymer to significantly enhance antigen-specific IFN-γ responses in splenocytes and lymph compared to antigen alone (Figure 4.31 a-b).
Figure 4.31 C100 is the optimal adjuvant for promoting Th1 immune responses in splenocytes and inguinal lymph nodes.

C57BL/6 mice were immunised s.c on day 0 with PBS or H56 (0.5 μg/mouse), alone or in combination with chitin-derived polymers (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, inguinal lymph nodes and spleens were collected. (a) Inguinal lymph nodes (1x10^6 cells/mL) and (b) Splenocytes (2x10^6 cells/mL) were restimulated ex-vivo with either media or H56 (2 μg/mL or 10 μg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one-way ANOVA. Antigen alone Vs Antigen + Chitin-derived polymer. ****p<0.0001
4.2.10. C100 induced cellular immunity requires STING, IFNAR and NLRP3 signalling.

Studies were carried out to address the role of STING, the IFNAR receptor and the NLRP3 inflammasome in C100-induced cellular immunity. C57BL/6 WT and Tmem173\(^{+/−}\), Ifnar1\(^{-/−}\) and Nlrp3\(^{-/−}\) mice were vaccinated s.c. on day 0 and on day 14 as previously outlined with PBS, antigen, or C100 and antigen. A second clinically relevant antigen, chlamydial major outer membrane protein (MOMP) was used to monitor Th1 immune responses. The Th1 enhancing effects of C100 seen with H56 translated to the MOMP antigen where vaccination with C100 and MOMP significantly enhanced antigen-specific IFN-γ responses in splenocytes compared to PBS or antigen alone (Figure 4.32). Vaccination of Tmem173\(^{-/−}\) mice with antigen and C100 failed to promote splenic MOMP-specific IFN-γ responses. There was no global defect in IFN-γ responses by cells from these mice as the cells responded similarly to WTs when stimulated with anti-CD3 (Figure 4.32).

Similarly, vaccination of Ifnar1\(^{-/−}\) mice with H56 and C100 failed to enhance antigen-specific IFN-γ responses in splenocytes and lymph nodes compared to WT mice immunised with antigen alone. Secretion of IFN-γ by splenocytes in response to stimulation with anti-CD3 was comparable between WT and Ifnar1\(^{-/−}\) mice, whereas in the inguinal lymph nodes, anti-CD3 induced IFN-γ secretion was reduced in Ifnar1\(^{-/−}\) compared to WT mice (Figure 4.33 a-b). In order to further confirm these results, the same approach was repeated using the antigen MOMP. As with H56, the enhanced splenic Th1 responses measured following vaccination of C57BL/6 mice with MOMP and C100 was not seen in vaccinated Ifnar1\(^{-/−}\) mice. In response to re-stimulation with anti-CD3, there was no difference in IFN-γ secretion between groups (Figure 4.34).

A similar trend was observed for NLRP3, where MOMP-specific Th1 responses induced following vaccination with MOMP and C100 were markedly reduced or completely abolished in the splenocytes and lymph nodes of Nlrp3\(^{-/−}\) mice compared to WT controls. In contrast, IFN-γ secretion in anti-CD3 stimulated cells did not differ between groups in the spleen or lymph nodes (Figure 4.35 a-b)
Figure 4.32 C100-induced antigen-specific IFN-γ requires STING

C57BL/6 WT and Tmem173−/− mice were immunised s.c on day 0 with PBS or MOMP (5 µg/mouse), alone or in combination with C100 (200 µg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens were collected. (a) Splenocytes (2x10⁶ cells/mL) were restimulated ex-vivo with either media, MOMP (5 µg/mL) or anti-CD3 (0.25 µg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one-way ANOVA. ****p<0.0001.
Figure 4.33 C100-induced H56-specific IFN-γ requires IFNAR

C57BL/6 WT and Ifnar1<sup>−/−</sup> mice were immunised s.c on day 0 with PBS or H56 (0.5 μg/mouse) alone or in combination with C100 (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. (a) Inguinal lymph nodes (1x10<sup>6</sup> cells/mL) and (b) Splenocytes (2x10<sup>6</sup> cells/mL) were restimulated ex-vivo with either media or H56 (10 μg/mL) or anti-CD3 (0.25 μg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one-way ANOVA with Tukey’s multiple comparisons test. **p<0.01, ***p<0.001, ****p<0.0001.
Figure 4.34 C100-induced MOMP-specific IFN-γ requires IFNAR
C57BL/6 WT and Ifnar1−/− mice were immunised s.c on day 0 with PBS or MOMP (5 µg/mouse), alone or in combination with C100 (200 µg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens were collected. (a) Splenocytes (2x10⁶ cells/mL) were restimulated ex-vivo with either media, MOMP (10 µg/mL) or anti-CD3 (0.25 µg/mL). Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one-way ANOVA with Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ****p<0.0001.
Figure 4.35 C100-induced antigen-specific IFN-γ requires NLRP3
C57BL/6 WT and Nlrp3−/− mice were immunised s.c on day 0 with PBS or H56 (0.5 μg/mouse), alone or in combination with C100 (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. (a) Inguinal lymph nodes (1x10^6 cells/mL) and (b) Splenocytes (2x10^6 cells/mL) were restimulated ex-vivo with either media or H56 (10 μg/mL) or anti-CD3 (0.25 μg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one-way ANOVA with Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
4.3. Discussion

There is a considerable need for the development of new and improved vaccines that induce strong antigen-specific cellular immunity. As most purified subunit antigens are poorly immunogenic on their own, adjuvants are included in vaccine formulations to potentiate the immune response of the antigen. To date, very few particulate adjuvants are included in licensed vaccines, these include alum, oil-in-water emulsions, AS04 and AS01. Chitosan, a cationic polysaccharide, is an attractive candidate given its defined performance as a Th1-skewing adjuvant, its biocompatible and biodegradable nature and its wide use as a biomaterial for drug delivery, as a wound healing material and as a scaffold for living cells [346]. This may appear bizarre, given that an adjuvant and a biomaterial require drastically different properties, with the former being pro-inflammatory and the latter being anti-inflammatory or inert. This work unravelled how chitosan polymers can achieve both functions and the parameters that can be measured to obtain a chitosan formulation with appropriate properties for each application. Consequently, C100 was identified as the optimal chitosan formulation for use in vaccine settings.

The study utilised oxidative mitochondrial stress as a readout for chitosan adjuvanticity, due to its defined role in chitosan-induced cGAS-STING activation and likely role in NLRP3 inflammasome activation. In doing so, the study unraveled a structure: function relationship between the degree and pattern of deacetylation and chitosan adjuvanticity. While high acetylated chitosan polymers such as C39, C49 and C72 caused no mitochondrial stress or IFNAR-dependent DC maturation, highly deacetylated chitosan polymers with uninterrupted clusters of amines were inflammatory, activating STING signalling and promoting IFNAR-dependent DC maturation. It is now clear that acetylation impinges strongly on the ability of chitosan polymers to trigger mitochondrial stress and explains why C100 is the optimal chitosan formulation in terms of mitochondrial stress and IFNAR-dependent DC maturation. Molecular weight within the range tested was not a crucial parameter for the C100 polymers investigated in this study. All four molecular weights drove similar levels of mitochondrial stress and DC maturation. This is likely due to the fact that the smallest C100 polymer has a sufficient number of positively chained amines to cause disruption.

In addition to activating the cGAS-STING pathway, highly deacetylated polymers were demonstrated to promote NLRP3 inflammasome activation in a mtROS-dependent
manner. It is thus, no surprise that C100 was the strongest inducer of IL-1β release. As with cGAS-STING activation, the molecular weight of C100 polymers had no dramatic effect on IL-1β release, further implicating deacetylation as the principal determinant in chitosan adjuvanticity. Intriguingly, oxidative stress induced by C100 was so high, even with low concentrations of C100, that it could not be fully quenched by saturating concentrations of the ROS scavenger, MitoTEMPO. Indeed, pre-treatment of cells with MitoTEMPO could abrogate protasan-induced IL-1β release, but only minimally reduced C100-induced IL-1β release. This work highlights mitochondrial stress as an attribute of chitosan-induced cGAS-STING and NLRP3 activation and identifies it as a likely point in which the pathways diverge. Furthermore, it refutes previous findings suggesting that chitosan induced type I IFN responses and inflammasome activation are mutually exclusive processes [343]. Future studies must identify whether the function of NLRP3 in chitosan-induced immunity is IL-1β or IL-18 dependent.

It is evident that the toxicity of chitosan formulations is dependent on the extent of deacetylation. C100 polymers were by far the most toxic chitin derivatives, with extensive cell death presenting as early as two hours after treatment, followed by C90 and then protasan. In contrast, C39, C49 and C72 were not toxic to BMDCs. These findings are consistent with a previous study showing a 98 % deacetylated chitosan polymer drives more LDH release than partly acetylated chitosan’s [343], [347]. Thus, it is clear that chitosan should not be generally classified as non-toxic. This study ruled out a role for lysosomal rupture in chitosan-induced cell death and demonstrated that only polymers which cause mitochondrial dysfunction trigger cell death. Whether these two processes are directly linked is unknown but plausible given the central role of mitochondrial dysfunction in mediating cell death [226].

The extent of in vitro type I IFN responses with highly deacetylated chitosan polymers was demonstrated to be highly reliant on IFNAR-feedback signalling and cell viability. Despite being the strongest inducer of mitochondrial stress and IFNAR-dependent DC maturation, C100 polymers drove minimal IFN-β and no CXCL10 secretion compared to C90 and protasan. This was a result of C100-induced toxicity that prevented STING-dependent type I IFNs from sustaining IFNAR feedback signalling. Importantly, C100-toxicity was not detrimental to its adjuvanticity in vivo, as when administered subcutaneously in combination with H56, C100 was found to induce the greatest antigen-specific IFN-γ response compared to the less deacetylated chitosan formulations. Critically, the antigen-specific IFN-γ response was STING, IFNAR and NLRP3-dependent. This highlights how mitochondrial stress, not cytokine secretion, should be
used as an *in vitro* screen to identify optimal chitosan polymers for different biomedical settings.

While this work did not delineate how chitosan deacetylation promotes BMDC toxicity, it implicated a role for autophagy in its regulation. Highly deacetylated chitosan polymers rapidly increased autophagic flux in primary and immortalised macrophages to such an extent that lysosomal degradation had to be blocked to visualise LC3-II lipidation. In contrast, C39, C49 and C72, failed to promote LC3-II lipidation. Importantly, when autophagy was impaired, either by pre-treatment with BafA or the use of DCs from ATG16−/− PKD mice, the toxicity of polymers increased and IFN responses decreased respectively. Thus, it appears autophagy regulates chitosan-induced type I IFN responses by removing dysfunctional mitochondria and promoting cell survival that allows sustained type I IFN signalling. These findings counter previous work suggesting that chitosan-induced lysosomal swelling blocks downstream steps in autophagy leading to cell death in macrophages [343].

This is not the first report of autophagy promoting type I IFN responses. The adjuvant c-di-AMP from *Listeria monocytogenes* triggers STING-dependent ER-phagy- a form of autophagy in response to ER stress- that appears to be necessary for type I IFN secretion by preventing cell death. Given that protasan triggers ER stress and mitochondrial oxidative stress (Chapter 3), it is possible autophagy limits chitosan-induced toxicity by removing both damaged organelles. Although autophagy is needed for optimal chitosan induced type I IFN responses *in vitro*, its contribution or benefit *in vivo* could differ. For instance, autophagy is a negative-regulatory feedback function of cGAS and STING activation that limits their activation by removing cytosolic DNA [354]. It also limits NLRP3 activation by degrading inflammasome components [168], [169]. Thus, future studies must address if autophagy is a beneficial feature of highly deacetylated chitosan polymers *in vivo* settings. This study did not address how chitosan triggers autophagy, although it is likely to occur in cGAS and/or STING dependent manner.

The discovery that STING occupies a central role in the activation of tumour-targeting immune responses has generated considerable interest in STING agonists (natural, synthetic CDNs and non-nucleotidyl STING agonists) for use in oncological indications. Despite pre-clinical evidence of efficacy, clinical translation has resulted into disappointingly modest efficacy. Specifically, their physiochemical properties render them poorly membrane permeable and prone to rapid enzymatic degradation [355]. In
addition, human STING is highly polymorphic, with implications for the selection of STING agonists that can activate multiple haplotypes. Highly deacetylated chitosan polymers represent an attractive alternative to conventional STING agonists. As with anti-cancer agents (radiotherapy, chemotherapy), they trigger activation of the cGAS-STING pathway through the accumulation of endogenous DNA, bypassing limitations of membrane permeability and STING polymorphism. Unlike, cGAMP or DMXAA, highly deacetylated chitosan polymers are toxic to BMDCs, and if toxicity extends to tumour cells, these polymers present an additional benefit of direct tumour killing alongside induction of anti-tumour responses upon intratumoral injection.

At first glance, chitosan polymers appeared to have slower type I IFN enhancing kinetics than conventional STING agonists, as the first wave of ifna and ifnb mRNA expression arose 8-10 hours after treatment compared to 1 and 2 hours with DMXAA and cGAMP respectively. However, given that cGAMP and DMXAA induced IFN-β secretion preceded CXCL10 production and protasan and C90 induced IFN-β and CXCL10 production presented simultaneously, it’s likely that chitosan polymers trigger an earlier wave of IFNs that are not detected by qPCR. In support of this, protasan-induced cxcl10 mRNA expression displayed identical kinetics to ifna and ifnb (Chapter 3). Altogether this would suggest that chitosan polymers trigger more sustained type I IFN signalling. Future studies should address the clearance rates of highly deacetylated chitosan polymers in vivo, in particular in tumour environments. Of interest, while chitosan polymers can be degraded by a variety of enzymes including chitinases, chitosanases and most commonly lysozymes, studies have shown the lysozymes selectively target chitosan-containing acetyl groups. Thus, chitosan polymers with a high DD have a much slower degradation rate than polymers with a low DD [356], [357]. Given chitosan’s success as a drug delivery system (DDS) [358], [359], C100 is likely to have a good retention profile in tumours that could allow continuous STING activation.

As with 2’3’-cGAMP and DMXAA, optimal chitosan-induced type I IFN signalling required IFNAR-dependent feedback amplification. Despite being much weaker inducers of IFN-β secretion than DMXAA or cGAMP, C90 and protasan triggered comparable levels of CXCL10 secretion. This is of significant value, given that CXCL10 not only attracts CD8* and CD4* effector T cells to tumour sites and but also directs the polarization and potentiates the biological function of these cells [360].

In addition to IRF3 activation, a less understood function of STING is mediating a pro-inflammatory cytokine response (TNF-α, pro-IL-1β, and IL-6) through the activity of NF-
κB [140]. To our knowledge, protasan is the only reported STING activator that does not elicit NF-κB signalling. This inability likely extends to C100 and C90 given that LPS priming is required for IL-1β secretion and explains why CXCL10 secretion and DC maturation are entirely IFNAR dependent. The role of STING-mediated NF-κB activation in anti-tumour immunity is unclear, yet given its role in tumour initiation, metastasis, cell proliferation and survival, it is possible that NF-κB signalling diminishes the full potential of conventional STING agonists as therapeutic adjuvants [151]. However, while this problem is bypassed by chitosan polymers, unlike DMXAA or cGAMP, chitosan polymers demonstrate robust inflammasome enhancing activity, which could be a limitation given the pro-tumour roles of IL-1β and IL-18 [51], [85].

This work identified mitochondrial oxidative stress as a readout for monitoring the adjuvanticity of chitosan polymers. It provides solid evidence that biomaterials should focus on chitosan polymers with minimal and homogeneous deacetylation while adjuvants should focus on chitosan polymers with extensive if not complete deacetylation. This is the first demonstration of a potential role for C100 as a vaccine adjuvant. Given its well characterised, scalable and cost-effective manufacturing process and its highly defined structure and mode of action, it should be promptly investigated in clinical settings. In addition, the unique properties of C100 as a STING activator support its investigation as an adjuvant therapy in cancer settings.
5. The chitin-derivative and STING activator C100 is an effective anti-cancer agent

5.1. Introduction, hypothesis and aims

The cGAS–STING signalling axis has emerged as a crucial regulator of type I IFN responses. GAS recognises dsDNA independently of its sequence, bestowing the ability to sense DNA from both microbial and endogenous origins. Upon DNA binding, cGAS catalyses the synthesis of the CDN 2′3′-cGAMP, which serves as a second messenger that binds to and activates STING [312]. STING traffics from the ER to perinuclear sites, where it activates TBK1 and IRF3 to induce the production of type I IFNs. STING also triggers a prominent pro-inflammatory cytokine response (e.g., TNF-α, IL-1β, and IL-6) through the activity of NF-κB [126], [361]. NF-κB, along with other transcription factors, synergizes with IRF3 by collectively binding a specific enhancer region within the IFN-β promoter, termed the enhanceosome, resulting in maximal ifnb expression [362], [363].

It is unclear how STING activates NF-κB signalling, but recent studies have implicated a kinase-independent function between proteins TBK1 and IKKε. Considering that TBK1 and IKKε kinase activity occurs after STING translocation to perinuclear sites, it is possible that NF-κB activation predominately occurs at the ER [143].

Cellular DNA is under constant threat of damage by exogenous and endogenous factors. Exposure to ionizing radiation, chemotherapeutic agents (e.g., platinum drugs) or products of cellular metabolism (e.g., aldehydes and ROS) can inflict dsDNA breaks, which are particularly lethal to cancer cells with defective DNA damage repair machinery [364]. Accumulating evidence implicates dsDNA breaks and defective DNA repair in activation of the cGAS-STING pathway and induction of anti-tumour immunity. Tumour-derived damaged DNA activates cGAS to produce cGAMP, resulting in STING-mediated type I IFNs and pro-inflammatory cytokines. Type I interferons stimulate cross-presentation of tumour antigens and mobilization of anti-tumour immunity [365]. Additionally, cGAS impedes tumorigenesis by detecting DNA damage in premalignant cells or in cancer cells treated with classic anti-cancer therapies (radiotherapy/chemotherapy). Thus, therapies that trigger DNA damage are not merely cytotoxic treatments, but additionally immune stimulants. While it is clear that type I IFNs are beneficial in limiting cancer progression by priming T cells, the role of STING-induced NF-κB activation in cancer is poorly defined.
Since the discovery that STING occupies a central role in the activation of tumour-targeting immune responses, there has been a surge in research aimed at identifying natural or synthetic CDNs and non-nucleotidyl STING agonists for use in oncological settings. Despite pre-clinical evidence of efficacy, no widely applicable, clinically effective and safe agonist has been identified or completed phase III trials [355]. The primary barriers to clinical translation are low cellular uptake and intracellular accessibility, poor pharmacokinetics, and STING variability, necessitating personalised STING agonists.

Highly deacetylated chitosan polymers present as attractive alternatives to conventional STING agonists due to their unusual mode of STING activation. The 86 % deacetylated chitosan salt protasan triggers indirect activation of the cGAS-STING pathway through the induction of mitochondrial stress and subsequent release of mtDNA into the cytosol, bypassing limitations of STING variability and poor intracellular accessibility. The previous chapter identified C100, a chitosan polymer with no remaining acetyl groups, as the most potent chitosan formulation- it triggered the highest levels of mitochondrial oxidative stress, IFNAR-dependent DC maturation, and STING-dependent cellular immunity. Thus, it was hypothesised that if chitosan polymers were to be successful anticancer agents, C100 would be the optimal formulation.

The specific aims of the chapter were as followed:

- Investigate unique features of highly deacetylated chitosan polymers as STING activators, such as their mode of activation, requirement for cellular uptake and the role of NF-κB signalling.
- Compare the kinetics and magnitude of STING activation between highly deacetylated chitosan polymers and natural or synthetic CDNs
- Investigate the potential of highly deacetylated chitosan polymers as cancer therapeutics in a B16 murine model of melanoma
5.2. Results

5.2.1. C100-induced STING activation is not dependant on mtDNA

In the previous chapter, it was demonstrated that C100 is the most potent chitosan formulation for the induction of mitochondrial stress and IFNAR-dependent DC maturation. While it has been verified that protasan activates the cGAS-STING pathway through the release of mtDNA into the cytosol, this has not yet been shown for C90 and C100. As such, BMDCs from WT mice were cultured with or without low dose EtBr to deplete mitochondrial DNA (referred to as Ctrl and EtBr DCs). On day 10, mtDNA depletion was confirmed by qPCR analysis of mitochondrial gene expression compared to nuclear gene expression (Figure 5.1 a). EtBr DCs displayed a significant reduction in expression of dloop compared to tert and non-numt compared to β2m (Figure 5.1 b & c). Ctrl and EtBr BMDCs were then stimulated for 24 hours with protasan, C90, C100 or DMXAA for 3 hours. As before, protasan-induced ifnb mRNA was significantly reduced in EtBr DCs and DMXAA induced gene expression was unaffected. C90-induced ifnb expression was similarly reduced in EtBr DCs, albeit to a lesser extent than demonstrated with protasan. In contrast, C100-induced ifnb expression was significantly increased in EtBr DCs, with mRNA levels increasing more than 3-fold compared to the Ctrl DCs. Of note, the amplified ifnb mRNA expression in EtBr DCs stimulated with C100 was higher than DMXAA-induced ifnb expression in Ctrl DCs (Figure 5.1 c).
Figure 5.1 C100-induced *ifnb* mRNA transcription does not require mtDNA.

(a) Experimental schematic on the method used to deplete mitochondrial DNA (mtDNA) in BMDCs (b-c) On day 10, relative total mtDNA amounts were quantified by qPCR with primers specific for the mitochondrial D-loop region (*dloop*) or a region of mtDNA that is not inserted into nuclear DNA (*non-numt*) and primers specific for nuclear DNA (*tert, β2m*). Statistical analysis was determined by unpaired t test with welch’s correction. ****p <0.0001. (d) Ctrl and EtBr DCs were stimulated with chitin-derived polymers (8 µg/mL) for 24 hours or DMXAA (10 µg/mL) for 3 hours. *ifnb* mRNA levels were calculated by qPCR with respect to *actb* and *rps18*. Data are expressed as mean ± SD of mRNA levels in technical triplicates with respect to *actb* and are representative of 5 independent experiments. Statistical analysis was performed by two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. ***p<0.001; ****p<0.0001.
5.2.2. STING is required for the enhanced *ifnb* expression resulting from EtBr culture and C100 treatment

The use of EtBr to deplete mtDNA is an established means to implicate mtDNA in an immune response. However, it is not without caveats. For example, EtBr intercalates all DNA and RNA species within the cell, which in theory could lead to nuclear mutations that alter immune responses. To ensure the amplified IFN responses observed in EtBr DCs treated with C100 was STING-dependent and not an off-target effect, WT and *sting*⁻/⁻ BMDCs were cultured with or without EtBr and stimulated with C100 (2, 4 or 8 µg/mL) or controls (DMXAA and LPS). As expected, the expression of *dloop* and *non-numt* were significantly reduced in the EtBr DCs compared to Ctrl DCs, whereas expression of *tert* and *β2m* did not change. WT and *sting*⁻/⁻ EtBr DCs expressed equivalent levels of *dloop* and *non-numt* (Figure 5.2 a -b). As before, culturing cells in EtBr amplified the C100-induced *ifnb* response, particularly with high concentrations of C100. C100-induced *ifnb* mRNA expression was STING dependent, as was the EtBr-dependent amplification of C100 responses Unsurprisingly, DMXAA-induced *ifnb* mRNA expression was STING dependant. *sting*⁻/⁻ DCs treated with LPS displayed a modest reduction in *ifnb* expression compared to the WT control. EtBr culturing had no effect on DMXAA and LPS-induced *ifnb* expression in WT cells (Figure 5.2 c).
Figure 5.2 STING is required for the enhanced *ifnb* expression induced by EtBr culture and C100 treatment.

(a & b) Relative total mtDNA amounts in Ctrl and EtBr WT and *sting*<sup>-/-</sup> BMDCs were quantified by qPCR with primers specific for the mitochondrial D-loop region (*dloop*) or a region of mtDNA that is not inserted into nuclear DNA (*non-numt*) and primers specific for nuclear DNA (*tert, β2m*). Statistical analysis was determined by one-way ANOVA with multiple comparisons. **** *p*<0.0001. (c) Ctrl and EtBr WT and *sting*<sup>-/-</sup> BMDCs were stimulated with C100 (2, 4 or 8 µg/mL) for 24 hours or DMXAA (10 µg/mL) or LPS (10 ng/mL) for 3 hours. *ifnb* mRNA levels were calculated by qPCR with respect to *actb* and *rps18*. Data are expressed as mean ± SD of mRNA levels in technical triplicates with respect to *actb* and are representative of 3 independent experiments.
5.2.3. C100 drives nuclear DNA fragmentation in BMDCs

Under normal circumstances, cellular DNA is confined to the nucleus or mitochondria, however in response to cellular stress, mitochondrial or nuclear DNA can enter the cytoplasm to trigger cGAS activation [366]. Unlike protasan and C90, C100-induced type I IFN responses were not mtDNA dependent. It was hypothesised that rampant mitochondrial stress induced by C100 was causing damage to the nuclear compartment to trigger activation of STING. The APO-tunnel labelling Kit was used to detect dsDNA breaks (DNA fragmentation) by flow cytometry. This system works by incorporating bromodeoxyuridine (BrdU) into the 3'-OH ends of dsDNA breaks in a reaction catalysed by exogenous terminal deoxynucleotidyl transferase (TdT). The incorporated BrdU is then immunocytochemically detected by a FITC labelled anti-BrdU antibody. To allow the evaluation of DNA fragmentation as a percentage of total DNA in live cells, BMDCs were stained with PI after permeabilization to label total cellular DNA and with A700 viability dye prior to BrdU-labelling of 3'-OH ends to identify live cells (Figure 5.3 a).

The APO-BrdU TUNEL assay kit provides fixed samples of positive and negative control cells for assessing assay performance. As expected, the negative control cells drove virtually no DNA damage while roughly one fifth of positive control cells displayed enhanced FITC-BrdU fluorescence representative of DNA fragmentation. The BMDC media treatment displayed equivalently low levels of DNA fragmentation to the negative control. In contrast, C100 treatment drove robust DNA fragmentation, with roughly half of the single cell population displaying fragmented DNA. More than half of the cells presenting fragmented DNA were dead, as when DNA fragmentation as a percentage of total DNA was measured in single, live cells, the fragmented population dropped to 22.5 % (Figure 5.3 b-c).
Figure 5.3 **C100 drives nuclear DNA fragmentation.** BMDCs were treated for 24 hrs with media or C100 (5 µg/mL). Human lymphoma cell lines were used as positive and negative controls for DNA fragmentation. DNA fragmentation was measured as a (b) percentage of total DNA in single cells or as a (c) percentage of total DNA in single live cells. Note DNA fragmentation as a percentage of live cells cannot be monitored with the human lymphoma controls as the cells are fixed and permeabilised. n=2.
5.2.4. Incubation of cells with C90 and protasan polymers has a limited impact on nuclear DNA

In comparison to C100, C90 and protasan are weak inducers of mitochondrial stress. Thus, it was assumed they would drive little, if any nuclear DNA damage. To investigate, BMDCs were stimulated for 3 or 9 hours with C90, C100 or protasan and then monitored for DNA fragmentation in single and single live cells. The efficacy of the APO-BrdU Tunnel assay labelling kit was verified in the previous experiment using the positive and negative control cell lines. As such a more appropriate control, etoposide was used that drives DNA fragmentation via the inhibition of topoisomerase II.

As before, media treated DCs demonstrated very little DNA fragmentation in single and single live cells. In contrast, 24-hour etoposide treatment drove extensive DNA damage, with 88.3 % and 83.4 % of single and single live cells, respectively displaying fragmented DNA (Figure 5.4 a-b). C100-induced DNA damage was apparent 3 hours after treatment, with 10.9 % of single cells displaying fragmented DNA compared to the 4.63 % in the media control. Protasan and C90 did not cause significant DNA damage with 1.34 % and 6.24 % of single cells displaying fragmented DNA after 3 hours of treatment. C100-induced DNA fragmentation was most apparent in single cells 9 hours after treatment, with 39.3 % of cells displaying fragmented DNA compared to 10.6 % and 9.88 % in C90 and protasan-treated cells. (Figure 5.4 a). For C100 treatments, the percentage of DNA fragmentation in single live cells was less than that in single cells due to the exclusion of dead cells. C90 and protasan drove minimal cell death (Figure 5.4 c), explaining why the percentage of DNA fragmentation in single and single live cells were comparable (Figure 5.4 a-b).
Gated on total DNA in single cells
Gated on total DNA in single, live cells
Figure 5.4 Unlike C100, short term protasan and C90 stimulation does not cause DNA fragmentation or cell death.

BMDCs were treated with 5 µg/mL of protasan, C90 or C100 for indicated times or 100 µM of etoposide for 24 hours. DNA fragmentation was measured as a (a) percentage of total DNA in single cells or as a (b) percentage of total DNA in single live cells (c) % cell death in single cells. n=2.
Protasan and C90 trigger high levels of mitochondrial stress resulting in the egress of mitochondrial DNA. mtDNA binds to cGAS, generating cGAMP, which then ligates and activates STING. C100 drives rampant mitochondrial stress that damages nDNA, resulting in the accumulation of cytosolic nDNA and activation of STING, presumably through cGAS.

*Note the role of Ca^{2+} in C90 and C100-induced mitochondrial stress and cGAS in C90 and C100-mediated STING activation have not been verified. Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA.

Figure 5.5 C100-induced cGAS-STING activation is dependent on mtROS-mediated nuclear DNA damage
5.2.5. An unknown actin polymerisation event, that is not endocytosis, promotes mitochondrial stress with highly deacetylated chitosan polymers

A major drawback in the translational use of STING agonists is their restricted access to the cytoplasm of target cells and limited understanding of how this is achieved. It is thought that chitosan uptake is needed for type I IFN responses and IL-1β release as pre-treatment of BMDCs with cytochalasin B blocks the secretion of both cytokines (Dr. Liz Carroll, PhD). To delineate the timecourse of chitosan uptake, BMDCs were treated with FITC-labelled protasan for 30 minutes, 1 hour, 4 hours or 6 hours and assessed by flow cytometry. Trypan blue was used to quench external FITC fluorescence and distinguish between surface bound and internalised chitosan-FITC. The total FITC-fluorescence was analysed in 10,000 single, live events, trypan blue was then added to quench external FITC fluorescence and a further 10,000 single, live events were recorded (Figure 5.6 a). To demonstrate the efficacy of trypan blue quenching, BMDCs were treated with 1 mg/mL of FITC-labelled protasan immediately before sample acquisition. There was a 100 % drop in FITC-fluorescence after trypan blue addition, signifying complete external FITC quenching. As expected, media-treated cells displayed no difference in FITC fluorescence before or after trypan blue quenching. While BMDCs could engulf protasan within 30 minutes, the percentage of cells doing so was low (5.53 %) and a 6-hour treatment appeared to be optimal for chitosan uptake with 19.7 % of cells marked as FITC⁺ (Figure 5.6 b).

In light of the fact that optimal chitosan uptake requires 6 hours, but chitosan-induced mitochondrial stress appears after 1 hour, it was speculated that an uptake-independent actin polymerisation event is required for the induction of mitochondrial stress. To investigate, BMDCs were pre-treated with Latrunulin B (LatB), and then stimulated with indicated concentrations of protasan, C90 or C100 for 1.5 hours. Additionally, BMDCs were stimulated with zymosan for 2 hours or rotenone for 6 hours as a negative control. Rotenone is lipophilic and as expected, LatB treatment did not affect its ability to drive mitochondrial stress. Zymosan increased MitoSOX fluorescence compared to the media control and while MitoSOX MFI values would suggest LatB did not enhance zymosan-induced mtROS, the overlay histograms clearly depict its ability to augment zymosan-induced mtROS production. In contrast to zymosan, blocking actin polymerisation dramatically reduced the MitoSOX fluorescence in response to all concentrations of C90, C100 and protasan. While all samples showed a dramatic reduction in MitoSOX fluorescence upon LatB treatment, it was only in samples that drove weak induction of
mtROS that the response could be fully abrogated by LatB. For instance, LatB treatment fully blocked the induction of mitochondrial stress in response to 2.5 µg/mL of protasan, 5 µg/mL of protasan and 2.5 µg/mL of C90, but only partially reduced mitochondrial stress when chitin-derivatives drove MitoSOX fluorescence above an MFI of 380 (Figure 5.7).

Figure 5.6 Optimal chitosan uptake requires 6 hours of treatment
BMDCs were treated with media, 4 µg/mL FITC-labelled protasan for indicated times or 1 mg/mL FITC-protasan immediately before acquisition. (a) Live cells were analysed for FITC fluorescence. (b) Percentage of FITC+ cells before and after trypan blue quenching. Representative of n=2.
Figure 5.7 Actin polymerisation is required for C90, C100 and protasan-induced mitochondrial stress

BMDCs were pre-treated for 40 minutes with LatB (2 µM) and then stimulated for 1.5 hours with indicated concentrations of protasan, C90 or C100. As controls, cells were stimulated with zymosan (10 µg/mL) for 2 hours or Rotenone (5 µM) for 6 hours. (a) Representative histograms of the MitoSOX fluorescence in single, live cells. Each black line corresponds to the media untreated control, each green line represents a stimulation, and each red line represents a sample that was pre-treated with LatB before stimulation. Representative of $n=2$. 

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5.2.6. Protasan does not activate NF-κB signalling

Despite the fact that chitosan, DMXAA and 2’3’-cGAMP are all STING agonists, chitosan-induced CXCL10 secretion was demonstrated to be entirely IFNAR-dependant, while cGAMP and DMXAA-induced CXCL10 secretion was only partially dependent on IFNAR (section 4.2.2). It was speculated that the differences in dependency on IFNAR were due to the presence of both NF-κB and IFN-stimulated response element (ISRE) in the CXCL10 promoter [367]. It is documented that chitin-derived polymers cannot drive NF-κB activation in macrophages [305]. This was verified in BMDCs by stimulating cells for 24 hours with indicated concentrations of protasan. As positive controls, BMDCs were additionally stimulated with CpG and LPS. As expected, CpG and LPS treated BMDCs secreted significant concentrations of IL-12p70, IL-23, IL-6 and TNF-α. Protasan treatment failed to induce the secretion of IL-12p70, IL-23, IL-6 or TNF-α at all concentrations tested. Untreated BMDCs displayed high basal levels of TNF-α, which all concentrations of protasan could marginally reduce (Figure 5.8 a-d).

To confirm that protasan does not drive NF-κB-dependent pro-inflammatory responses, a time course experiment was set up to monitor NF-κB activation. IκBα is one of three components in the IκB complex that interacts with NF-κB transcription factor dimers, being responsible for their retention in the cytoplasm. Upon phosphorylation by IκB kinases (IKK), IκB undergoes proteasome-dependent degradation and NF-κB is translocated to the nucleus, where it drives inflammatory gene expression. DMXAA was used a positive control, given its capacity to drive IκBα degradation downstream of STING. As expected, DMXAA treatment reduced the relative expression of IκBα compared to the media control. In contrast, stimulation of cells with protasan for 3, 6, 9 or 12 hours did not reduce basal IκBα expression (Figure 5.9 a-b). Although cells treated for 16 or 20 hours with protasan had slightly reduced IκBα expression compared to medium controls, this was not statistically significant when densitometry readings from biological triplicates were analysed (Figure 5.9 b).

In addition, a time course experiment was set up to monitor and compare pro-inflammatory cytokine mRNA responses in BMDCs treated with protasan, DMXAA, 2’3’-cGAMP or etoposide. Etoposide was included due to its demonstrated capacity to activate NF-κB to a greater degree than IRF3, downstream of STING in human keratinocytes [145]. Protasan stimulated DCs did not exhibit enhanced transcription of il6, tnfα or il12p40 at any of the timepoints tested (Figure 5.10 a-c). In contrast, DMXAA-
induced \textit{tnfa} and \textit{il-6} expression was apparent after 30 minutes of treatment and peaked after 4 hours (Figure 5.10 a &b). 2′3′-cGAMP-induced \textit{il6} mRNA expression was slower and weaker than that induced by DMXAA, with significant levels only appearing 12 hours after treatment (Figure 5.10 a). 2′3′-cGAMP-induced \textit{tnfa} mRNA expression peaked 8 hours after stimulation, with levels marginally higher than the 4-hour DMXAA-induced \textit{tnfa} mRNA peak (Figure 5.10 b). As with protasan, both DMXAA and 2′3′-cGAMP failed to upregulate \textit{il12p40} expression (Figure 5.10 c). Etoposide-treated DCs failed to upregulate \textit{tnfa} or \textit{il12p40} at any timepoint tested (Figure 5.10 b & c). While etoposide treatment marginally increased \textit{il6} mRNA expression after 20 hours, it was not statistically significant (Figure 5.10 a).

**Figure 5.8 Protasan does not drive pro-inflammatory cytokine secretion**

BMDCs were stimulated with media, protasan (1.5, 3 or 6 µg/mL), LPS (10 ng/mL) or CpG (4 µg/mL) for 24 hours. (a) Concentrations of IL-12p70, (b) IL-23, (c) IL-6 and (d) TNF-α were measured in the supernatants of treated cells by ELISA. Results show mean ± SD for technical triplicates and represent two independent experiments. Statistical analysis determined by one-way ANOVA. Media vs treatment *\(p<0.05\), ***\(p<0.001\), ****\(p<0.0001\).
Figure 5.9 Protasan does not drive IκBα degradation
(a) Time course analysis of IκBα and β-actin expression in BMDCs treated with protasan (5 µg/mL) or DMXAA for 3 hours. (b) Densitometry of IκBα expression normalised to β-actin and compared to the media control. Results show mean ± SD for biological triplicates. Mouse 1 in blue, mouse 2 in green and mouse 3 in red. Statistical analysis was determined by one-way ANOVA. Media vs treatment. ***p<0.001.
Figure 5.10  Protasan does not upregulate pro-inflammatory cytokine gene expression

BMDCs were stimulated with protasan (5 µg/mL), DMXAA (10 µg/mL), 2’3’-cGAMP (1 µM) or etoposide (50 µM) for indicated times. mRNA levels were calculated by qPCR for (a) *il6*, (b) *tnfa* and (c) *il12p40* with respect to *actb* and *rps18*. Data shows technical triplicate mRNA levels with respect to *actb* and is representative of three independent experiments. Data was analysed by multiple one-way ANOVA. Media vs treatment. *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001. For simplicity, ns not shown on graphs.

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5.2.7. C90 and C100 do not drive inflammatory-NF-κB-dependent responses

It was assumed that C90 and C100, like protasan, would not activate the NF-κB arm of STING signalling. To verify, BMDCs were stimulated for 24 hours with indicated concentrations of C90, C100 and protasan and then monitored for IL-6 and TNF-α secretion by ELISA. As positive controls, cells were stimulated with CpG, LPS, DMXAA, 2’3’-cGAMP and etoposide [262]. As expected, CpG, LPS DMXAA and 2’3’-cGAMP treatment caused significant secretion of IL-6 and TNF-α. DMXAA was a stronger driver of IL-6 and TNF-α secretion than 2’3’-cGAMP. In line with the *il6* and *tnfa* mRNA data, etoposide drove little IL-6 secretion and did not increase basal levels of TNF-α. As seen with protasan, C90 and C100 treatment failed to drive the secretion of IL-6 protein at any concentration tested or increase the secretion of TNF-α above the medium control (Figure 5.11 a-b).

These results were confirmed by monitoring IκBα degradation. C90 and C100, like protasan, failed to drive IκBα degradation across a broad range of timepoints tested. In contrast, DMXAA-induced IκBα degradation was apparent within 30 minutes with almost complete degradation after 1 hour. While 2’3’-cGAMP-induced IκBα degradation was slower and weaker than DMXAA, the degradation spanned over a longer timeframe, with degradation occurring after 2 hours and was sustained up to 10 hours later. An intrinsic autoregulatory feedback loop in NF-κB signalling is the NF-κB-dependant expression of IκBα. This was evident 4 hours after treatment with DMXAA and 24-hours after treatment with 2’3’-cGAMP. Similar to *il6* mRNA expression, etoposide-induced IκBα degradation was only visualised 24 hours after treatment (Figure 5.12 a-b).
Figure 5.11 Highly deacetylated chitin-derived polymers do not drive IL-6 or TNF-α secretion

BMDCs were stimulated with C90, C100 or protasan (1.5, 3 or 6 µg/mL), LPS (10 ng/mL), CpG (4 µg/mL), etoposide (50 µM), DMXAA (10 µg/mL) or 2′3′-cGAMP (10 µM) for 24 hours. (a) IL-6 and (b) TNF-α concentrations were measured in the supernatants of treated cells by ELISA. Results show mean ± SD for technical triplicates and represent two independent experiments. Statistical analysis determined by one-way ANOVA. Media vs treatment *p<0.05, ***p<0.001, ****p<0.0001.
**Figure 5.12 Highly deacetylated chitin-derived polymers do not drive IκBα degradation in BMDCs**

BMDCs were stimulated with C90, C100, protasan (5 µg/mL), DMXAA (10 µg/mL), 2’3’-cGAMP (10 µM) or Etoposide (50 µM) for indicated times. (a) Cells were lysed and IκBα levels determined by Immunoblot with β-actin used as a loading control. (b) Densitometry of IκBα expression normalised to β-actin and compared to the media control. n=3
5.2.8. Highly deacetylated chitin-derived polymers do not cause STING degradation or drive detectable IRF3 activation.

Chitosan-induced CXCL10 production is entirely IFNAR-dependent yet appears concurrent with IFN-β responses (section 3.2.1 & 4.2.5). This would suggest that chitosan-induced STING activation occurs earlier than previously thought, but triggers such low type I IFN responses that it has not been detected by PCR. In an attempt to elucidate the kinetics of chitosan-induced STING activation and type I IFN signalling, BMDCs were treated with C90, C100 and protasan for 0.5, 1, 2, 4, 8, 12 or 24 hours and then monitored for degradation and phosphorylation of STING and IRF3 phosphorylation over a range of timepoints. Of note, while STING expression is stable when localised to the ER, translocation results in rapid degradation, and represents a major rate-limiting step in STING signalling. As a positive control and to further understand how chitosan-induced signalling deviates from other STING agonists, BMDCs were additionally treated with DMXAA, 2′3′-cGAMP and etoposide over the same time period.

As expected, DMXAA and 2′3′-cGAMP drove STING degradation and IRF3 phosphorylation. For DMXAA, STING degradation and phosphorylation began as quickly as 30 minutes after stimulation, with complete degradation achieved by 8 hours and sustained for up to 24 hours. The kinetics of 2′3′-cGAMP-induced STING degradation were slower, with degradation appearing after 1 hour but taking 24 hours for complete reduction. Of note, phosphorylated STING was not detected in response to 2′3′-cGAMP at any timepoints tested (Figure 5.13 a-b). DMXAA-induced p-IRF3 was detectable after 30 minutes and peaked within 2 hours of treatment. The signal was still detectable up to 12 hours after stimulation. 2′3′-cGAMP-induced p-IRF3 was apparent after 4 hours, peaked after 12 hours and remained detectable 24 hours later (Figure 5.13 a & c). As reported in human keratinocytes [145], etoposide failed to drive STING phosphorylation or degradation in BMDCs across the range of timepoints tested (Figure 5.13 a-b). Additionally, etoposide treatment failed to induce detectable IRF3 phosphorylation (Figure 5.13 a & c). Similarly, despite driving STING-dependent type I IFN expression and secretion, C90, C100 and protasan failed to induce STING phosphorylation and degradation or detectable IRF3 phosphorylation at all timepoints tested (Figure 5.13 a-c).

It was hoped that although chitosan-induced IRF3 phosphorylation was not detected, upstream phosphorylation of TBK1 would be. BMDCs were treated with protasan or DMXAA as a positive control for 1, 2, 4, 8, 12, 16 or 20 hours and monitored for
degradation and phosphorylation of STING and phosphorylation of IRF3 and TBK1. DMXAA drove rapid STING phosphorylation and degradation that was accompanied by TBK1 and IRF3 phosphorylation. In contrast, protasan drove no detectable TBK1 phosphorylation across all timepoints and failed to trigger STING degradation and IRF3 phosphorylation as before (Figure 5.14).

To ensure that chitin-derived polymers were not degrading STING or phosphorylating TBK1 and IRF3 in the period between time gaps, a new time-course experiment was set up where BMDCs were treated for 3, 6, 9, 12, 16 and 20 hours with protasan. As a positive control BMDCs were treated with DMXAA for 4 hours. In the representative blot, protasan appeared to increase STING expression, with increases beginning as early as 3 hours, followed by a dip to basal levels at 9 hours and a second sustained increase after 16 hours. Of note, this increase was not significant when STING expression was normalised to β-actin and compared to the media control in three biological replicates. In contrast, DMXAA dramatically reduced the expression of STING, with all three independent experiments demonstrating low densitometry readings of near 0 compared to media controls. Unlike DMXAA, protasan treatment failed to drive detecte TBK1 or downstream IRF3 phosphorylation at all timepoints tested (Figure 5.15 a).
Figure 5.13 Highly deacetylated chitin-derived polymers and etoposide do not drive STING degradation in BMDCs

BMDCs were stimulated with C90, C100, protasan (5 µg/mL), DMXAA (10 µg/mL), 2’3’-cGAMP (10 µM) and Etoposide (50 µM) for indicated times. (a) Cells were lysed and measured for STING and p-IRF3 levels by Immunoblot with β-actin used as a loading control. (b) Densitometry of STING expression normalised to β-actin and compared to the media control. (c) Densitometry of p-IRF3 expression normalised to β-actin and compared to the media control. n=3
Figure 5.14 Protasan does not drive detectable STING degradation or TBK1 and IRF3 phosphorylation.

BMDCs were stimulated with protasan (5 µg/mL) or DMXAA (10 µg/mL) for indicated times. (a) Cells were lysed and measured for STING, p-TBK1 and p-IRF3 levels by Immunoblot with β-actin used as a loading control. n=2.
Figure 5.15 Protasan can marginally increase STING expression in dendritic cells
(a) Time course analysis of STING expression in cells treated with protasan (5 µg/mL) or DMXAA for 3 hours. (d) Densitometry of STING expression normalised to β-actin and compared to the media control from three independent experiments. Results show mean ± SD for biological triplicates. Treatments from mouse 1 in blue, mouse 2 in green and mouse 3 in red. Statistical analysis determined by one-way ANOVA.
5.2.9. Brefeldin A limits chitosan-induced IFN responses in BMDCs

A lack of visible STING degradation can be indicative of one of two things, STING trafficking is dispensable for chitosan-induced type I IFN signalling or low-level STING translocation and degradation is occurring, but is just not detected by western blot. STING exits the ER in the form of COP-II vesicles and ERGIC [137]. Brefeldin A (BrefA) blocks ER-to Golgi-trafficking of COP-II vesicles by inhibiting ARF GTPases and is often used to investigate the importance of STING translocation in type I IFN responses [152]. Given the toxicity of BrefA, assessing cytokine secretion was precluded. Instead, cells were pre-treated with the inhibitor for 30 minutes, stimulated with DMXAA (positive control), LPS (negative control) or protasan and then analysed for expression levels of `ifna` and `ifnb`. The kinetics of optimal DMXAA (2-4 hrs) and LPS (3 hrs)-induced IFN mRNA responses are rapid compared to protasan (16 Hrs) in BMDCs. To prevent lengthy and toxic BrefA treatments, BMDCs were treated with protasan for only 8 hours. As a result, protasan-induced `ifna` and `ifnb` expression was much lower than that of DMXAA. As expected, pre-treatment with BrefA significantly reduced the expression of `ifna` and `ifnb` in response to DMXAA but not LPS. Importantly, protasan-induced `ifna` and `ifnb` expression was significantly reduced in BMDCs pre-treated with BrefA. Of note, BrefA did not completely abolish DMXAA or protasan-induced responses, suggesting that low level STING trafficking was still occurring (Figure 5.16 a-b).
Figure 5.16 Brefeldin A pre-treatment reduces protasan and DMXAA-induced *ifna* and *ifnb* transcription.

BMDCs were pre-treated for 30 minutes with BrefA (0.5 µM) and then stimulated for 8 hours with protasan (4 µg/mL) or etoposide (50 µM) or 3 hours with DMXAA (10 µg/mL) or LPS (10 ng/mL). mRNA levels were calculated by qPCR for (a) *ifna* and (b) *ifnb* with respect to *actb* and *rps18*. Data are expressed as mean ± SD for technical triplicate mRNA levels with respect to *actb* and are representative of three independent experiments. Statistical significance was measured using two-tailed unpaired student’s *t* tests with the Holm-sidak method for multiple comparisons. **p<0.01, ***p<0.001.
5.2.10. Brefeldin A triggers low grade NF-κB signalling upon chitosan treatment.

Several reports have highlighted that the expression of NF-κB-dependent genes can proceed in response to STING activators in the absence of STING trafficking and TBK1 function \[136\], \[144\], \[350\]. Additionally, it has been shown that STING can undergo ligand-independent translocation in macrophages when ER Ca\(^{2+}\) levels are reduced \[138\]. In chapter 3, protasan was suggested to drive an ER Ca\(^{2+}\) efflux event in BMDCs, that was necessary for mtROS induction, the release of mtDNA and activation of the cGAS-STING pathway. This led to the hypothesis that chitin-derived polymers fail to drive TBK1-independent NF-κB activation as a result of premature Ca\(^{2+}\)-induced STING translocation from the ER (Figure 5.17 a). To investigate, BMDCs were pre-treated with low doses of BrefA and stimulated with protasan to address whether this promoted NF-κB and pro-inflammatory cytokines and blocked type I IFN responses. DMXAA was used as a control for IRF3 and NF-κB activation given the demonstrated role of p-TBK1 in both responses. Etoposide was used as a control for NF-κB activation as it promotes IL-6 in the absence of STING trafficking and TBK1 function in human keratinocytes \[145\]. LPS was used as a control for IRF3 and NF-κB activation as activation occurs independently of STING and TBK1. As demonstrated previously, BrefA treatment reduced DMXAA-induced \(i\!f\!n\!a\) and \(i\!f\!n\!b\) mRNA responses (Figure 5.17 d-e). BrefA additionally reduced DMXAA induced \(i\!l\!6\) and \(t\!n\!f\!a\) expression (Figure 5.17 b-c). Etoposide drove little expression of \(i\!l\!6\), \(t\!n\!f\!a\), \(i\!f\!n\!a\) or \(i\!f\!n\!b\) so conclusive observations could not be drawn (Figure 5.17 b-e). As expected, BrefA treatment did not reduce LPS-induced \(i\!l\!6\) or \(t\!n\!f\!a\) expression. In fact, \(i\!l\!6\) and \(t\!n\!f\!a\) mRNA expression was significantly increased with BrefA treatment (Figure 5.17 d-e). As before, BrefA treatment slightly increased \(i\!f\!n\!a\) and \(i\!f\!n\!b\) mRNA expression in response to LPS (Figure 5.17b-c). BrefA treatment slightly increased \(i\!l\!6\) and \(t\!n\!f\!a\) mRNA expression in response to protasan, albeit not to a significant extent (Figure 5.17 d-e). As before, retention of STING at the ER reduced chitosan-induced \(i\!f\!n\!a\) and \(i\!f\!n\!b\) mRNA expression (Figure 5.17 b-c).
Figure 5.17 Brefeldin A promotes low level expression of *il6* and *tnfa* in response to protasan.

(a) Chitosan-induced opening of IP₃-gated Ca²⁺ channels trigger mitochondrial fission, mitochondrial ROS and STING translocation from the ER to the ERGIC. Mitochondrial stress prompts the release of mtDNA into the cytosol where it engages cGAS to produce 2′3′-cGAMP. 2′3′-cGAMP binds STING at the ERGIC, allowing exclusive activation of type I IFNs.
(b-e) BMDCs were treated for 30 minutes with Brefeldin A (0.5 µM) and then stimulated for 8 hours with protasan (4 µg/mL) or 3 hours with DMXAA (10 µg/mL) or LPS (10 ng/mL). mRNA levels were calculated by qPCR for (b) *il6*, (c) *tnfa*, (d) *ifna* and (e) *ifnb* with respect to *actb*. Data are expressed as mean ± SD for technical triplicates and are representative of three independent experiments. Statistical significance was measured using two-tailed unpaired student’s *t* tests with the Holm-sidak method for multiple comparisons. **** *p*<0.0001.

5.2.11. Highly deacetylated chitosan polymers have tumoricidal activity.

Chitin-derived polymers present as interesting alternatives to traditional STING agonists- (1) they drive the endogenous release of DNA into the cytosol to activate STING, (2) they are the only STING activators defined to drive an exclusive type I IFN profile, (3) they drive no observable STING degradation or TBK1 and IRF3 phosphorylation by immunoblot, (4) despite not activating NF-κB they drive similar levels of anti-tumour CXCL10 to DMXAA and 2'3'-cGAMP and (5) deacetylation dictates their ability to drive cell death in BMDCs.

It was investigated whether the toxicity of chitosan polymers in BMDCs extends to tumour cells. B16 melanoma cells were cultured *in vitro* for 20 hours with C90, C100 or protasan. For comparison, cells were treated with etoposide, DMXAA or CpG. As expected, CpG and DMXAA treatments were not toxic to B16 melanoma cells. Etoposide moderately increased cell death from 14.8 % in the medium control to 20.7 %. In contrast, all three chitosan polymers were tumoricidal in a dose-dependent manner. Surprisingly C90 was the most toxic chitosan polymer, with the highest concentration driving 51.3 % cell death compared to 48.5 % and 40.9 % after C100 and protasan treatments respectively (Figure 5.18).
Figure 5.18 Highly deacetylated chitosan polymers are toxic to B16 melanoma cells

B16 Melanoma cells were treated for 20 hours with 10, 20 or 40 µg/mL of chitin-derived polymers, 4 µg/mL of CpG, 10 µg/mL of DMXAA or 100 µM of etoposide. (a) Gating strategy determining toxicity in single cells. (b) Representative dot plots of single, dead cells. n=1.
The subcutaneous B16 melanoma model was used to evaluate the \textit{in vivo} anti-tumour potential of highly deacetylated chitin-derived polymers. It is a well-established model, notoriously difficult to treat as a result of its immune-suppressive tumour microenvironment. Melanoma cell lines often render the cGAS-STING pathway defective by blocking STING trafficking to sites conducive to type I IFN signalling and by hypomethylating STING promoter regions and decreasing STING expression [146]. As such, expression of STING protein in B16 and B16-expressing OVA (B16-OVA) melanoma cell lines was assessed. B16 and B16-OVA cell lines were treated with media, DMXAA and CpG. Both cell lines expressed STING, with slightly higher expression in B16 than in B16-OVA cells. Signifying functionality, DMXAA treatment drove complete STING degradation, with very faint phosphorylation bands appearing for both cell lines. As expected, CpG did not drive STING degradation in either cell line. STING expression appeared slightly higher after CpG stimulation in B16 cells (Figure 5.19 a).

![B16 and B16 expressing OVA melanoma cell lines express functional STING.](image)

\textbf{Figure 5.19 B16 and B16 expressing OVA melanoma cell lines express functional STING.}

B16 and B16-melanoma cells were stimulated with media, CpG (4 µg/mL) or DMXAA (10 µg/mL) for 4 hours. (a) Cells were lysed and measured for STING levels by Immunoblot with β-actin used as a loading control. \( n=1 \)
5.2.13. Intratumoral injection of C100 reduces tumour growth and extends mouse survival

C57BL/6 mice were injected subcutaneously with $3.5 \times 10^5$ B16-OVA cells on the upper left femur. When tumour size reached 0.5 to 0.7 cm (roughly 75 mm$^3$) in the largest diameter, mice were randomized into five different experimental groups and injected i.t with PBS, C90, C100, Protasan or CpG as a positive control. CpG is currently being tested by i.t injection in patients as a single agent and in combination with other therapeutic modalities (NCT02927964, NCT02266147, NCT01745354, NCT02254772, and NCT02521870). Compounds were injected i.t in order to focus activation on APCs acquiring tumour antigens. Four days later, mice were injected i.t as before. Tumour size was monitored with a digital calliper every day and mice were sacrificed when tumour size reached 1.5 cm in the largest diameter as per guidelines (Figure 5.20 a).

PBS treated mice displayed aggressive tumour growth, with doubling time of approximately 2-3 days (Figure 5.20 b). By day 15 all mice had reached endpoint, with a median survival of 11 days (Figure 5.20 d). CpG treatment significantly reduced tumour growth and extended mouse survival, with a median survival of 25 days after the initial i.t injection (Figure 5.20 b, c & d). Protasan treatment drove initial rapid increases in tumour size, which for the majority of mice reduced or plateaued for a number of days before exponentially increasing (Figure 5.20 b & c). Of note, the median survival time increased, albeit not significantly to 14 days (Figure 5.20 d). C90 also increased survival time from 11 days in the PBS control group to 14 (Figure 5.20 d). In terms of tumour growth, C90 treatment displayed virtually identical kinetics to the PBS control until day 8, when growth began to moderately lag behind (Figure 5.20 b & c). In contrast, the inhibitory effects of C100 on tumour growth were obvious as early as one day after the initial injection. Growth kinetics were identical to the control CpG up to 9 days after injection. Beyond this point, C100-induced tumour growth inhibition remained apparent and significant but to a lesser extent than the CpG treatments (Figure 5.20 b & c). C100 treatment increased median survival time by a week, with an average survival time of 18 days after the initial injection compared to the PBS control (Figure 5.20 d).
Figure 5.20 Intratumoral delivery of C100 delays tumour growth and promotes survival.

Mice were injected s.c with 3.5 x 10^5 B16 melanoma cells. When tumours reached 75 mm^3 they were injected i.t with 30 µL of PBS, chitin-derived polymers (200 µg/mouse), or CpG (50 µg/mouse). 3 days later tumours were injected as before. a) Schematic of experimental design. (b) Tumour growth rate at challenge site displayed as mean tumour volume. An unpaired two-tailed t test was used to determine statistical significance between PBS and C100 treated mice, where *p<.05 and **p<0.01(c) Spider plots of
individual tumour growth. (d) Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between PBS and C100 treated mice where **p<0.01.

5.2.14. Ethidium bromide culturing amplifies C100-induced DNA damage in DCs

It was hypothesised that the C100 and EtBr-amplified IFN response in BMDCs demonstrated in section 5.2.2 was dependent on cumulative DNA damage. If so, it suggests a potential for C100 to synergise with DNA damaging agents (radiotherapy or chemotherapy) in the treatment of cancer. To investigate, Ctrl and EtBr-cultured DCs were stimulated with media, C100 for 8 or 16 hours or etoposide for 16 hours and analysed for DNA fragmentation by flow cytometry. Culture of cells in EtBr resulted in a small but clear population of cells with fragmented DNA (Figure 5.21 a & c). Short term C100 treatment was more damaging to the DNA of EtBr-cultured DCs than WTCtrls (Figure 5.21 a-e). For instance, C100-treatment for 8 hours increased DNA fragmentation in single cells from 8.2 % in Ctrl DCs to 37 % in EtBr DCs (Figure 5.21 a). Likewise, the MFI of single cells after C100 treatment rose to 416 in EtBr DCs compared to 258 and 178 in media-treated EtBr DCs and C100-stimulated Ctrl DCs respectively (Figure 5.21 e). This cumulative DNA damage relative to media was not apparent after 16 hours of C100 treatment, with the percentage of DNA fragmentation in single cells rising from 1.68 % to 24.8 % in Ctrl DCs and 12.4 % to 37.7 % in EtBr DCs (Figure 5.21 a & c). However, the MFI of fragmented DNA in single and single live cells after 16-hour C100 treatment was much higher than media-treated EtBr DCs and C100-treated Ctrl DCs (Figure 5.21 b & d). Finally, etoposide drove clear DNA fragmentation in single and single live cells (Figure 5.21 a,c & e). Similar to both C100 timepoint treatments, EtBr culturing amplified etoposide-induced DNA damage, albeit to a weaker extent (Figure 5.21 b & d).
a

Media

C100 8 Hrs

C100 16 Hrs

Etoposide

FSC-A

BrdU-FITC

Gated on total DNA in single cells

b

DNA fragmentation

DNA fragmentation

DNA fragmentation

DNA fragmentation

DNA fragmentation

DNA fragmentation

DNA fragmentation

DNA fragmentation

1.88

12.4

8.20

37.0

24.6

37.7

11.8

26.6

MF-FITC-BrdU

C100 8 Hrs

C100 16 Hrs

Etoposide

Ctrl | EtBr

Ctrl | EtBr

Ctrl | EtBr

Ctrl | EtBr

Ctrl | EtBr
c

Media

DNA fragmentation

Ctrl 1.01

EtBr 3.51

C100 8 Hrs

DNA fragmentation

10.9

DNA fragmentation

23.1

C100 16 Hrs

DNA fragmentation

21.1

DNA fragmentation

29.5

Etoposide

DNA fragmentation

11.2

DNA fragmentation

23.5

3rdU-FITC  Gated on total DNA in single, live cells
Figure 5.21 Ethidium Bromide treatment enhances C100-induced DNA fragmentation.

Ctrl and EtBr BMDCs were treated with C100 (5 µg/mL) for 8 or 16 hours or etoposide (100 µM) for 16 hours. The BrdUTP /FITC-anti-BrdUTP TUNEL labelling system was used to monitor DNA fragmentation. (a) DNA fragmentation as a percentage of total DNA in single cells (b) MFI of fragmented DNA (BrdU-FITC) in single cells (d) DNA fragmentation as a percentage of total DNA in single live cells. (e) MFI of fragmented DNA (BrdU-FITC) in single live cells. MFI= mean fluorescence intensity
5.2.15. Long term C100 treatment drives enhanced H2AX phosphorylation in EtBr cultured DCs.

To substantiate the APO-TUNNEL data, Ctrl and EtBr DCs were stimulated with media, C100 or etoposide for 8 hours and then lysed and blotted for p-H2AX, a marker of DNA damage. EtBr culture alone did not drive H2AX phosphorylation. C100 treatment increased p-H2AX compared to media alone in Ctrl DCs but drove no detectable phosphorylation in EtBr DCs. Etoposide drove H2AX phosphorylation in Ctrl DCs and to a lesser degree in EtBr DCs (Figure 5.22 b).

The experiment was repeated with an additional 16-hour C100 treatment group. Similar to before, the EtBr culture method did not drive detectable p-H2AX, 8-hour C100 treatment drove noticeable p-H2AX that was less visible in EtBr DCs and etoposide drove vast H2AX phosphorylation in Ctrl DCs and no phosphorylation in EtBr DCs. Long term C100 treatment drove less H2AX phosphorylation than short term treatment in Ctrl DCs. However, in contrast, 16-hour C100 treatment triggered much stronger H2AX phosphorylation in EtBr DCs (Figure 5.22 c).
Figure 5.22 Prolonged C100 treatment augments Ethidium bromide-induced H2AX phosphorylation

Ctrl and EtBr DCs were stimulated with C100 (8 µg/mL) for 8 or 16 hours or with etoposide (100 µM) for 8 hours. Cells were then lysed and blotted for expression of p-H2AX. β-actin was used as a loading control. n=2.
Figure 5.23 C100 treatment augments EtBr-induced nuclear DNA damage and elicits synergistic cGAS-STING activation.

1. C100 triggers high levels of mitochondrial stress in control BMDCs that damages nDNA causing its accumulation in the cytosol. nDNA triggers STING activation, presumably through cGAS.
2. EtBr intercalates nuclear DNA causing low grade DNA damage that is not sufficient to trigger cGAS-STING signalling.
3. EtBr culture sensitises BMDCs to oxidative DNA damage by C100. Extreme high levels of nDNA accumulate in the cytosol, triggering STING-dependent type I IFN responses.

Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; EtBr, Ethidium Bromide.
5.2.16. Intratumoral delivery of C100 outperforms, but does not synergise with cisplatin treatment

It was hoped that the amplified DNA damage and type I IFN responses in C100-treated EtBr DCs would be translatable to the B16 melanoma model. Cisplatin is a clinically approved chemotherapy and like EtBr, intercalates DNA to drive dsDNA breaks. C57BL/6 mice were injected s.c with 3.5 x 10^5 B16-OVA cells on the upper left femur. When tumour size reached 0.4-0.5 cm in the largest diameter, mice were randomized into the five different experimental groups, PBS (i.t), Cisplatin (intraperitoneal (i.p)) and PBS (i.t), C100 (i.t), cisplatin (i.p) and C100 (i.t) and the positive control CpG (i.t). Groups receiving cisplatin were injected i.p when tumours measured 0.4-0.5 cm in the largest diameter (day 0) and injected the following day with PBS or C100 i.t (day 1). Of note, all cisplatin treated mice had tumours between 0.5-0.7 cm in diameter on day 1 upon initial i.t injection. On day 3, appropriate groups received a second dose of cisplatin i.p. On day 4, mice were injected i.t as before (Figure 5.24 a).

As before, PBS treated mice displayed aggressive tumour growth (Figure 5.24 b and c). By day 12, all mice had reached endpoint, with a median survival of 9 days (Figure 5.24 d). CpG treatment significantly reduced tumour growth and extended mouse survival, with a median survival of 22.5 days after the initial i.t injection (Figure 5.24 b-d). C100 dramatically stunted tumour growth compared to the PBS controls, with identical growth kinetics to the control CpG, up to 7 days after the initial i.t injection. Beyond this point, C100-induced tumour growth inhibition remained apparent and significant but to a lesser extent than the CpG treatments (Figure 5.24 b & c). Of note, there was no statistically significant difference between growth rates in C100 and CpG treated mice up to 9 days after injection, however the CpG treated mice had a median survival of 22.5 days compared to 13.5 for C100 (Figure 5.24 b-d). Cisplatin was less effective at stunting tumour growth than C100. Despite C100 and cisplatin separately retarding tumour growth, there was no additive or synergistic effect when treatments were combined. Cisplatin and C100 treated mice displayed faster tumour growth kinetics than C100, and slower tumour growth kinetics than cisplatin alone (Figure 5.24 b & c). Similarly, Cisplatin treatment slightly reduced C100-dependent mouse survival from 13.5 to 13 days while increasing cisplatin-alone mouse survival from 12 to 13 days (Figure 5.24 d).
Figure 5.24 Systemic cisplatin treatment does not boost C100-induced anti-tumour protection

Mice were injected s.c with $3.5 \times 10^5$ B16-OVA melanoma cells. On day 0 when tumours were 0.4-0.5 cm in diameter mice were injected i.p with cisplatin (3 mg/kg). On day 1, when tumours were 0.5-0.7 cm in diameter (roughly 75 mm$^3$), they were injected i.t with PBS, CpG (5 µg/mouse) or C100 (200 µg/mouse). On day 3, mice were injected i.p as
before. On day 4, tumours were injected as before. (a) Schematic of experimental design. (b) Tumour growth rate at challenge site displayed as mean tumour volume. An unpaired two tailed $t$ test was used to determine statistical significance between groups. *$p<0.05$, **$p<0.01$. (c) Spider plots of individual tumour growth. C) Kaplan-Meier survival analysis of challenged mice, a Mantel-Cox test was used to determine statistical significance between PBS and treated mice. *$p<0.05$, **$p<0.01$, and ***$p<0.001$. 
5.3. Discussion

The crucial role of STING in cancer immune surveillance has motivated the development of 2′3′-cGAMP and other structurally-related CDNs as therapeutics that activate innate immunity to enhance anti-tumour T cell responses. However, similar to other nucleic acid therapeutics, CDNs suffer from poor intracellular bioavailability that restricts their activity and clinical utility. Addressing this problem, this work identifies tumoricidal chitosan polymers that trigger the endogenous release of DNA to activate STING, stimulating anti-tumour immunity that inhibits tumour growth and improves survival in murine models of melanoma.

Chitosan-induced tumour protection occurred in a deacetylation-dependent manner, identifying C100 polymers as the optimal chitosan formulation for tumour settings. While C90 and protasan induced STING activation was dependent on mtDNA, C100-induced activation involved damage to the nuclear compartment. In this sense C100 functions similarly to radiotherapy or chemotherapeutic agents, whereby nuclear DNA damage triggers the accumulation of cytosolic DNA that activates STING. Critically, this mode of activation is not restrained by STING variability within the human population, unlike direct STING agonists that can demonstrate variable affinity for different STING alleles [148]. How damaged nuclear DNA triggers the accumulation of cytosolic DNA is unclear and beyond the scope of this study (see section 1.5.5). Future work must confirm cGAS as the sensor upstream of C100-induced STING activation.

Unlike electronegative and hydrophilic CDNs, chitosan polymers are readily taken up by BMDCs. Previous work suggested this uptake was required for chitosan-induced cGAS-STING and NLRP3 activation, as cytochalasin B blocked protasan-induced secretion of IL-1β and IFNβ in BMDCs (Dr. Liz Carroll, PhD). However, this work identified chitosan-induced mitochondrial stress as an event upstream of polymer engulfment. Given that actin polymerisation was pivotal for C90, C100 and protasan-induced mtROS production, this work proposes that actin polymerisation facilitates chitosan-induced mitochondrial stress by promoting ER-mitochondria contact and mitochondrial Ca^{2+} uptake. In support of this, actin polymerisation promotes ER-mitochondria contact during mitochondrial fission and facilitates efficient Ca^{2+} transfer between organelles [213].

Blocking trafficking-mediated STING degradation provides a therapeutic target to boost STING signalling. Chemical inhibition of STING degradation functionally augments
cGAMP-mediated immune activation and anti-tumour responses in vivo, allowing substantially reduced or less frequent cGAMP dosing [152]. Highly deacetylated chitosan polymers are unusual in that they do not trigger observable STING degradation or IRF3 phosphorylation upon activation in DCs. In fact, the expression of STING appears to increase with treatment. This is bizarre given the in vitro and in vivo dependency of protasan and C100 polymers on STING and the ability of brefeldin A to reduce protasan-induced ifna and ifnb mRNA responses. It is proposed that highly deacetylated polymers are triggering low-level STING activation that is just enough to initiate IFNAR-dependent DC maturation and IFN feedback amplification. Future studies should determine the role of IFNAR in increased STING expression upon chitosan treatment. This could have significance given that STING expression is commonly decreased in human colon adenocarcinoma and melanoma cell lines as a method of immune evasion [368], [369].

This work clearly demonstrates the inability of chitosan polymers to initiate NF-κB signalling downstream of STING activation and proposes a model whereby chitosan-induced IP$_3$-mediated ER Ca$^{2+}$ release triggers premature translocation of STING to the ERGIC where it engages cGAMP, promoting type I IFN production and simultaneously limiting NF-κB activity. In support of this, ubiquitination of lysine residues on STING that are necessary for translocation are required for type I IFN but not NF-κB signalling [136], [144]. The MCMV protein M152 that perturbs STING translocation from the ER to the Golgi compartment, blocks anti-viral type I IFN production while keeping the early pro-viral NF-κB response intact [144]. Finally, Etoposide-induced STING-dependent IRF3 but not NF-κB responses are blocked in keratinocytes by forced retention of STING at the ER [145]. While the role of STING-induced NF-κB in cancer is not well defined it is widely documented that NF-κB can be constitutively activated in cancer cells to promote proliferative and anti-apoptotic genes [150]. Indeed, the NF-κB targets, TNF-α and IL-6 are the best studied pro-tumorigenic cytokines. Their expression is commonly elevated in tumours and correlates with poor prognosis [370], [371]. On the other hand, NF-κB can synergize with IRF3 to increase maximal expression of IFN-β [362]. Thus, it’s difficult to conclude whether a lack of NF-κB activity is a positive or negative attribute of C100. Future work must address the role of STING-induced NF-κB in cancer and determine if C100’s inability to activate NF-κB contributes to or diminishes its intratumoral efficacy.

This work demonstrated an advantage of C100 polymers over DMXAA and 2′3′-cGAMP in their ability to drive in vitro tumour cell death. The mechanism of C100-induced cell death is unclear but likely involves mitochondria and nuclear compartments given the extensive damage it bestows on both. Persistent DNA damage in cells triggers the
induction of senescence or apoptosis, thus acting as a barrier to tumorigenesis [242]. Critically, cGAS is required for senescence and promotes the secretion of various cytokines, chemokines, growth factors and proteases through STING that trigger a pro-inflammatory programme referred to as SASP [245], [372]. SASP enforces senescence growth arrest in neighbouring cells and promotes clearance of senescent cells by immune cells [243], [244]. Thus, future studies should address the role of senescence and SASP in C100-mediated tumour protection.

A limitation of STING-directed therapies that also applies to C100 is the difficulty in determining the precise source of cGAMP production, be it direct production of cGAMP in APCs, indirect production of cGAMP in APCs after engulfment of tumour cells containing damaged DNA, or tumour-derived cGAMP that is transferred to neighbouring cells via transporters, gap junctions or exosomes. Future studies should attempt to address the sources of cGAMP production upon C100 injection and confirm the in vivo role of type I IFNs by monitoring tumour growth in cgas−/−, sting−/− and ifnar−/− mice. Additionally, studies must characterise the tumour infiltrates that afford C100 protection. Although the anti-tumour effect of STING agonists are primarily attributed to CD8+ T cells [373], type I IFNs additionally act on NK cells to boost activation and anti-tumour cytotoxicity [374].

Chitosan polymers are most commonly degraded by lysozymes that selectively target the acetyl groups of the chitosan chains. This affords highly deacetylated chitosan polymers a much slower degradation rate than polymers with low degrees of deacetylation [356], [357]. The intratumoral dosing regimen used in the B16 melanoma models was based on studies with nucleotides that exhibit poor pharmacokinetic profiles, largely due to rapid degradation by host enzymes such as ENPP1 [39], [374], [375]. Future studies should address the degradation rates of C100 polymers within the tumour microenvironment, as this may allow a lower and less frequent dosage. For instance, repetitive and/or high doses of the ENPP1-resistant STING agonist ADU-S100, while effective in clearing the injected tumour, diminishes tumour-specific T cell responses and limits durable immunity [373]. Similarly, high doses of radiation hamper optimal activation of the cGAS-STING pathway through Trex1 induction, that degrades DNA in the cytosol of irradiated cancer cells [376].

Ethidium bromide culture was utilised in this chapter to implicate mtDNA in protasan, C90 and C100-induced IFN responses in BMDCs. However, it serendipitously revealed a unique role for nuclear damage in C100-induced STING activation. Strikingly despite
failing to trigger \textit{ifnb} expression alone, EtBr culture amplified C100-induced \textit{ifnb} mRNA responses. This amplification required STING and was dependent on synergistic DNA damage between both compounds. Unfortunately, the response did not translate to the \textit{in vivo} setting, with C100 treatment outperforming cisplatin treatment alone and cisplatin and C100 combinational therapy. It is hoped that the dose of cisplatin was too high, triggering Trex1 induction that limited cytosolic DNA accumulation. Future studies should use Trex1 expression to identify the optimal chemotherapeutic dose and additionally investigate a synergy between low dose radiotherapy and C100. Additionally, studies should investigate C100 combined with immune checkpoint inhibitors given the dramatic synergies that have been observed between STING agonists and ICI in murine cancer models [355], [377].

In summary, this work identified C100 polymers as attractive alternatives to current STING therapies that sidestep common barriers to clinical translation such as poor cellular uptake, low cytosolic availability and variable affinity for different STING alleles. In addition, it prompts in-depth investigation into the understanding of STING-mediated NF-\kappa B activation and its role in cancer.
6. General Discussion

There is a clear need for new adjuvants that more effectively promote cell-mediated immunity for widespread diseases including HIV-AIDS, malaria, tuberculosis and cancer. Alum adjuvants fail to induce strong Th1-biased cellular immune responses critical to anti-viral and anti-tumour immune responses [378] and MF59, AS03 and AF03 preferentially promote Th2 polarisation and antibody responses that affords little protection against viral and intracellular bacterial pathogens [379]. While it is clear that innate immunity governs the induction of protective adaptive immune responses, the precise mechanisms by which licensed adjuvants promote and direct adaptive immunity are only partially understood. Hence, there is a clear need to develop new adjuvants with defined mechanisms of promoting durable cellular immunity. Dendritic cells bridge innate and adaptive immunity, and determine both the magnitude and type of adaptive immune responses that are induced, in part through the upregulation of maturation markers and secretion of cytokines [33]. Consequently, understanding mechanisms of adjuvant induced DC maturation and cytokine secretion is crucial for optimal adjuvant design and a critical feature for identifying new and improved adjuvants.

The adjuvant potential of chitosan has been demonstrated for vaccine administration by injection and mucosal routes with a number of different co-administered [11], [71], [292], [304]. In particular, chitosan displays significant potential as an adjuvant for the promotion of cellular immunity. While the Lavelle lab have identified a definitive role for cGAS-STING and the NLRP3 inflammasome in chitosan-induced cellular immunity [71], there are hugely conflicting reports regarding the direct innate immune modulating properties of chitosan polymers. Chitosan has been proposed to induce cytotoxicity in macrophages, promote NK cell activation, and enhance non-specific host resistance to bacterial infection [305], [306], [380]. Furthermore, it has been shown to promote NO production in peritoneal macrophages [381] and TNF-α secretion in human monocytes and BMDCs [382], [383]. Conversely, chitosan has been demonstrated to promote the anti-inflammatory cytokines, IL-1ra, IL-10 and TGF-β and more recent reports suggest an inability of chitosan polymers to elicit the production of inflammatory cytokines in macrophages [305], [383], [384]. Discrepancies between studies are due to poor polymer characterisation in terms of chitosan source, method of purification, size and purity.
This study monitored the adjuvanticity of a series of highly characterised and purified chitosan polymers derived from crustaceans, each with a different DD or molecular weight, prepared in the same laboratory. In doing so, it unravelled the mechanistic basis underlying the differential immunomodulatory effects of chitin-derived polymers. **Acetylation was found to dictate the ability of chitosan polymers to promote Th1 responses against the TB antigen H56 and suppress tumour growth in B16 melanoma models, thus identifying C100 polymers as optimal chitosan formulations.** Mechanistically, polymer acetylation blocked the induction of mitochondrial oxidative stress in DCs that was pivotal to chitosan-induced cGAS-STING signalling and NLRP3 inflammasome activation. Indeed, highly acetylated chitosan polymers such as C39, C49 and C72 were relatively inert, while highly deacetylated chitosan polymers with uninterrupted clusters of amines were inflammatory in comparison. This is in line with a nearly three-decade old study exemplifying how a 70 % deacetylated chitosan polymer displayed more efficacy than a 30 % deacetylated polymer in non-specifically supressing meth-A tumour growth in BALB/c mice when injected *i.p.* [380]. In addition, it provides an explanation for the failure of phase I/IIa clinical trial investigating a *Haemophilus influenzae* type b glycoconjugate vaccine (Act-Hib) adjuvanted with a 50 % deacetylated chitosan, referred to as Viscogel. While Viscogel was safe and well tolerated, it had modest effects on cellular or humoral immune responses in vaccinated subjects compared to the antigen alone (NCT01578070) [293], [294]. It must be noted that although C100 is the best chitosan adjuvant, its ability to damage nuclear DNA does raise concerns regarding its safety in prophylactic settings. Additional work is needed to evaluate C100’s mutagenicity, carcinogenicity and safety pharmacology before it can be translated to clinical settings.

**STING activators are heavily investigated in oncological settings due to the pivotal and natural anti-tumour role of the cGAS-STING pathway.** Tumour-derived DNA triggers cGAS-STING signalling and type I IFN production that promotes tumour-antigen presentation on DCs, DC maturation and cross presentation to CD8+ T cells for anti-tumour immunity. Alternatively, tumours can produce 2’3’-cGAMP which can transfer to non-tumour cells. To date, gap junctions, folate transporter SLC19A1, P2X7R, and the volume-regulated anion channel (VRAC) LRRC8 have been reported to transmit 2’3’-cGAMP from cell to cell or from the extracellular region to cells [122], [123], [385]–[387]. These processes are critical in preventing neoplastic progression and limiting tumorigenesis. Despite the plethora of STING agonists that have been and are being developed, clinical translation has resulted in disappointingly modest efficacy. For instance, the synthetic STING ligand DMXAA achieved robust anti-tumour immune
responses in B16 melanoma models but failed in Phase III trials (NCT00738387 and NCT00662597), likely due to its inability to bind human STING [355]. Intratumoral administration of natural CDNs such as mammalian 2’3’-cGAMP and c-di-GMP, c-di-AMP and 3’3’-cGAMP from prokaryotes display anti-tumour potential in mouse models of colon, brain, skin, pancreatic, breast, and B cell malignancies. However, intrinsic characteristics such as size and charge render these molecules poorly membrane permeable and prone to rapid enzymatic degradation, resulting in low drug bioavailability in tumour tissues [377]. Furthermore, 2’3’-cGAMP is the only naturally-derived CDN with high affinity for human STING. Various approaches are underway to address limitations, such as the development of CDN drug delivery systems (liposomes, polymers and hydrogels), synthetic STING agonists with greater affinity for human STING (ADU-S100, MK1454, E7766, MK-2118, BMS-986301, SB-11285, IMSA-101), small molecule STING agonists (GSK3745417) and ENPP1 inhibitors (MAVU-104). However, despite huge investment, these approaches are still showing underwhelmingly low response rates and poor pharmacokinetics. Merck’s trial data reported a 0% overall response rate with MK-1454 monotherapy and only 24% when combined with pembrolizumab (anti-PDL1) (NCT03010176) and data from the ADU-S100 clinical trial showed that only 1/53 patients achieved a complete response in the spartalizumab (anti-PD1) combination group (NCT03172936). Findings from E7766, MK-2118, BMS-986301, SB-11285, IMSA-101 and GSK3745417 are not yet disclosed and the MAVU-104 Phase I trial has not yet begun. Much hope is now placed on DDSs, in particular polymers given their ability to profoundly alter and improve drug pharmacokinetics, provide local and controlled STING agonist release into tumours and synergise with ICI therapies [377]. Of significance, chitosan polymers are efficacious DDSs in diseases such as cancer. Characteristics including hydrophilicity, cationic net charge, biocompatibility, and their biodegradable nature allow sustained release and enhanced bioavailability of contents. Future investigations should explore the use of C100 as a DDS to encapsulate negatively charged CDN compounds [358], [359]. In this setting, the delivery system and the drug itself would function to activate anti-tumour STING signalling.

Unique features of highly deacetylated chitosan polymers, particularly C100, present them as attractive alternatives to conventional STING agonists. As with anti-cancer agents (radiotherapy, chemotherapy), chitosan polymers promoted STING-dependent type I IFN responses through the accumulation of endogenous DNA, bypassing limitations of membrane permeability and STING polymorphism. C100 polymers functioned as indirect DNA damaging agents, causing rampant mitochondrial oxidative stress that triggered dsDNA breaks and the accumulation of nDNA in the
cytosol. It is likely that mtDNA also contributes to C100-induced STING activation, but this could not be demonstrated with the EtBr culture method given the nature of the drug. EtBr intercalates all DNA/RNA entities in the cell, and despite successfully depleting mtDNA it also caused low-grade nuclear DNA damage that synergised with C100-induced DNA damage, masking any potential contribution of mtDNA in STING activation. In contrast, C90 and protasan caused mitochondrial stress that imposed minimal damage on the nuclear compartment, preventing synergy with EtBr and highlighting the dependence of mtDNA for STING activation. Future studies should address the contribution of mtDNA in C100-induced STING activation using mice that specifically lack transcription factor A, mitochondrial (TFAM) in myeloid cells [57]. The molecular details that allow mtDNA to escape from the mitochondria require further investigation, but presumably involve permeabilisation of the outer and inner mitochondrial membranes [221], [318]. It is not uncommon for mitochondrial stress to trigger cGAS-STING activation- microbial pathogens, such as HSV and dengue virus can indirectly trigger cGAS–STING activation through the induction of mitochondrial stress that results in mtDNA leakage [388], [389]. A similar scenario occurs in malignant settings. Cancer cells undergoing oxidative stress and mitochondrial dysfunction release mitochondrial DNA (mtDNA) into the cytosol that promotes cGAS-STING signalling [111], [368]. Furthermore, in adipose tissue, a mitochondrial stress-activated cGAS–STING pathway functions as a sentinel to suppress thermogenesis and energy expenditure [390]. Further research should address the theory that C90 and C100, like protasan activate STING via cGAS. Until a null phenotype is observed in cGAS<sup>−/−</sup> mice vaccinated with C90 or C100 and no type I IFNs are detected in BMDCs from cGAS<sup>−/−</sup> mice stimulated with C90 and C100, the contribution of other DNA sensors (e.x IFI16 and DDX41) toward C90 and C100-induced STING activation cannot be ruled out. Interestingly, mtDNA and genomic DNA may not be synonymous with respect to activating cGAS. Recent evidence suggests that nucleosome-bound chromatin promotes cGAS sequestration instead of activation [259], [391]. However, this notion remains controversial, as another study suggests that cytoplasmic nucleosomes increase cGAS activity [392]. Curiously, despite the fact that C100-induced type I IFNs and cellular immunity are STING and IFNAR dependent, this work did not detect features of STING activation such as STING degradation, STING phosphorylation or TBK1 and IRF3 phosphorylation. Additional studies must address this contradiction by monitoring STING and IRF3 translocation upon chitosan treatment in BMDCs.

As a method to evade DNA-sensing machinery, tumours harbouring mitochondrial dysfunction or chromosomal instability can reduce STING expression to ablate
downstream IFN signalling [111], [368]. However, the fact that only 1-2 % of cancers completely block expression of STING, implies that complete silencing of STING is not advantageous for tumour growth. Indeed, STING activation is associated with increased metastasis in tumours with low antigenicity [147]. It is well known that endogenous type I IFNs are beneficial in limiting cancer progression by priming T cells and driving anti-cancer immune responses. For instance, IFNAR signalling has been shown to be critical for STING-mediated tumour suppression in murine models of cancer [375], [393], [394]. In contrast, the role of STING-dependent NF-κB signalling in cancer is less clear with recent reports implying a pro-tumour role. STING and downstream NF-κB signalling in astrocytes increases brain metastasis [395]. STING agonists markedly increase PD-L1 expression in cancer cells through a mechanism involving NF-kB activation [396]. Radiation-induced STING-dependent non-canonical NF-κB activation inhibits type I IFN production and anti-tumour immunity [397]. In line with this, chromosomally unstable tumours exclusively activate STING-dependent non-canonical NF-κB signalling to drive secretion of pro-inflammatory cytokines and metastasis [398]. Finally, STING-mediated induction of SASP is NF-κB dependent and chronic SASP-correlated inflammation relates to malignant behaviours such as immune-suppression and oncogene-driven senescence evasion [243], [245], [399]. These reports are unsurprising given the large body of evidence demonstrating the role NF-κB plays in tumour initiation, metastasis and angiogenesis and the number of anti-cancer agents that target NF-κB signalling [151]. Thus, the inability of C100 to promote STING-dependent NF-κB activation likely contributes to its efficacy in the B16 melanoma models. To our knowledge, highly deacetylated chitosan polymers are the only STING activators to promote exclusive type I IFN profiles.

Unlike electronegative CDNs, cationic chitosan polymers are readily taken up by BMDCs. It was previously thought that uptake was required for chitosan-induced cGAS-STING and NLRP3 activation, as cytochalasin B blocked protasan-induced secretion of IL-1β and IFN-β in BMDCs (Dr. Liz Carroll, PhD). However, this work identified chitosan-induced mitochondrial stress as an upstream event of polymer engulfment and instead suggests that actin polymerisation facilitates chitosan-induced mitochondrial stress by promoting ER-mitochondrial contact and mitochondrial Ca^{2+} uptake. In support of this, actin polymerisation promotes ER-mitochondria contact during mitochondrial fission and facilitates efficient Ca^{2+} transfer between organelles [18]. The identification of a receptor(s) mediating the recognition and/or phagocytosis of chitosan has eluded researchers, but links have been established between dectin-1 and chitosan in mediating signalling events from the cell surface rather
than uptake. Dectin-1 is required for optimal type I IFN induction in response to low concentrations of protasan and pharmacological inhibition of Syk, the tyrosine kinase recruited following engagement of Dectin-1, compromises the induction of type I IFNs in response to low concentrations of protasan (Dr. Liz Carroll, PhD). In light of the fact that Dectin-1 ligation induces ROS in a Syk-dependent manner and ER Ca\(^{2+}\) flux in a Syk-PLCy2-dependent manner [400], [401], it is tempting to speculate that chitosan is sensed by Dectin-1 leading to signalling via Syk and PLC to trigger IP\(_3\)-mediated opening of Ca\(^{2+}\) channels on the ER. However, an obvious flaw in this theory is that chitosan's dependency on Dectin-1 and Syk is overridden with high concentrations. Thus, it is more likely that several cell surface receptors work in association with each other to sense chitosan. Moreover, given the cationic nature of highly deacetylated chitosan polymers, it cannot be ruled out that intracellular signalling stems from electrostatic interactions with the DC membrane, especially since this may explain the efficacy of C100 over less positive charged chitosan formulations. Regardless, future studies should investigate a role for Dectin-1 in C100 adjuvanticity's.

It is evident that the **toxicity of chitosan formulations is dependent on the extent of deacetylation.** While toxicity limited C100-induced IFNAR feedback signalling *in vitro*, it was not detrimental to its adjuvanticity *in vivo*, as when administered subcutaneously in combination with H56, C100 was found to induce the greatest STING, IFNAR and NLRP3-dependent antigen-specific IFN-γ response compared to the less deacetylated chitosan formulations. In fact, **C100's direct tumoricidal activity** likely contributed to the polymers ability to retard tumour growth in the therapeutic B16 vaccination model. It appears that only polymers that cause mitochondrial dysfunction trigger cell death. Whether these two processes are directly linked is unknown but plausible given the central role of mitochondrial dysfunction in mediating cell death [226]. Alternatively, it is possible that positively charged amine groups interact strongly with the cell, rupturing the membrane and thus triggering cell death. The ionic interaction between the polymer and the cell membrane would be disrupted by neutral acetyl groups, minimising membrane disruption and accounting for reduced toxicity with less deacetylated polymers. Future work should address the form of cell death and its *in vivo* contribution to chitosan-induced immune responses.

This work highlights **mitochondrial stress as an attribute of chitosan-induced cGAS-STING and NLRP3 activation and identifies it as a likely point in which the pathways diverge.** Critically, it refutes previous findings suggesting that chitosan induced type I IFN responses and inflammasome activation are mutually exclusive
processes [343]. Future work could address crosstalk between the PRR pathways. For instance, in response to various PAMPs, IFN-induced STAT1 gene products repress NLRP3 inflammasome activation and IL-1β release [402], [403]. Critically, the clear dependency on mtROS implicates Ca<sup>2+</sup> in chitosan-induced NLRP3 inflammasome activation. Though there is substantial literature supporting an involvement of Ca<sup>2+</sup> in NLRP3 inflammasome activation, many of these studies have involved the use of 2-ABP, an inhibitor of Ca<sup>2+</sup> signalling that since has been demonstrated to block the inflammasome in a Ca<sup>2+</sup>-independent manner or BAPTA-AM, a Ca<sup>2+</sup> chelator that inhibits the inflammasome in the absence of Ca<sup>2+</sup>, suggesting off-target effects [65], [66], [404], [405]. Hence, this study did not utilise Ca<sup>2+</sup> inhibitors to monitor the role of Ca<sup>2+</sup> in chitosan-induced IL1β release. It can't be overlooked that although NLRP3 is critical for C100-induced Th1 responses, IL-1β and IL-18 can exert pro-tumorigenic effects [406]. Both are commonly upregulated in tumours and are thought to contribute to tumour invasiveness and metastasis by inducing the expression of angiogenic genes and growth factors [85]. Future studies should address whether the function of NLRP3 in chitosan-induced immunity is IL-1β or IL-18 dependent and determine whether these cytokines are limiting C100s anti-tumour protection.

ER stress was monitored after chitosan stimulation as an indirect method of monitoring whether a Ca<sup>2+</sup> efflux event was required for the induction of mitochondrial stress. However, seeing as ER stress is a newly defined consequence of STING activation [138], [179], [341], future work must verify that chitosan-induced ER stress occurs independently of STING. This should be achieved by monitoring chitosan-induced ER stress and ER Ca<sup>2+</sup> release in mtDNA depleted DCs and DCs from cgas<sup>-/-</sup> mice. Critically, ER stress has a negative role in the induction of anti-tumour immune responses. Several reports have demonstrated that ER stress affects the cell surface expression of MHC class I molecules [205]–[208]. Notably, DCs in the tumour environment exhibit ER stress and robust IRE1α activation [209], ER-stress imprinted DCs promote tumour growth in mice [208][209], and relieving ER stress in DCs extends host survival by enhancing T cell anti-tumour immunity [209]. Furthermore, ER stress is harmful to T cell viability and responsible for T cell cytopenia in SAVI patients. SAVI (STING-N154S) mice have chronically elevated ER stress through disrupted Ca<sup>2+</sup> homeostasis that renders T cells hyperresponsive to TCR-signalling-induced ER stress and tips the balance toward cell death. Pharmacological inhibition of ER stress with the chemical chaperone TUDCA or point mutation of residues R331 and R334 in the UPR motif protects STING-N154S mutants from T cell cytopenia [139]. Several ER stress inhibitors are US Food and Drug Administration–approved drugs with an excellent safety profile; thus, future work should
investigate whether alleviating ER stress can amplify C100-induced Th1 immune responses.

Beyond the pro-tumour role of ER stress, **C100 polymers were demonstrated to trigger autophagy** that may well diminish its anti-tumour effect. For instance, autophagy is a negative-regulatory feedback function of cGAS and STING activation that limits their activation by removing cytosolic DNA [354]. It also limits NLRP3 activation by degrading inflammasome components and supports tumor cells by providing cellular building blocks and energy [168], [169]. That being said, autophagy is linked to the induction of CD4⁺ and CD8⁺ T cell responses through increased MHCcl and MHCcII antigen processing and presentation [180]–[183] and suppression of tumorigenesis by triggering cell death in chromosomally unstable cells [407]. Future work must delineate the mechanism of autophagy induction by chitosan polymers and its in vivo impact on C100-induced responses in prophylactic and therapeutic vaccine settings.

The presence and activity of T cells within the tumour microenvironment is modulated through stimulatory and inhibitory receptors, with established tumours tipping the balance toward immunosuppression. The inhibitory signals on T cells are delivered through surface molecules such as CTLA4 and PD1 by interaction with their respective ligands expressed on cancer cells and/or APCs. Immune checkpoint inhibitor therapy attempts to relieve the negative checkpoints on T cells within the TME. However, checkpoint blockers work most effectively on T cell rich tumours, an uncommon occurrence that is likely responsible for ICI’s success in only a fraction of patients. Drug-induced STING activation is an increasingly common approach to sensitise patients to ICI. Stimulation of STING results in the production of the crucial Th1 recruiting cytokines, CXCL9 and CXCL10 and Type I IFNs [360]. The promise of combined ICI therapy and STING agonists is clear in the finding that of 10 current clinical trials investigating STING agonists, 9 are under evaluation in combination with ICI [377]. Given the potent induction of CXCL10 by DCs stimulated with highly deacetylated chitosan polymers, future studies should address the anti-tumour potential of C100 polymers in combination with ICI.

Altogether this work unravelled how chitosan polymers can possess inert and inflammatory properties and the parameters that can be measured to obtain a chitosan formulation with appropriate properties for each application. In doing so, it identified C100, as the optimal chitosan formulation for use in prophylactic vaccines and therapeutic cancer settings. Unlike many empirically developed licensed adjuvants, C100 displayed a clear in vitro and in vivo mechanism of action, activating two major
PRR pathways in the cell, the cGAS-STING pathway and NLRP3 inflammasome. Critically, C100 polymers sidestep common barriers to clinical translation of STING therapies and possess unique tumoricidal activity. Potential pitfalls for C100 include induction of autophagy and ER stress. However prior knowledge gives the potential to optimise C100 to its full potential. Finally, an underappreciated point in adjuvant design is the benefit of an adjuvant having multiple biomedical applications as chitosan does. Indeed, the majority of adjuvants that are licensed had alternative medical uses prior to use in vaccines [349]. C100 is cheap, readily available, derived from biomedically safe chitin and has a well-defined manufacturing process. All in all, this supports progress towards clinical evaluation of C100 as a therapeutic and/or prophylactic vaccine adjuvant.
Figure 6.1 Proposed mechanism of C100-induced effects on DCs leading to Th1 polarisation

C100 triggers Th1 responses as a consequence of cGAS-STING and NLPR3 activation. Mechanistically, C100 treatment initiates opening of IP$_3$R-gated Ca$^{2+}$ channels, causing ER stress and STING translocation from the ER to the ERGIC. An actin polymerisation event promotes the flow of Ca$^{2+}$ into the mitochondria, resulting in mitochondrial fission,
reduced mitochondrial mass and mitochondria stress. Mitochondrial stress triggers activation of the NLRP3 inflammasome, the egress of mtDNA into the cytosol and damage to the nuclear compartment. nDNA accumulates in the cytosol and engages with cGAS generating cGAMP. cGAMP binds to STING at the ERGIC, allowing exclusive type I IFN production. IFN-β binds the IFNAR receptor driving ISGF3-dependent gene transcription. DCs subsequently express increased levels of CD80 and CD86 on their surface, increase IRF7 expression and secrete IFN-β and CXCL10. Newly secreted IFN-β binds the IFNAR receptor, initiating IFN feedback amplification. Viability is required for positive feedback signalling in vitro. Solid Arrow: Supported by data. Dotted Arrow: Speculative.
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Appendix

Chitin-derived polymer deacetylation regulates mitochondrial reactive oxygen species dependent cGAS-STING and NLRP3 inflammasome activation

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Key words

Chitosan; vaccine adjuvant; mitochondrial stress; STING; IFNAR; NLRP3

Abbreviations

DCs, dendritic cells; BMDC, bone marrow derived DC; DDA, degree of deacetylation; mtROS, mitochondrial reactive oxygen species; GlcNAc, N-Acetyl-glucosamine; Glc, glucosamine; IFN; interferon; IFNAR; IFN-α/β receptor; H56, hybrid 56; MOMP, Major outer membrane protein; Act-Hib, Haemophilus influenzae type b glycoconjugate vaccine; s.c; subcutaneous; i.m; intramuscular.
Abstract

Chitosan is a cationic polysaccharide that has been evaluated as an adjuvant due to its biocompatible and biodegradable nature. The polysaccharide can enhance antibody responses and cell mediated immunity following vaccination by injection or mucosal routes. However, the optimal polymer characteristics for activation of dendritic cells (DCs) and induction of antigen-specific cellular immune responses have not been resolved. Here, we demonstrate that only chitin-derived polymers with a high degree of deacetylation (DDA) enhance generation of mitochondrial reactive oxygen species (mtROS), leading to cGAS-STING mediated induction of type I IFN. Additionally, the capacity of the polymers to activate the NLRP3 inflammasome was strictly dependent on the degree and pattern of deacetylation and mitochondrial ROS generation. Polymers with a DDA below 80% are poor adjuvants while a fully deacetylated polyglucosamine polymer is most effective as a vaccine adjuvant. Furthermore, this polyglucosamine polymer enhanced antigen-specific Th1 responses in a NLRP3 and STING-type I IFN-dependent manner. Overall these results indicate that the degree of chitin deacetylation, the acetylation pattern and its regulation of mitochondrial ROS are the key determinants of its immune enhancing effects.
Introduction

The cationic polysaccharide, chitosan is 1-4-β-linked polymer comprised of N-Acetyl-glucosamine (GlcNAc) and glucosamine (Glc) units that is being investigated as vaccine adjuvants. The polymer is generated by the chemical deacetylation of chitin which is found in the exoskeleton of crustacea, insects, and some fungi. Deacetylation of chitin leads to the conversion of acetyl groups to amine groups, which are distributed along a glucosamine backbone [1].

Polymer properties including molecular weight, DDA and the distribution of acetyl groups strongly influence the biological properties of chitosan, but their impact on chitosan’s adjuvant properties remain poorly defined. Much of the literature to date has focused on chitosan salts with a DDA between 80 and 90%. Such preparations generally have a blockwise distribution of the remaining acetyl groups along the glucosamine backbone, and are produced under heterologous conditions. Alternative polymers with a much lower DDA, ranging from 35-70%, can be produced under homogenous conditions and have a random distribution of acetyl groups. The main advantage of chitosan preparations with a lower DDA is that solubility is significantly improved under physiological conditions and biodegradation is faster [2], but this may be at the expense of their immunostimulatory properties.

Despite the efforts made to address the effects of polymer deacetylation on the immunostimulatory properties of chitosan, the literature is overall inconclusive due to issues such as endotoxin contamination and inconsistencies between formulations obtained from different sources [3], [4]. Addressing these inconsistencies is essential to provide a basis for polymer optimisation for multiple applications from vaccine adjuvants to biomaterials and drug delivery systems. In this study we compared a series of highly characterised and purified polymers, in which the DDA and the deacetylation pattern have been varied under controlled conditions. In doing so, we defined the mechanistic basis underlying their differential immunomodulatory effects with a particular emphasis on their adjuvant properties.

The maturation of DCs is a key determinant in the immunostimulatory effects of vaccines [5], [6]. Adjuvants can activate DCs either directly by engaging with pathogen recognition receptors (PRRs) or indirectly through the delivery of danger signals to the cell which engage PRRs located both externally and internally in the cells. This activation leads to enhanced expression of MHC and costimulatory molecules on the DC surface in addition to the secretion of cytokines which direct T cell polarisation and differentiation. The activation and polarisation of T cells is influenced by multiple factors including the nature of the antigen presenting DC and specific combination of cytokines secreted by DCs and other cells [7]. We have shown that the chitosan salt, protasan CL213 activated DCs and enhanced T cell responses through engagement of the cGAS-STING pathway and NLRP3 inflammasome [8]. Here we demonstrate that the ability of chitin-derived adjuvants to promote activation of the NLRP3 inflammasome, the cGAS-STING pathway and antigen-specific cellular immunity thorough the activation of DCs is dictated by the degree of chitin deacetylation and the distribution of acetyl groups. This study establishes the precise
characteristics of chitin-derived polymers that dictate the extent of DC activation and the mechanistic basis by which polymer physicochemical properties regulate immune activation, paving the way for the design of chitin-derived polymers as vaccine adjuvants.

Materials and Methods

C38, C49 and C72 (ViscosansTM: randomly or homogenously deacetylated chitosans) were produced from chitin (ChitiNor AS, CN141107) in a proprietary process at Viscogel AB, here provided by Flexichem AB [9]. To prepare C90, chitin was soaked in 40 % NaOH over night at room temperature and then heated for six hours at 90 °C. Subsequent washes with water (until neutral) and a final wash with ethanol followed by drying produced a Chitosan polymer with a DD of 90 %. C100 was produced similarly through repeated treatments with 40 % NaOH at 90 °C. C100 polymers of varying molecular weight were prepared as in [10]. In brief, chitin was hydrolysed in 3M HCl at room temperature for 6.5 h to 96 h. After washing and drying, these chitin preparations were subjected to deacetylations in 40 % NaOH as described above. The pattern and degree of chitosan deacetylation was determined by 1H-NMR. Molecular weights are estimations based on relative viscosity (Brookfield 1% HoAc, 20°C). Alhydrogel was from Brenntag Biosector, CpG ODN 1826 from Oligos etc and LPS (from Escherichia coli LPS, Serotype R515) from Enzo life sciences. Bafilomycin A and Rotenone were purchased from Sigma.

Table 1

<table>
<thead>
<tr>
<th>Chitosan</th>
<th>Estimated Molecular weight (kDa)</th>
<th>DDA (%)</th>
<th>Deacetylation pattern</th>
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<td>86</td>
<td>Heterogenous</td>
<td>Novamatrix, Norway</td>
</tr>
<tr>
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<td>38</td>
<td>Homogenous</td>
<td>Viscogel AB</td>
</tr>
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<td>49</td>
<td>Homogenous</td>
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<tr>
<td>C72</td>
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<td>72</td>
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<td>Viscogel AB</td>
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<tr>
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<td>Heterogenous</td>
<td>Primex, Island</td>
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<td>350</td>
<td>100</td>
<td>n.a</td>
<td>Viscogel AB</td>
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</table>

DDA; Degree of deacetylation
Mice

C57BL/6 mice were obtained from Harlan Olac (Bicester, UK). Nlrp3\(^{-/-}\) mice were provided by the late Prof. Jurg Tscopp (Department of Biochemistry, University of Lausanne, Switzerland). Ifnar1\(^{-/-}\) mice were kindly provided by Paul Hertzog (Centre for Innate Immunity and Infectious Diseases MIMR-PHI and Monash University, Clayton, Victoria, Australia). Tmem173\(^{-/-}\) mice, deficient in STING were provided by Dr. Lei Jin (Department of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, University of Florida, USA). All mice (aged 8-16 weeks) were bred in house and maintained according to the regulations of the EU and the Irish Health Products Regulatory Authority (HPRA). Procedures were conducted under animal license number AE19136/P079 and were approved by TCD Animal Research Ethics Committee (Ethical Approval Number 091210).

Isolation and culture of bone-marrow derived dendritic cells (BMDCs)

Isolated bone marrow cells were seeded at 0.45 x 10\(^6\) cells/ml in complete RPMI 1640 medium supplemented with 20 ng/ml of murine GM-CSF (peprotech) and incubated at 37 °C with 5% CO\(_2\). On day 3 of culture, cells were supplemented with 30 mL of complete RPMI 1640 medium containing 20 ng/mL GM-CSF. On day 6, non-adherent cells were removed from flasks by gentle pipetting and 30 ml of complete RPMI 1640 medium containing 20 ng/ml GM-CSF was added. Two days later, cells were supplemented for the final time with 30 mL of complete RPMI 1640 medium containing 20 ng/mL GM-CSF. On day 10, loosely adherent cells were harvested by gentle pipetting and plated in complete RPMI 1640 medium supplemented with 10 ng/ml GM-CSF.

Measurement of mitochondrial reactive oxygen species

BMDCs were seeded at 1x10\(^6\) cells/mL in 12 well round flat bottom plates. After 1.5 hours of treatment, plates were centrifuged and supernatants were discarded. As a positive control for mitochondrial superoxide (mtROS), cells were treated with rotenone (5 µM) for 12 hours. Cells were removed from wells using ice cold PBS, transferred to labelled FACs tubes and washed with 2 mL PBS. Once washed and spun down, cells were co-stained with fixable viability stain 510 (200 µL: 1 in 1000 Dilution) and (MitoSox Red ThermoFisher #M36008) (500 µL: 1 in 5000 dilution) in PBS for 15 minutes. Cells were then washed twice in PBS and re-suspended in 200 µL FACS buffer and acquired immediately.

Detection of costimulatory molecule expression on BMDCs

Cells were stained with Fixable Viability Stain 510, centrifuged at 400 x g for 5 min at 4 °C and re-suspended in 100 µL of FACS buffer containing purified anti-mouse CD16/CD32 to block FcyRII/III. Cells were then surface stained by the addition of 10 µL of fluorochrome-conjugated
antibodies against CD40, CD80 and CD86. Samples were acquired using a BD FACSCANTO II with summit software (Dako, Colorado) and the data analysed using Flowjo™ software (Treestar, Oregon).

**Measurement of cytokine secretion by ELISA**
Concentrations of IL-6 were measured using antibodies obtained from BD Biosciences (BD 555240). IFN-β was detected using antibodies from Santa Cruz Biotechnology Inc.(Capture antibody #sc-57201) and R&D systems (detection antibody #32401-1). IFN-γ (DY485) CXCL10 (DY466) and IL-1β (DY401) ELISA kits were purchased from R&D systems.

**Q-PCR**
RNA was isolated using High Pure RNA Isolation Kit (Roche) and reverse transcribed into complementary DNA (cDNA) with an M-MLV reverse transcriptase, RNase H minus, point mutant (Promega). Quantitative PCR was performed using KAPA SYBR FAST qPCR Kit Master Mix (2X) (KAPA Bio- systems) in accordance with the instructions provided by the manufacturer using Aligent Technologies Stratagene Mx3005P technology. The The following primers were used (5'->3'). Actb forward, TCCAGCCTTTCTTGGT, Actb reverse, GCACTGTGTGGCATAGAGGTGTC, Rps18 forward, CTTAGGGGACAAGTGCGG, Rps18 reverse, GGACATCTAAGGCGATCACA, Ifnb forward, ATGGTGGTCCGA GCAGAGAT, Ifnb reverse, CCACCCTACTCTGAGGCA, Ifna forward, ATGGCTAGGCTCTGCTTCCT, Ifna reverse, AGGGCTCTCCAGAGGTCT GCTCTG. RNA expression was normalized to Actb and Rps18 expression and to expression in the relevant untreated control sample.

**Vaccination of mice and assessment of humoral and cellular immunity**
For the vaccination studies, two clinically relevant antigens were used, the *Mycobacterium tuberculosis* vaccine antigen, hybrid 56 (H56) (Statens Serum Institute) a fusion protein composed of Ag85B-ESAT6-Rv2660c and the *Chlamydia trachomatis* major outer membrane protein (MOMP) (Statens Serum Institute) [11]–[13]. Mice were vaccinated by the subcutaneous route or intramuscular route on day 0 with PBS, H56 (5 µg/mouse), or H56 with adjuvants, MOMP (5 µg/mouse) or MOMP with adjuvants. On day 14, mice were boosted with the same treatments. On day 21, mice were sacrificed and spleens and inguinal lymph nodes were collected to allow for the measurement of antigen-specific responses. Cells were restimulated ex vivo with H56 (2 µg/mL and 10 µg/mL) or MOMP (5 µg/mL) for 72 hours and antigen-specific IFN-γ was determined by ELISA.

**2.3 Statistical Analysis**
Statistical analysis was performed using Graphpad Prism 5 software. Specific tests performed are specified in figure legends for individual experiments.
Results

High levels of deacetylation are required for chitosan-induced mitochondrial stress

We previously reported that a commercially available 86 % deacetylated chitosan chloride salt, referred to as protasan CL213, activates the cGAS-STING pathway resulting in IFNAR-dependent DC maturation and Th1-immune responses [8]. While this finding gave great impetus for its translational use as an adjuvant to promote cellular immunity, it was at odds with a phase I/IIa clinical trial containing a novel adjuvant called Viscogel and *Haemophilus influenzae* type b glycoconjugate vaccine (Act-Hib). Viscogel was a chitosan-based formulation containing water and 50 % deacetylated chitosan, and while it was safe and well tolerated, it had a modest effect on cellular or humoral immune responses in vaccinated subjects compared to antigen alone [14], [15]. We speculated that this was a result of the adjuvant’s low DDA and/or its homogenous deacetylation pattern and subsequent inability to activate the cGAS-STING pathway. To prove this, we generated a panel of chitosan polymers 38%, 49%, 72%, 90%, and 100% deacetylation, herein referred to as C38, C49, C72, C90 and C100 (Table 1). We used mitochondrial stress as the initial readout for cGAS-STING activation given its crucial role in the adjuvanticity of protasan. The mitochondrial specific ROS indicator MitoSOX was used to selectively detect superoxide in the mitochondria of live cells after stimulation with C38, C49, C72, C90, C100 and the protasan chloride salt CL213 (herein referred to as protasan) (Fig. 1S a). As a positive control, cells were treated with the complex I mitochondrial inhibitor, Rotenone, which as expected promoted an increase in mitochondrial ROS, visualised as an increase in MitoSOX fluorescence and the percentage of mtROS\(^+\) cells. C38, C49 and C72 failed to enhance MitoSOX fluorescence or percentage of mtROS\(^+\) cells compared to media treatment alone. In contrast, protasan, C90 and C100 drove robust mitochondrial ROS (Fig.1 a & b). C100 was the strongest mitochondrial stress inducer, particularly when cells were treated with low concentrations of polymers (Fig. S1 b & c).

C100 differs from the other chitosan polymers in that chains of positively charged amines are not disrupted by acetyl groups. We speculated that long sequences of amines are responsible for chitosan-induced mitochondrial ROS production. To prove this, two chitosan polymers were generated with identical DDA but alternative distribution of acetyl groups. While both polymers had 83 % of acetyl groups removed, the remaining 17 % of acetyl groups were either clustered together to leave long chains of amines free (referred to as heterogenous distribution) or distributed evenly over the polymer (homogenous distribution) (Fig. S1 d). As expected, homogenous chitosan polymers failed to drive mitochondrial stress, while heterogenous chitosan significantly enhanced MitoSOX fluorescence and the percentage of mtROS\(^+\) cells compared to media alone (Fig.1 c, d & e).
Figure 1 Highly deacetylated chitosan polymers enhance production of mitochondrial reactive oxygen species.

(a-b) Analysis of mtROS production in single, live BMDCs treated with indicated concentrations of chitin-derived polymers (5 µg/mL) for 1.5 hours or rotenone (5 µM) for 6 hours. (a) FSC-W vs MitoSOX (b) Unit area vs MitoSOX. Data are representative of four independent experiments (c-e) Analysis of mtROS production in single, live BMDCs treated with homogenous and heterogenous 83% deactylated chitosan polymers (8 µg/mL) for 1.5 hours or rotenone (5 µM) for 6 hours. (c) FSC-W vs MitoSOX (d)
Unit area vs MitoSOx. (e) Data combined from the two independent experiments were analysed by one-way ANOVA. *\( p<0.05 \) **\( p<0.01 \). MFI= Mean fluorescence intensity.

**High levels of deacetylation are required for cGAS-STING activation and IFNAR-dependent DC maturation**

Protasan-induced mitochondrial stress activates the cGAS-STING pathway, resulting in type I IFN production and IFNAR-dependent DC maturation [8]. We speculated that polymers which failed to drive mitochondrial ROS would not induce production of type I IFNs or cause DC maturation, and that C90 and C100 would be the strongest inducers of type I IFN secretion and DC maturation. The TLR9 agonist, CpG and TLR4 agonist, LPS were used as positive controls for IFN-\( \beta \) and CXCL10 secretion and DC maturation. As expected, both drove significant IFN-\( \beta \) and CXCL10 production (Fig. 2 a & S2 a) and enhanced the expression of CD80 and CD86 compared to media controls (Fig. 2 b & S2 a). C38, C49 and C72 treated cells failed to produce IFN-\( \beta \) or CXCL10 (Fig. 2 a & S2 a) or express more CD80 or CD86 compared to media controls (Fig. 2S f).

Incubation of cells with protasan upregulated \( Ifna \) and \( Ifnb \) mRNA levels (Fig. S2 d) and drove the secretion of IFN-\( \beta \) and CXCL10 across a range of concentrations tested (Fig. 2 a & S2 a) [8]. While C90 drove higher levels of \( Ifna \) and \( Ifnb \) mRNA and IFN-\( \beta \) and CXCL10 secretion than protosan CL213 at all concentrations tested, C100 did not (Fig. 2 a & S2 a,d). Of note, protosan, C90 and C100-induced upregulation of \( Ifnb \) mRNA and IFN-\( \beta \) and CXCL10 secretion were STING-dependent (Fig. S2 b & c). Despite driving lower levels of IFN-\( \beta \) secretion than protasan and C90, C100 promoted the highest expression of CD80 and CD86 and this process was entirely IFNAR-dependent (Fig. 2 b). In contrast, CpG and LPS-induced CD80 and CD86 upregulation was IFNAR-independent (Fig.2 b). Finally, in line with the mitochondrial stress data (Fig. 1 c-e), polymers with a heterogenous distribution of acetyl groups drove high levels of IFN-\( \beta \) secretion while those with a homogenous distribution of acetyl groups did not (Fig 2 c), further supporting the concept that long chains of positively charged amine groups, not interrupted by acetylated sugar moieties, are critical for cGAS-STING activation.
Figure 2 The degree and pattern of polymer deacetylation dictates the extent of type I IFN production and IFNAR-dependent DC maturation. (a) ELISA analysis of IFN-β secretion in the supernatants of BMDCs stimulated for 24 hours with indicated concentrations of chitin-derived polymers, CpG (4 µg/mL) or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by one-way ANOVA. Media vs Treatment. (b) BMDCs from WT and Ifnar1-/- mice were treated with C90,
C100 or protasan (2, 4 or 8 µg/mL), CpG (4 µg/mL) or LPS (10 ng/mL) for 24 hours. BMDCs were collected and stained, gated as single, live, CD11c\(^+\), MHC\(\text{II}^\text{Hi}\) cells and analysed for expression of CD80 and CD86. Figures show representative histograms and Mean fluorescence intensity values for CD80 and CD86; n=3. (c) ELISA analysis of IFN-β secretion in the supernatants of BMDCs stimulated for 24 hours with indicated concentrations of homogenous and heterogenous chitosan polymers or CpG (4 µg/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\), ****\(p<0.0001\).

**High levels of deacetylation are required for NLRP3 inflammasome activation.**
Given that the NLRP3 inflammasome, alongside the cGAS-STING pathway is critical for protasan-induced cellular immunity, we sought to determine the effect of polymer deacetylation on chitosan-induced NLRP3 inflammasome activation. While chitosan alone cannot induce NF-\(\kappa\)B activation and subsequent secretion of IL-6, it can promote activation of the NLRP3 inflammasome in vitro in LPS or CpG primed DCs, as measured by enhanced secretion of IL-1\(\beta\). Similar to protasan, the entire panel of chitin-derived polymers failed to induce IL-6 secretion and so were co-treated with CpG to provide signal 1 for NLRP3 inflammasome activation in vitro (Fig. S3 a). As a positive control, cells were treated with alum and LPS. As expected, alum and LPS co-treatment drove high amounts of IL-1\(\beta\) release. C38, C49, and C72 did not drive IL-1\(\beta\) release. In contrast, C90, C100 and protasan were robust inducers of IL-1\(\beta\) release, with C100 outperforming C90 at all concentrations tested, and C90 similarly outperforming protasan (Fig. 3 a). C90, C100 and protasan-induced IL-1\(\beta\) release was significantly reduced in the absence of NLRP3 (Fig. S3 b). Finally, while heterogenous polymers that drive mitochondrial stress enhanced IL-1\(\beta\) release, homogenous polymers that do not drive mitochondrial stress did not (Fig 3 b).

It seemed plausible that NLRP3 activation, like cGAS, is dependent on mitochondrial stress since the optimal polymers for both pathways are the strongest inducers of mtROS. Indeed, pre-treatment of BMDCs with saturating concentrations of MitoTEMPO abrogated protasan-induced IL-1\(\beta\) secretion at all concentrations tested and C90-induced IL-1\(\beta\) release at low concentrations. However, MitoTEMPO inhibition of IL-1\(\beta\) release was only partial with high C90 concentrations. A similar outline held true for C100-treated DCs, with low concentrations more susceptible to ROS scavenging than higher concentrations (Fig. 3 c). Of note, MitoTEMPO had little effect on alum-induced IL-1\(\beta\) release. It was postulated that residual IL-1\(\beta\) release was due to unscavenged mtROS after MitoTEMPO treatment. To investigate, we directly monitored the ability of MitoTEMPO to scavenge chitosan-induced mtROS. MitoTEMPO reduced protasan-induced MitoSOX fluorescense at all concentrations tested, with the highest concentration displaying a drop in MFI from 2129 to 778. C90-induced mitochondrial stress was less sensitive to MitoTEMPO treatment, particularly at high concentrations and C100-induced MitoSOX fluorescense was unaffected by MitoTEMPO treatment at all concentrations tested (Fig. S3 c).

Chitosan polymers are reported to disrupt and escape lysosomes through a proton-sponge effect [16]. The DDA dictates the extent of lysosomal disruption, as chitosan amine groups have a pKa value of
6.5, and become protonated in acidifying lysosomal lumens, leading to the accumulation of chloride ions and water and subsequent membrane rupture. Recent work suggests that chitosan-induced inflammasome activation and type I IFN responses are mutually exclusive and rely on varying levels of lysosomal rupture [16]. This is not inkeeping with our findings given that highly deacetylated polymers are concurrently activating type I IFN responses and the NLRP3 inflammasome in DCs. To address these discrepancies, we investigated the impact of lysosomal rupture on chitosan-induced mitochondrial stress by blocking lysosomal acidification with the v-ATPase inhibitor bafilomycin A (BafA). Critically, pretreatment of BMDCs with BafA had no effect on C100 or protasan-induced mitochondrial stress (Fig S3 d).
The immunostimulatory effects of C100 apply across a range of molecular weights

The data thus far highlight the importance of polymer deacetylation and acetylation pattern for mitochondrial stress and subsequent activation of the cGAS-STING pathway and the NLRP3 inflammasome. As C100 was the strongest inducer of mitochondrial stress we decided to focus on this polymer. Before translating this adjuvant to an in vivo setting, we investigated the effect of molecular weight on the ability of C100 to activate DC, as increasing the molecular weight would increase the length of deacetylated chitosan chains. BMDCs were treated with C100 polymers of four molecular weights, 115 kDA, 140 kDA, 250 kDA and 350 kDA and analysed for mitochondrial stress, DC maturation and inflammasome activation. All four C100 polymers were stronger than C90 and protasan at driving mitochondrial ROS, evident in the larger increase in percentage of mtROS$^+$ cells and MitoSOX fluorescence. There was no significant difference in mitochondrial stress triggered by the four C100 polymers of different molecular weights (Fig. 4 a). Similarly, all four C100 polymers were stronger drivers of IL-1β release than protasan and C90 and drove equivalent levels of IL-1β release to one another (Fig. 4 d). As shown in Figure 2 b, 115 kDA C100 outperformed protasan and C90-induced upregulation of CD80 and CD86. While all four C100 polymers upregulated CD80 and CD86, the higher molecular weights slightly outperformed 115 kDA C100 (Fig. 4 b & c).
Figure 4 C100 induces mitochondrial ROS, DC maturation and Inflammasome activation across a broad range of molecular weights. (a) BMDCs were treated with chitin-derived polymers (8 µg/mL) for 1.5 hours or Rotenone (5 µM) for 6 hours. Single live cells were analysed for % of mtROS+ cells and MitoSOX fluorescence. Data combined from the two independent experiments was analysed by one-way ANOVA with Tukeys multiple comparison test. MFI= Mean fluorescence intensity. (b) BMDCs were treated with C90, C100 or protasan (2, 4 or 8 µg/mL), CpG (4 µg/mL) or LPS (10 ng/mL) for 24 hours. BMDCs were collected and stained, gated as single, live, CD11c+, MHCIIhi cells and analysed for expression of CD80 and CD86. Figures show representative histograms and MFI values for CD86 and CD80. n=3. (d) BMDCs were stimulated with medium or LPS (10 µg/mL) alone or in combination with chitin-derived polymers (6.25 µg/mL) or alum (10 µg/mL). Levels of IL-1β were measured in supernatants 24 hours later. Results are expressed as the mean ± SD for technical triplicates and are representative of three independent experiments. Statistical analysis was performed by two way ANOVA with Sidak’s multiple comparison test. ****p<0.0001.

C100 is the optimal polymer formulation for driving cellular immunity.

The previous section has demonstrated the necessity of clustered amine groups on chitosan polymers for inducing mtROS and subsequent type I IFN-dependent DC maturation and NLRP3 inflammasome activation in vitro. We sought to translate these findings to an in vivo setting to validate the use of mitochondrial stress as a readout for the adjuvanticity of chitosan polymers and to confirm C100 as the optimal chitosan formulation. The clinically relevant TB antigen, H56 was used to assess the capacity of the adjuvants to promote antigen specific Th1 cell type responses. C57BL/6 mice were immunized subcutaneously (s.c.) on day 0 with PBS, antigen alone, or antigen and C38, C49, C72, C90, C100 or protasan. On day 14, mice were boosted in the same manner and on day 21 mice were sacrificed and spleens and lymph nodes collected to allow for the measurement of antigen-specific IFN-γ. Restimulation with H56 at two different concentrations revealed a higher antigen specific IFN-γ response in splenocytes and lymph nodes from mice vaccinated with H56 and C100 compared to those immunized with antigen alone. Importantly, C100 was the only chitosan polymer to significantly upregulate antigen-specific IFN-γ responses in splenocytes and lymph nodes compared to antigen alone (Fig. 5 a & b).
Figure 5 C100 is the optimal adjuvant for promoting Th1 immune responses in splenocytes and inguinal lymph nodes. C57BL/6 mice were immunised s.c on day 0 with PBS or H56 (0.5 μg), alone or in combination with chitin-derived polymers (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. (a) Splenocytes (2x10^6 cells/mL) and (b) inguinal lymph nodes (1x10^6 cells/mL) were restimulated ex-vivo with either media or H56 (2 μg/ml or 10 μg/ml). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean + SD for 5 mice per experimental group. Statistical analysis was determined by one way ANOVA. Antigen alone Vs Antigen + Chitin-derived polymer. ****p<0.0001
C100 induced cellular immunity requires STING, IFNAR and NLRP3 signalling.

Studies were carried out to address the role of STING, IFNAR and the NLPR3 inflammasome in C100-induced cellular immunity. C57BL/6 WT and Tmem173-/-, Ifnar1-/- and Nlrp3-/- mice were vaccinated s.c. on day 0 and on day 14 as previously outlined with PBS, antigen, or C100 and antigen. A second clinically relevant antigen, chlamydial major outer membrane protein (MOMP) was used to monitor Th1 immune responses. The Th1 enhancing effects of C100 seen with H56 translated to the MOMP antigen where vaccination with C100 and MOMP significantly enhanced antigen-specific IFN-γ responses in splenocytes compared to PBS or antigen alone (Fig 6 a). Vaccination of Tmem173-/- mice with antigen and C100 failed to promote splenic MOMP-specific IFN-γ responses. There was no global defect in IFN-γ responses by cells from these mice as the cells responded similarly to WT when stimulated with anti-CD3 (Fig 6 a & S4 a).

Similarly, vaccination of Ifnar1-/- mice with H56 and C100 failed to enhance antigen-specific IFN-γ responses in splenocytes and lymph nodes compared to WT mice immunised with antigen alone. (Fig 6 b). Secretion of IFN-γ by splenocytes in response to stimulation with anti-CD3 was comparable between WT and Ifnar1-/- mice, whereas in the inguinal lymph nodes, anti-CD3 induced IFN-γ secretion was reduced in Ifnar1-/- compared to WT mice (Fig. S5 a & b). In order to further confirm these results, the same approach was repeated using the antigen, MOMP. As with H56, the enhanced splenic Th1 responses measured following vaccination of C57BL/6 mice with MOMP and C100 was not seen in vaccinated Ifnar1-/- mice. In response to re-stimulation with anti-CD3, there was no difference in IFN-γ secretion between groups (Fig S5 c).

A similar trend was observed for NLRP3, where MOMP-specific Th1 responses induced following vaccination with MOMP and C100 were markedly reduced or completely abolished in the splenocytes and lymph nodes of Nlrp3-/- mice compared to WT controls (Fig 6 c). In contrast, IFN-γ secretion in anti-CD3 stimulated cells did not differ between groups in the spleen or lymph nodes (Fig. S6 a).
Figure 6 C100-induced antigen-specific IFN-γ requires STING, IFNAR and NLRP3 signalling
(a) C57BL/6 WT and Tmem173−/− mice were immunised s.c on day 0 with PBS or MOMP (5 μg), alone or in combination with C100 (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens were collected. (a) Splenocytes (2×10⁶ cells/mL) were restimulated ex-vivo with either media, MOMP (5 μg/mL) or anti-CD3 (0.25 μg/mL). (b & c) C57BL/6 WT and (b) Ifnar1−/− and (c) Nlrp3−/− mice were immunised s.c on day 0 with PBS or H56 (0.5μg), alone or in combination with C100 (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. Splenocytes (2×10⁶ cells/mL) and
inguinal lymph nodes (1x10^6 cells/mL) were restimulated ex-vivo with either media or H56 (10 μg/ml) or anti-CD3 (0.25 μg/mL). (a-c) Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean + SD for 5 mice per experimental group. Statistical analysis was determined by one way ANOVA with Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Intramuscular immunization with antigen and C100 induces antigen-specific IFN-γ production.**

While subcutaneous immunization was chosen as the optimal route for chitosan vaccination in these studies, it was important to address whether the induction of Th1 responses by C100 was unique to s.c. injection alone. Intramuscular (i.m) vaccination is the most widely used route of injection in humans, associated with similar immune outcomes with a lower rate of adverse events [17]. Consequently, mice were immunized via the i.m. route with PBS, antigen alone (H56) or antigen with C100 on day 0. On day 14, mice were boosted in the same manner and 7 days later, were sacrificed. Spleens and inguinal lymph nodes were isolated to assess antigen-specific cellular immune responses. I.M. vaccination with H56 and C100 enhanced antigen-specific Th1 responses in both spleens and draining lymph nodes (Fig. S7 a & b).
Discussion

There is a considerable need for the development of new and improved vaccines that induce strong antigen-specific cellular immunity. As most purified subunit antigens are poorly immunogenic on their own, adjuvants are included in vaccine formulations to potentiate the immune response of the antigen. To date, a limited number of adjuvants have been included in licensed vaccines although this has increased over recent years. These include alum, oil-in-water emulsions, AS04 and AS01. Chitosan, a cationic polysaccharide, is an attractive candidate given its potential as a Th1-skewing adjuvant, its biocompatible and biodegradable nature and its wide use as a biomaterial for drug delivery, as a wound healing material and as a scaffold for living cells [2]. This may appear counterintuitive, given that an adjuvant and a biomaterial are designed for different outcomes, with the former being pro-inflammatory and the latter being anti-inflammatory or inert. The current study has unravelled the physicochemical properties of chitosan polymers that control activation of innate immune responses and validate assessment of a single parameter, mitochondrial ROS to optimise polymers for diverse applications. Consequently, we identified C100 as a highly effective chitosan formulation for use in vaccine settings.

The study utilised oxidative mitochondrial stress as a readout for chitosan adjuvanticity, due to its defined role in chitosan-induced cGAS-STING activation and likely role in NLRP3 inflammasome activation. In doing so, we identified a structure:function relationship between the degree and pattern of deacetylation and chitosan adjuvanticity. While homogenously and highly acetylated chitosan polymers such as C39, C49 and C72 caused no mitochondrial stress or IFNAR-dependent DC maturation, highly deacetylated chitosan polymers with uninterrupted clusters of amines were inflammatory, activating STING signalling and promoting IFNAR-dependent DC maturation. It is now clear that acetylation impinges strongly on the ability of chitosan polymers to trigger mitochondrial stress and explains why C100 is the optimal chitosan formulation in terms of mitochondrial stress and IFNAR-dependent DC maturation. Despite being the strongest inducer of mitochondrial stress and IFNAR-dependent DC maturation, C100 polymers drove less robust IFN-β and no CXCL10 secretion in vitro compared to C90 and protasan but did enhance DC maturation. This highlights a need to monitor the use of mitochondrial stress induction rather than cytokine secretion for the primary in vitro screening of chitosan polymers for different biomedical settings.

In addition to activating the cGAS-STING pathway, highly deacetylated polymers were demonstrated to promote NLRP3 inflammasome activation in a mtROS-dependent manner. It is thus, no surprise that C100 was the strongest inducer of IL-1β release. Intriguingly, oxidative stress induced by C100 was so high, even with low concentrations of C100, that it could not be fully quenched by saturating concentrations of the ROS scavenger, MitoTEMPO. Indeed, pre-treatment of cells with MitoTEMPO could abrogate protasan-induced IL-1β release, but only minimally reduced C100-induced IL-1β release. This demonstrates mitochondrial stress as a key inducer of chitosan-induced cGAS-STING and NLRP3 activation and identifies it as a likely point at which the pathways diverge.
Molecular weight within the range tested was not a crucial parameter for the C100 polymers investigated in this study. All four molecular weights drove similar levels of mitochondrial stress, DC maturation and NLRP3 activation. This is likely due to the fact that the smallest C100 polymer has a sufficient number of positively chained amines to cause disruption.

Finally, linking \textit{in vitro} observations to an \textit{in vivo} vaccination setting, C100 was found to induce the greatest H56-specific Th1 response compared to other chitosan formulations. C100’s efficacy as an adjuvant was STING, IFNAR and NLRP3-dependent and further confirmed using a second clinically relevant administration route (intramuscular) as well as in combination with a second clinically relevant antigen (MOMP). Critically, this work provides an explanation for the failure of phase I/IIa clinical trial investigating 50 % deacetylated chitosan (Viscogel) as an adjuvant in combination Act-Hib. C49, a similarly deacetylated chitosan to viscogel showed no adjuvant activity \textit{in vitro} and \textit{in vivo}, failing to trigger mitochondrial stress, cGAS-STING activation, NLRP3-dependent IL-1β release and H56-specific Th1 responses. This emphasises how future vaccine studies should focus on highly deacetylated chitosan polymers with heterogenous distribution [324], [325]. It must be noted that while the chitosan salt, protasan CL213, was ineffective in promoting Th1 responses when administered by the subcutaneous route, it is an effective adjuvant when administered by other routes (intraperitoneal, intranasal). However, the current study demonstrates the superior efficacy of C100 for the most clinically relevant subcutaneous and intramuscular vaccination routes.

Of interest, STING activators are under evaluation in oncological settings due to the anti-tumour role of the cGAS-STING pathway [18]. Despite pre-clinical evidence of efficacy, no widely applicable, clinically effective and safe agonist has been identified or completed phase III trials. The primary barriers to clinical translation are low cellular uptake and intracellular accessibility, poor pharmacokinetics, and STING variability, necessitating personalised STING agonists [18]. While chitosan polymers can be degraded by a variety of enzymes (chitinases, chitosanases and most commonly lysozymes), studies have shown that lysozymes selectively target chitosan-containing acetyl groups. Consequently, chitosan polymers with a high DD have a much slower degradation rate than polymers with a low DD [19], [20] and given their success as a drug delivery system (DDS) [21], [22], are likely to have good retention profiles in tumours that could allow sustained activation. Furthermore, chitosan polymers are the only reported STING activator that do not elicit NF-κB signalling. C90, C100 and protasan failed to promote IL-6 secretion and required LPS priming for NLRP3-dependent IL-1β secretion in BMDCs. The role of STING-mediated NF-κB activation in anti-tumour immunity is unclear, yet considering its role in tumour initiation, metastasis, cell proliferation and survival [23], protasan, C90 and C100 present as an attractive adjuvant for use in therapeutic cancer vaccines.

To conclude, we have identified mitochondrial oxidative stress as a readout for monitoring the adjuvanticity of chitosan polymers. The ability of acetylation to diminish the adjuvanticity of C100 addresses a longstanding mystery of how chitosan polymers can achieve both inert and inflammatory properties. It is evident that biomaterials for wound healing and scaffold applications should focus on
chitosan polymers with minimal and homogeneous deacetylation while adjuvants should focus on chitosan polymers with extensive if not complete deacetylation. This is the first demonstration of a potential role for C100 as a vaccine adjuvant. Given its well characterised, scalable and cost-effective manufacturing process and its highly defined structure and mode of action, it should be promptly investigated in clinical settings. In addition, the unique properties of C100 as a STING activator support its investigation as an adjuvant therapy in cancer settings.
Supplemental Figures

a

b

C90

C100

Protasan

C

Media  C90  C100  Protasan

Media  Rotenone
Figure S1. Related to Figure 1 (a) Gating strategy for measuring mtROS production in single, live BMDCs. FMO, Fluorescence minus one. (b-c) mtROS production in BMDCs treated with indicated concentrations of chitin-derived polymers (2, 4 or 8 µg/mL) for 1.5 hours or rotenone (5 µM) for 6 hours. (b) FSC-W vs MitoSOX (c) Unit area vs MitoSOX. (c) Values correspond to mean fluorescence intensity. Data is representative of four independent experiments. (d) Deacetylation of chitin can be homogenous or heterogenous. Polymers with a homogenous pattern of deacetylation have uniform distribution of acetyl and amine groups along the polyglucosamine backbone. Heterogenous chitosan polymers have clusters of acetyl groups separated by long chains of positively charged amine groups along the polyglucosamine backbone.
Figure S2. Related to Figure 2 (a) ELISA analysis of CXCL10 secretion in the supernatants of BMDCs stimulated for 24 hours with indicated concentrations of chitin-derived polymers, CpG (4 µg/mL) or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by one-way ANOVA. (b-c) ELISA analysis of (b) IFN-β and (c) CXCL10 secretion in the supernatants of WT and Tmem173−/− BMDCs stimulated for 24 hours with indicated concentrations of chitin-derived polymers, CpG (4 µg/mL) or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical significance was determined by Two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. (d) BMDCs were stimulated with indicated concentrations of chitosan for 24 hours, CpG (4 µg/mL) for 3 hours or LPS (10 ng/mL) for 2 hours. mRNA levels were calculated by qPCR for Ifnb with respect to Actb and Rps18. Data shows technical triplicate mRNA levels with respect to Actb and is representative of three independent experiments. Statistical analysis was performed by one-way ANOVA. (e) BMDCs from WT and Tmem173−/− mice were treated with chitin-derived polymers (8 µg/mL) for 24 hours or LPS (10 ng/mL)
for 2 hours. mRNA levels were calculated by qPCR for Ifna and Ifnb with respect to Actb and Rps18. Data shows technical triplicate mRNA levels with respect to Actb and is representative of three independent experiments. Statistical significance was determined by Two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons. (a-e) *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001. (f) BMDCs from WT mice were treated with C90, C100 or protasan (8 µg/mL), CpG (4 µg/mL) or LPS (10 ng/mL) for 24 hours. Cells were stained for flow cytometry and gated as single, live, CD11c+, MHCIIhi cells and then analysed for expression of CD80 and CD86. Values correspond to Mean fluorescence intensity. n=5.
Figure S3 Related to Figure 3  
(a) ELISA analysis of IL-6 secretion in the supernatants of BMDCs stimulated for 24 hours with indicated concentrations of chitin-derived polymers or LPS (10 ng/mL). Results are expressed as the mean ± SD for technical triplicates and represent data from three independent experiments. Statistical analysis was performed by one-way ANOVA. Media Vs Treatment. ***p<0.001.  
(b) BMDCs from WT and Nlrp3\textsuperscript{-/-} mice were treated with medium, CpG (4 µg/mL) or LPS (10 ng/mL) alone or together with chitin-derived polymers (0.8-6.25 µg/mL) or Alum (10 ng/mL). Levels of IL-1β were measured in supernatants 24 hours later. Results are expressed as the mean ± SD for technical triplicates and are representative of three independent experiments. Statistical analysis was performed by Two-tailed unpaired student’s t tests with the Holm-sidak method for multiple comparisons ***p<0.001.  
(c) BMDCs were pre-treated for 40 minutes with MitoTEMPO (500 µM* saturating concentration) and then stimulated with chitin-derived polymers (2, 4 or 8 µg/mL) for 1.5 hours. Single, live cells were analysed for MitoSOX fluorescence.  
(d) BMDCs were pre-treated for 1 hour minutes with Bafilomycin A (100 µM) and then stimulated with chitin-derived polymers (2.5, 5 or 7.5 µg/mL) for 1.5 hours. Single, live cells were analysed for MitoSOX fluorescence.  
Values on graph correspond to mean fluorescence intensity.  
(c-d) Values on graph correspond to mean fluorescence intensity.  
n=2.
Figure S4. Related to Figure 6 C57BL/6 WT and Tmem173<sup>−/−</sup> mice were immunised s.c on day 0 with PBS or MOMP (5 µg/mouse), alone or in combination with C100 (200 µg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens were collected. (a) Splenocytes (2x10<sup>6</sup> cells/mL) were restimulated <i>ex-vivo</i> with either media, MOMP (5 µg/mL) or anti-CD3 (0.25 µg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one way ANOVA with Tukey’s multiple comparisons test. **p<0.01, ***p<0.001.
**Figure S5. Related to Figure 6**

(a-c) C57BL/6 WT and *Iflar1*−/− mice were immunised s.c on day 0 with PBS, (a) MOMP (5 µg/mouse) or (b-c) H56 (0.5 µg/mouse), alone or in combination with C100 (200 µg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. (a & c) Splenocytes (2x10⁶ cells/mL) and (b) inguinal lymph nodes (1x10⁶ cells/mL) were restimulated ex-vivo with either media, MOMP (5 µg/mL), H56 (10 µg/mL) or anti-CD3 (0.25 µg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one way ANOVA with Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure S6. Related to Figure 6 (a) C57BL/6 WT and Nlrp3<sup>-/-</sup> mice were immunised s.c on day 0 with PBS or H56 (0.5μg/mouse), alone or in combination with C100 (200 μg/mouse). On day 14, mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. Splenocytes (2x10<sup>6</sup> cells/mL) and inguinal lymph nodes (1x10<sup>6</sup> cells/mL) were restimulated ex-vivo with either media or H56 (10 μg/mL) or anti-CD3 (0.25 µg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ±SD for 5 mice per experimental group. Statistical analysis was determined by one-way ANOVA with Tukey’s multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure S7. Intramuscular vaccination with H56 and C100 promotes Th1 responses. C57BL/6 mice were immunised i.m. on day 0 with PBS or H56 (0.5 μg/mouse), alone or in combination with C100 (200 μg/mouse). On day 14 mice were immunised with the same formulations and on day 21, spleens and inguinal lymph nodes were collected. (a) Splenocytes (2x10^6 cells/ml) and (b) lymph node cells (1x10^6 cells/ml) were restimulated ex vivo with either medium, H56 (10 μg/mL), or anti-CD3 (0.25 μg/mL). Levels of IFN-γ were measured in supernatants by ELISA after 72 hours. Data represent mean ± SD for 5 mice per experimental group. Statistical analysis was determined by one way ANOVA with Tukey’s multiple comparisons test. *p<0.05, **p<0.01.
Data availability: The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.


