

Cyclosporine A modulates CD103⁺ dendritic cell responses to fungal PAMPs

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Declaration of authorship

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A handwritten signature in black ink on a light orange background. The signature is written in a cursive style and reads "Alex Liddicoat".

Alex Liddicoat

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Abstract

Cyclosporine A (CsA) is an immunosuppressant that protects against inflammatory diseases and graft rejection. Despite its strong efficacy, one side-effect of CsA is an increased risk of fungal infection. To minimise this, further research is required to determine the mechanisms by which CsA impacts on innate immune cell subsets that mediate antifungal immunity. Using both *in vivo* and *in vitro* models, this work examined the impact of CsA on migratory CD103⁺ DCs and type I IFNs, both emerging mediators of antifungal immunity. During intranasal challenge with fungal Zymosan, a clinically-available oral CsA formulation inhibited CD103⁺ DC trafficking to the lung-draining lymph nodes. In an *in vitro* CD103⁺ DC culture model, CsA inhibited Zymosan-induced migration and reduced the expression of the chemokine receptor CCR7, as well as co-stimulatory molecules. Intriguingly, CsA also inhibited CD103⁺ DC secretion of type I IFNs and IFNAR signalling *in vitro*, which was important for maturation and expression of CCR7, as well as migration of activated CD103⁺ DCs to the lung-draining lymph nodes in the *in vivo* model. These data suggest that CsA inhibits type I IFN-dependent activation and migration of CD103⁺ DCs, providing further insight into how the drug increases susceptibility to fungal infection.

Abbreviations

AIM2, absent in melanoma 2

APCs, antigen-presenting cells

ASC, Apoptosis-associated speck-like protein containing a CARD

BMDC, bone-marrow-derived dendritic cell

BMDM, bone marrow-derived macrophages

CARD, C-terminal caspase-recruitment domain

CCR7, CC-chemokine receptor 7

CD, cluster of differentiation

CD40L, CD40 ligand

CFSE, carboxyfluorescein succinimidyl ester

cGAMP, cyclic guanosine monophosphate-adenosine monophosphate

cGAS, cyclic GMP-AMP synthase

CLRs, c-type lectin receptors

CNS, central nervous system

COX-2, cyclooxygenase-2

CsA, Cyclosporine A

DAG, diacylglycerol

DAMPs, danger-associated molecular patterns

DC, dendritic cell

DT, diphtheria toxin

EAE, experimental autoimmune encephalomyelitis

ERGIC, ER-golgi intermediate compartments

FITC, fluorescein isothiocyanate

Foxp3, forkhead box P3

GM-CSF, granulocyte-macrophage colony-stimulating factor

IDO, indoleamine 2,3-dioxygenase

IFNs, interferons

IFN β , interferon- β

IFN γ , interferon- γ
IKK, inhibitor of NF κ B kinase
iNOS, inducible nitric oxide synthase
IP₃, inositol-1,4,5-triphosphate
IRAK, IL-1R-associated kinase
IRF, interferon regulatory factor
ITAM, immunoreceptor tyrosine-based activation motif
LGP2, laboratory of genetics and physiology 2
Lin, lineage
LPS, lipopolysaccharide
LRRK2, leucine-rich repeat kinase 2
MAVS, mitochondrial antiviral signalling protein
MDA5, melanoma differentiation association gene 5
MHC, major histocompatibility complex
MLNs, mesenteric lymph nodes
MOG, Myelin oligodendrocyte glycoprotein
MPT, mitochondrial permeability transition
NETs, neutrophil extracellular traps
NFAT, nuclear factor of activated T cells
NF κ B, nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell, natural killer cell
NLRs, Nod-like receptors
NRON, non-coding repressor of NFAT
OVA, ovalbumin
PAMP, pathogen-associated molecular pattern
PBMC, peripheral blood mononuclear cell
PGE₂, prostaglandin E₂
PKC- δ , protein kinase C- δ
PLC- γ , phospholipase-c- γ
PLGA, poly(lactide-co-glycolic acid)

PMA, phorbol-12-myristate-13-acetate
Poly (I:C), polyinosinic:polycytidylic acid
PRR, pattern recognition receptor
RA, rheumatoid arthritis
RIG-I, retinoic acid-inducible gene I
RLRs, RIG-I-like receptors
ROS, reactive oxygen species
RNS, reactive nitrogen species
SLE, systemic lupus erythematosus
STAT, signal transducer and activator of transcription
STIM1, stromal interaction molecule 1
STING, stimulator of interferon genes
TGF β , transforming growth factor β
TIR, Toll/IL-1 receptor
TLR, toll-like receptor
TNF α , tumour necrosis factor- α
TRAF, TNF receptor associated factor
Treg, regulatory T cell.
TRIF, TIR domain-containing adaptor protein inducing IFN β

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Chapter 1: Introduction

1.1 Dendritic cells induce T cell proliferation and polarisation

Dendritic cells (DCs) were discovered in the early 1970s by Ralph Steinmann, who named these cells based on their branched morphology [1]. DCs have since been characterised as a key link between innate and adaptive immunity, being essential in priming pro- and anti-inflammatory T cell responses [2, 3]. Following engagement of pattern recognition receptors (PRRs), by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), DCs undergo maturation. This process is characterised by upregulated antigen processing and presentation by MHC molecules, facilitating antigen recognition by cognate T cell receptors. In addition, mature DCs upregulate co-stimulatory molecules, including CD80, CD86 and CD40. T cells express the co-stimulatory receptor CD28, which interacts with CD80 and CD86, as well as CD40L that binds to CD40. Together, these interactions induce T cells to produce IL-2 and proliferate (Fig. 1.1) [4].

In addition to triggering T cell expansion, DCs, along with other innate immune cells such as macrophages, NK cells and $\gamma\delta$ T cells, secrete cytokines that drive T cell polarisation within lymphoid tissues and maintain this differentiation in peripheral tissues [4]. For example, in response to PAMPs such as the Toll-like receptor (TLR) 9 ligand CpG, DCs produce IL-12, leading to the differentiation and priming of TH1 and cytotoxic CD8⁺ T cells. In addition, DCs can secrete IL-6, IL-23 and IL-1 β in response to fungal and extracellular bacterial PAMPs that induce TH17 cell differentiation. This process is also dependent on TGF β and IL-21 [4]. By contrast, DCs exposed to self-antigens in the absence of PAMPs or DAMPs can induce anti-inflammatory Treg cell responses, which facilitates self-tolerance, a process that is enhanced by DC production of IL-10 [4]. During autoimmunity, self-tolerance is disrupted, leading to DC maturation in response to self-antigens and detrimental priming of inflammatory self-

reactive T cells [5]. Overall, modulation of DC maturation or cytokine production can significantly impact both the early and late phases of T cell responses and the degree of inflammation within tissues [6].

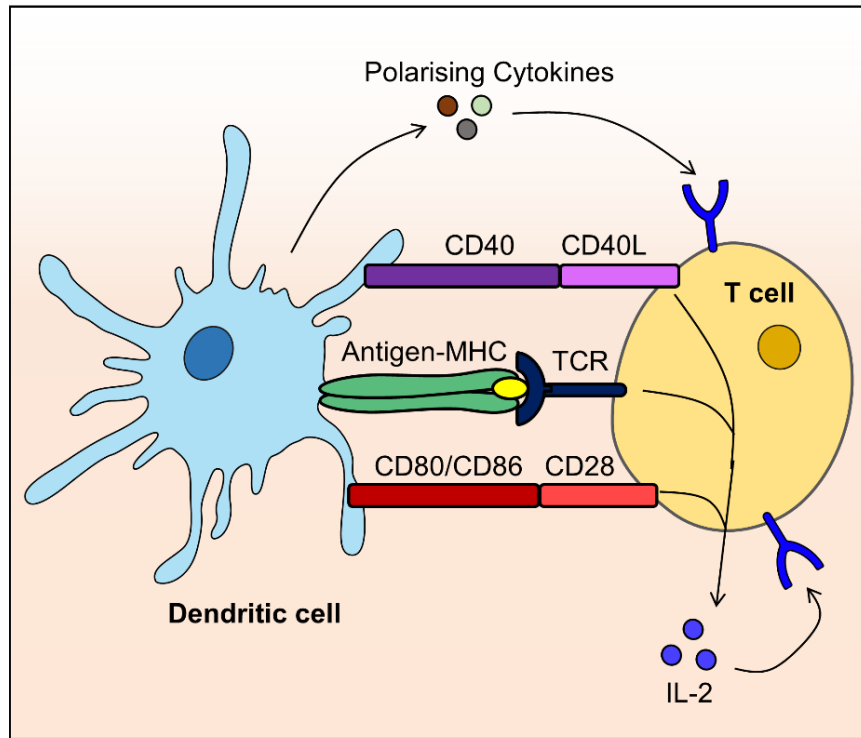


Fig. 1.1: Dendritic cells (DCs) drive naive T cell proliferation and polarisation. Following stimulation, DCs present antigens to the T cell receptor with MHC molecules. In addition, DCs engage T cell co-stimulatory receptors, such as CD40L and CD28, using the co-stimulatory molecules CD40, CD80 and CD86. Collectively, these signals trigger T cells to produce IL-2, driving clonal expansion. DCs also secrete cytokines that control polarisation of T cells into distinct subsets. For example, DC-derived IL-12 induces TH1 cells, while IL-6, IL-1 β and IL-23 drive TH17 cell polarisation. Abbreviation: TCR, T cell receptor. Taken from Liddicoat A.M. and E.C. Lavelle *Biochem Pharmacol*, 2019. 163: p. 472-480 [3].

1.2 The role of DCs during infection and autoimmunity

A number of studies have demonstrated that DCs are critical for the innate and adaptive immune response to pathogens. A useful model for studying DC function uses mice expressing simian diphtheria toxin receptor (DTR) exclusively in cells expressing CD11c (CD11c-DTR mice), a marker highly expressed by all DCs. This enables depletion of DCs upon injection of diphtheria toxin (DT), with no observable effect on cells with lower CD11c expression, such as macrophages [7, 8]. In these mice, DC depletion reduced priming of antigen-specific CD8⁺ T cells after intravenous *Listeria monocytogenes* or *Plasmodium yoelii* infection [8]. In addition, CD11c-DTR mice had increased susceptibility to Herpes simplex virus, due to impaired priming of CD4⁺ and CD8⁺ T cells and NK cells [9]. Moreover, CD11c-DTR mice failed to control *Mycobacterium tuberculosis* replication in the lung and spleen, which correlated with reduced antigen-specific IFN γ production by CD4⁺ T cells [10]. In a more specific model, whereby DT and DTR are used to deplete DCs through their exclusive expression of the transcription factor Zbtb46 (DTR-Zbtb46 mice), infection with *Toxoplasma gondii* resulted in greater susceptibility and impaired IFN γ responses in CD4⁺ T cells [11]. DTR-Zbtb46 mice also failed to prime antigen-specific CD4⁺ T cells in lung-draining lymph nodes during *Mycobacterium tuberculosis* infection [12].

Other studies have investigated the function of specific DC subsets (see section 1.6 for DC subset discussion). Indeed, BATF3-deficient mice, which lack lymphoid-resident CD8 α ⁺ and peripheral CD103⁺ DCs, failed to prime antigen-specific IFN γ production by CD8⁺ T cells in response to influenza or herpes simplex virus infection [13, 14]. BATF3-deficient mice also succumb to *Toxoplasma gondii* infection, due to defective IL-12 production and priming of IFN γ ⁺ T cell responses [15]. Using cre-lox recombination, IRF4 was knocked out exclusively in CD11c⁺ cells, to deplete IRF4-dependent CD11b⁺ DCs in mice and investigate their

function during *Aspergillus fumigatus* infection. These animals lacking CD11b⁺ DCs had increased fungal burden in the lungs, with defective IL-17 expression in CD4⁺ T cells in the lungs and draining lymph nodes [16]. Thus, DCs are crucial for inducing optimal T cell responses and providing host resistance to a range of pathogens.

In addition to their vital role in promoting adaptive immunity against infections, DCs are also involved in the induction and prevention of autoimmunity. For example, DCs accumulated in the inflamed central nervous system (CNS) of mice undergoing relapsing experimental autoimmune encephalomyelitis (EAE), promoting the differentiation of TH17 cells [17]. In a model of EAE induced by adoptive transfer of MOG-reactive T cells, MHCII-deficient mice failed to develop disease, indicating a role for antigen-presenting cells (APCs) [18]. Intriguingly, mice expressing MHCII exclusively in CD11c⁺ DCs were equally susceptible to WT mice, indicating that DCs are a key APC for engaging effector T cell function in this model [18]. In the Imiquimod-induced psoriasis model, CD11c-DTR mice lacking DCs failed to develop disease, as these cells were a key source of IL-23 and IL-36 [19]. IL-23 promotes development of pathological IL-17-producing cells, while IL-36 promotes keratinocyte proliferation and inflammatory chemokine production [19]. In MRL/MpJ-Fas^{lpr}/J mice, which develop systemic lupus erythematosus (SLE), DC deficiency reduced serum concentrations of IgG and IgM specific for nucleosomes, as well as glomerular and interstitial nephritis and proteinuria, all key clinical markers of SLE [20]. In addition, DC depletion during SLE reduced spontaneous priming of IFN γ expression by CD4⁺ and CD8⁺ T cells [20]. Despite contributing to pathology, DCs can also contribute to tolerance. Indeed, DC depletion in Flt3L-deficient mice led to a loss of Treg cells, while Flt3L administration protected against colitis, by expanding tolerogenic DCs and subsequently Treg cells [21]. In active EAE, one study reported increased disease severity in mice lacking DCs due to reduced Treg cell numbers [22]. Thus, DCs can contribute to both pathology and resolution in autoimmune

conditions. As such, future strategies that target DCs in autoimmunity may aim to selectively reduce pro-inflammatory responses, while maintaining self-tolerogenic functions in these cells.

1.3 DC activation by PRR signalling pathways

As mentioned, DC activation is dependent on PRRs that detect PAMPs or DAMPs. The PRR families that induce activation include TLRs, Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), cytosolic DNA sensors and c-type lectin receptors (CLRs). Upon ligation, PRRs trigger intracellular signalling pathways, many of which activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), a crucial transcription factor for DC expression of antigen presentation proteins, co-stimulatory molecules and cytokines. [4, 23].

1.3.1 TLRs

TLRs are expressed on the DC surface (TLR1/2, TLR4-6, human TLR10) or within endosomes (TLR3, TLR7-9, mouse TLR11-13). Structurally, all TLRs are divided into three domains – the ectodomain, which is composed of leucine-rich repeats (LRRs), a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain [24]. TLR ectodomains form homo- or hetero-dimers, adopting a horseshoe-like structure while binding to a corresponding PAMP or DAMP. Cell-surface TLRs bind to PAMPs on the outer membrane of microbes in the extracellular space. For example, in conjunction with the co-receptor MD-2, TLR4 recognises bacterial outer-membrane lipopolysaccharide (LPS) [25, 26]. TLR2 forms a heterodimer with TLR1 or TLR6 and can recognise a number of ligands, including bacterial peptidoglycans, lipoproteins and lipoteichoic acid, as well as fungal zymosan [27]. Furthermore, TLR5 was found to mediate the response to bacterial flagellin [28]. Finally, while human TLR10 and its precise ligands are poorly defined, it was found to

dimerize with TLR2 and aid recognition of *L. monocytogenes* [29]. In contrast to surface TLRs, endosomal TLRs predominantly recognise nucleic acids. Indeed, TLR3 and TLR7 recognise double-stranded and single-stranded RNA respectively, while TLR8 also engages RNA motifs [30-33]. TLR9 was identified to recognise DNA with unmethylated CpG motifs, a common feature of microbial DNA [34]. Independently of nucleic acids, TLR11/12 heterodimers were found to mediate DC responses to profilin from protozoa, while TLR11 also recognised flagellin from *E. coli* [35-37]. Finally, TLR13 was found to mediate pro-inflammatory cytokine responses to bacterial RNA in DCs [38]. Importantly, incubating DCs with TLR ligands leads to activation, with increased cytokine secretion, including TNF α , IL-6, IL-12 and IL-23, as well as enhanced expression of co-stimulatory molecules [4, 39, 40].

DC activation is initiated when the cytoplasmic TIR domain of the TLR binds to the TIR domain of an adaptor protein. This adaptor, MyD88, was initially discovered to bind the IL-1 receptor (IL-1R). Following this, the death domain of MyD88 was found to bind the death domain of IL-1R-associated kinase (IRAK4) in the cytosol, leading to engagement of IRAK2 and IRAK1 [41, 42]. Intriguingly, a number of studies found that this adaptor also signals from TLR2, TLR4, TLR5 and TLR7-9, leading to IRAK engagement, highlighting a common signalling pathway between the IL-1R and TLRs [28, 31, 34, 43-45]. An additional TIR adaptor, named MyD88 adaptor-like (MAL) protein, was later found to bridge MyD88 to TLR4 and TLR2 [46-48]. Downstream, IRAK1 associates with TNF receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase that triggers assembly of a multi-protein complex composed of TAK1 and the regulatory subunits TAB1-3 [27, 49]. In turn, TAK1 activates the inhibitor of NF κ B kinase (IKK) complex, which triggers activation of the transcription factor NF κ B [27]. Of note, NF κ B triggers DC activation by inducing expression of pro-inflammatory cytokines and co-stimulatory molecules. In addition to NF κ B, MyD88 signalling through endosomal TLRs7-9 engages TRAF3 and TRAF6, leading to activation of IKK α [50, 51]. In

turn, this kinase phosphorylates and activates IRF7, a transcription factor for type I IFNs, which are known to enhance DC activation [50, 51].

In contrast to other TLRs, TLR3 signals through a MyD88-independent pathway. Indeed, an alternative adaptor called TIR domain-containing adaptor protein inducing IFN β (TRIF) was found to bind the TIR domain of TLR3 [52]. Following this, a number of studies found that TRIF recruits TRAF3, which activates TBK1 and IKK ϵ . These kinases phosphorylate and activate IRF3, another key transcription factor for the expression of type I IFNs, which, as mentioned, enhance DC activation [50, 53-55]. In addition to MyD88-dependent activation of NF κ B, TLR4 binding to LPS also triggers IRF3 activation and type I IFN secretion [27]. This occurs independently of MyD88, when TLR4 is internalised to an endosome and engages a similar TRIF-dependent pathway to TLR3 [56, 57]. A fourth adaptor known as TRAM aids this process by binding to endosomal TLR4 and subsequently recruiting TRIF [58].

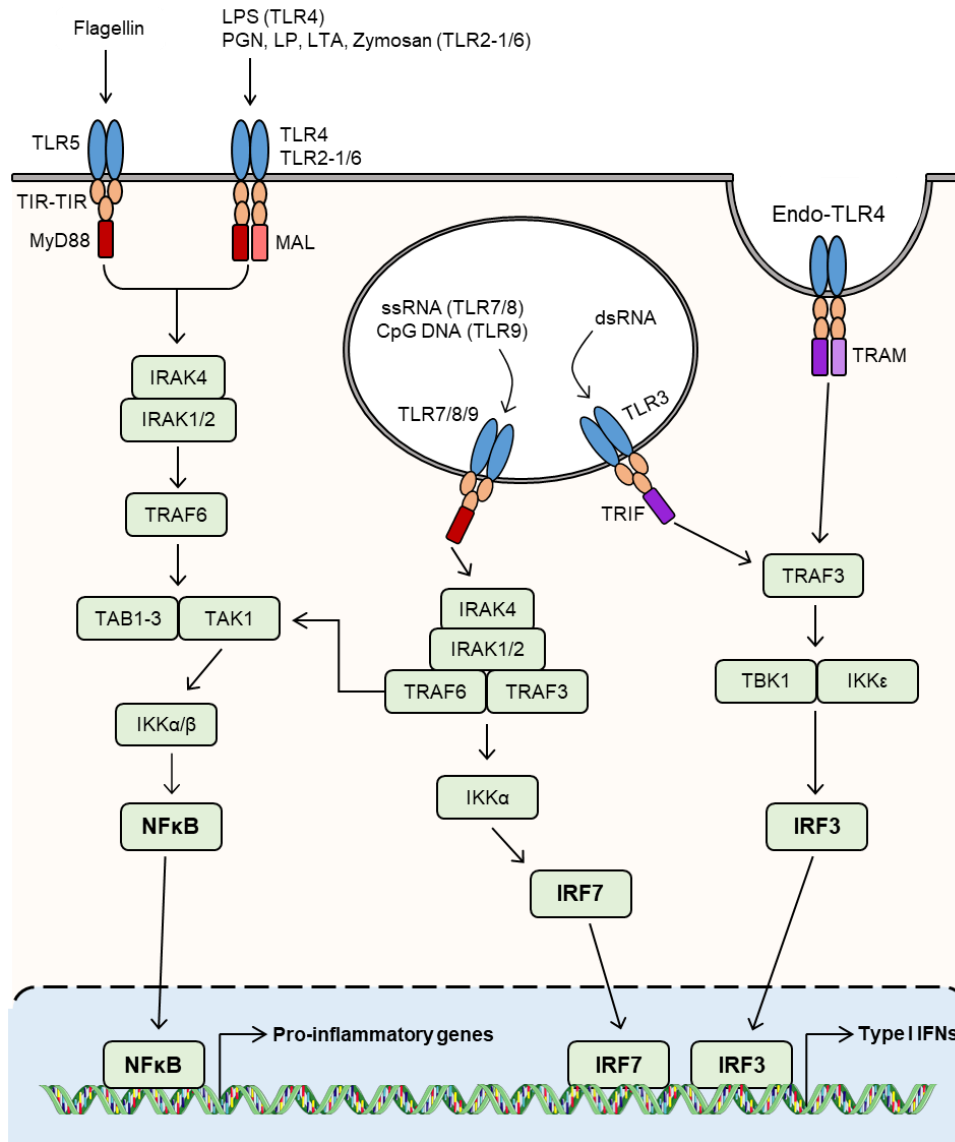


Fig. 1.2: TLR Signalling for upregulation of inflammatory genes. TLR2/1, TLR2/6, TLR4 and TLR5 bind to their ligands from the cell surface, while TLR3 and TLR7-9 recognise nucleic acids within endosomes. TLRs contain TIR domains that bind to the TIRs of adaptor proteins, including MyD88, MAL, TRIF and TRAM. MyD88 signals from all TLRs shown excluding TLR3 and engages both IRAKs and TRAF adaptors. This leads to activation of TAK1, the IKK complex and ultimately the transcription factors NFκB and IRF7. Meanwhile, TLR3 signals through the adaptor TRIF, as does endocytosed TLR4 (endo-TLR4), alongside the fourth adaptor protein TRAM. This leads to activation of TRAF3, which engages TBK1 and IKKε, a complex that activates the transcription factor IRF3. NFκB upregulates pro-inflammatory genes, while IRF3 and IRF7 induce expression of type I IFNs. Abbreviations: IKK, inhibitor of NFκB kinase; IRAKs, IL-1 receptor-associated kinases; IRF, Interferon regulatory factor; LTA, lipoteichoic acid; MAL, MyD88-adaptor like; MyD88, Myeloid differentiation primary response 88; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGN, peptidoglycans; TAB, TAK1-binding protein; TAK1, TGFβ-activated kinase 1; TBK1, TANK-binding kinase 1; TIR, Toll interleukin-1 receptor; TLR, Toll like receptor; TRAF, TNF receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing IFNβ.

1.3.2 NLRs

DCs express NLRs to detect both PAMPs and endogenous molecules in the cytosol. Structurally, NLRs possess an N-terminal protein-binding domain, a nucleotide-binding domain and a C-terminal LRR. The most characterised NLRs are NOD1 and NOD2, which possess a single and double CARD domain in the N-terminus respectively. NOD1 was identified as a sensor for gamma-D-glutamyl-meso-diaminopimelic acid, a gram-negative bacterial peptidoglycan motif [59]. Meanwhile, NOD2 detected a peptidoglycan motif found in all bacteria – muramyl dipeptide [60]. NOD1 or NOD2 stimulation leads to their oligomerization, allowing the N-terminal CARD domain to bind RICK, through its own CARD domain. RICK-deficient mice studies demonstrated that this kinase was essential for inflammatory cytokine production in response to NOD1 and NOD2 agonists [61]. After assembling with NOD1/2, RICK is ubiquitinated, allowing recruitment of TAK1. In a similar manner to TLR signalling, TAK1 activates the IKK complex, ultimately leading to NFκB activation [62]. A number of studies highlight the importance of NOD signalling for DC activation. In human monocyte-derived DCs, LPS-induced IL-12p70 secretion was significantly enhanced by combined stimulation with NOD1 or NOD2 ligands [63]. This led to increased priming of IFNγ⁺ T cells, highlighting synergism between NOD and TLR4 signalling in the induction of TH1 cell responses [63]. In addition, *ex vivo* culture with NOD ligands also enhanced CD40 and CD86 expression and antigen cross-presentation by isolated splenic DCs [64]. *In vivo*, intraperitoneal injection with NOD ligands increased expression of CD40 and CD86 in CD11b⁺CD11c⁺ DCs in the spleen [65]. Finally, in tolerogenic human monocyte-derived DCs, which were generated by culture with IL-10, siRNA-mediated knockdown of NOD1 enhanced the tolerogenic phenotype by decreasing CD86 and IL-12 expression [66].

Another group of NLRs are involved in the formation of inflammasomes, multi-protein complexes that enable processing and secretion of the cytokines IL-1 β and IL-18. The best characterised inflammasome is NLRP3 (see [67] for detailed inflammasome review). An initial priming signal such as TLR signalling induces NF κ B activation, which upregulates expression of pro-IL-1 β and NLRP3 itself. However, the active secretion of IL-1 β requires a second signal in the form of inflammasome activation. Indeed, the NLRP3 inflammasome processes pro-IL-1 β into its mature form [4, 23]. Structurally, NLRP3 contains a C-terminal LRR, central NACHT-binding domain and N-terminal Pyrin domain, which associates with the pyrin domain of the adaptor protein ASC. In turn, ASC links NLRP3 to caspase-1, through a CARD:CARD interaction [23, 68]. Following this, caspase-1 processes pro-IL-1 β [23].

A diverse range of stimuli activate NLRP3. For example, muramyl dipeptide, a known ligand for NOD2, also triggered NLRP3-dependent secretion of IL-1 β , as did transfection of DNA from adenovirus or *E. coli* [69, 70]. In addition to PAMPs, endogenous DAMPs such as uric acid crystals – a key mediator of the inflammatory condition gout – were found to activate the NLRP3 inflammasome [71]. Other key inflammasome-activating DAMPs include ATP, mitochondrial DNA and reactive oxygen species (ROS) [72-74]. Finally, *in vivo* studies found that NLRP3 was important for inflammatory responses to particulate matter, such as asbestos, silica and the vaccine adjuvant alum [75, 76].

Due to its diverse range of stimuli, it has been suggested that NLRP3 does not directly bind to these ligands but responds to a common cellular stress event downstream from each initial stimulus [67]. Proposed events include Ca²⁺ flux, lysosomal disruption, mitochondrial dysfunction, metabolic changes and trans-golgi disassembly [77]. However, a number of studies highlight that K⁺ efflux may constitute the common signal required for NLRP3 activation. Indeed, early studies indicated that ATP engaged NLRP3 by activating the cell-surface cation channel P2X7, which decreases intracellular K⁺ [72, 78]. In addition, the K⁺

ionophore nigericin potently activates NLRP3 by decreasing intracellular K^+ [72]. Consistent with these stimuli, Munoz-Planillo *et al* reported that NLRP3 activation in response to alum and silica particulates also required K^+ efflux [79]. Furthermore, studies using ROS scavengers found that alum-induced NLRP3-mediated IL-1 β secretion was maintained in the absence of ROS, further highlighting the importance of K^+ efflux [79]. Moreover, *in vitro* studies found that K^+ -low media cultures led to spontaneous IL-1 β secretion, which could be inhibited by exogenous addition of KCl [79, 80]. Thus, although a consensus model for NLRP3 activation remains to be elucidated, the importance of K^+ efflux is widely accepted.

Functionally, a number of studies highlight a role for NLRP3 in DC priming of TH17 cells. Indeed, co-culture of OVA-specific CD4 $^+$ T cells with *Nlrp3* $^{-/-}$ splenic DCs led to reduced priming of antigen-specific TH17 cells [81]. Meanwhile, in murine models of multiple sclerosis, a TH17 cell-mediated disease, myelin-specific CD4 $^+$ T cells primed in *Nlrp3* $^{-/-}$ mice failed to trigger disease upon adoptive transfer into T cell-deficient mice, a trend that was observed after initial priming in WT mice [81]. Thus, DC expression of NLRP3, which drives secretion of IL-1 β , is important for their induction of TH17 cell responses.

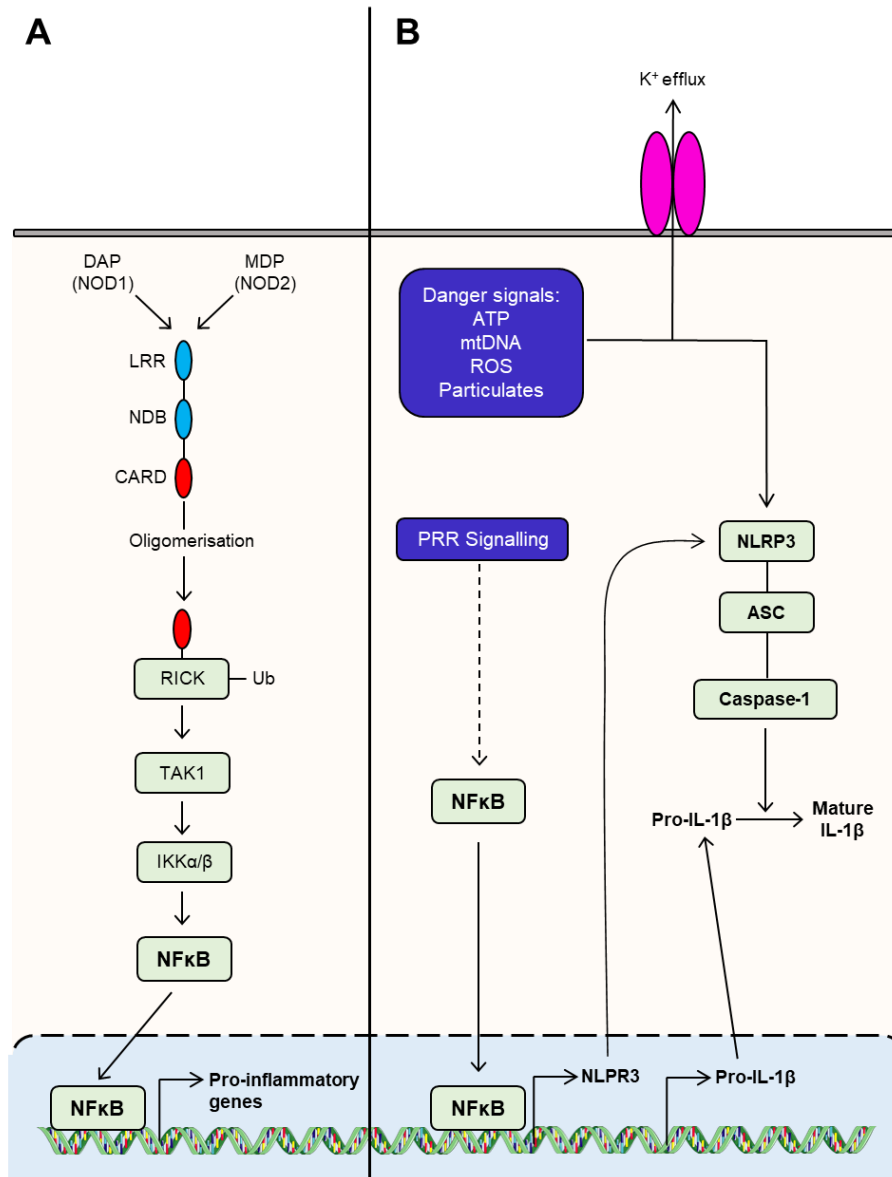


Fig 1.3: NLR Signalling induces antibacterial genes and inflammatory IL-1 β . (A) NOD1 and NOD2 are expressed in the cytosol and bind to DAP and MDP respectively. This triggers NOD oligomerisation and recruitment of RICK through a CARD-CARD interaction. Following this, RICK is ubiquitinated and recruits TAK1, allowing activation of the IKK complex and NF κ B, which upregulates pro-inflammatory genes. (B) NF κ B upregulates NLRP3 and pro-IL-1 β . Meanwhile, danger signals lead to a K⁺ efflux that activates NLRP3, triggering association with the adaptor ASC. This recruits Caspase-1, which processes pro-IL-1 β into its mature secretory form. Abbreviation: ASC, Apoptosis-associated speck-like protein containing a CARD; CARD, caspase activation and recruitment domain; DAP, gamma-D-glutamyl-meso-diaminopimelic acid; LRR, leucine-rich repeat; MDP, muramyl dipeptide; mtDNA, mitochondrial DNA; NBD, Nucleotide-binding domain; NLRP3, NLR Family Pyrin Domain Containing 3; NOD, Nucleotide-binding oligomerization domain-containing protein; Ub, ubiquitination.

1.3.3 RLRs

RLRs detect viral RNA in the cytosol. Members of this family include the prototypical retinoic acid-inducible gene I (RIG-I), melanoma differentiation association gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [82]. Each member consists of a central RNA helicase domain, which enables RNA recognition, as well as C-terminal repressor domain. In addition, RIG-I and MDA5 possess two N-terminal CARD domains that mediate downstream signalling [82]. LGP2 lacks these CARD domains; however, it remains able to bind RNA and has a regulatory role. Indeed, LGP2 was found to either enhance or reduce RIG-I and MDA5 signalling, depending on the type of virus and RNA ligand triggering activation [83, 84].

A number of studies highlight that RIG-I and MDA5 recognise distinct RNA ligands. For example, RIG-I was found to bind ssRNA with 5'-phosphate motifs, which are absent in viruses that trigger MDA5-dependent responses [85, 86]. Moreover, in studies using ssRNA genome fragments from hepatitis C virus, it was found that RIG-I binds homopolyuridine and homopolyriboadenine motifs, highlighting that RIG-I recognises specific nucleic acid sequences [87]. Regarding dsRNA, MDA5 was more important than RIG-I for the induction of type I IFNs in response to 4-8kbp ligands. Intriguingly, shortening of these ligands to 300bp – by nuclease digestion – led to a RIG-I-dependent response, highlighting how the length of dsRNA influences which receptor is engaged [88].

Following ligand recognition, RIG-I and MDA5 are released from their auto-inhibitory conformation. This allows the N-terminal CARD domains to interact with the CARD domains of mitochondrial antiviral signalling (MAVS) adaptor molecule [89, 90]. This triggers MAVS oligomerization on the outer mitochondrial membrane [82]. Here, aggregated MAVS was found to recruit TRAF2, TRAF3, TRAF5 and TRAF6, leading to activation of the IKK complex and TBK1/IKK ϵ , which engage NF κ B and IRF3 respectively [91, 92]. Consistent

with other PRR signalling pathways in DCs, RLR-induced NF κ B upregulates pro-inflammatory genes and IRF3 triggers expression of type I IFNs.

Studies using RIG-I- and MDA5-deficient mice or siRNA knockdown have highlighted the importance of these receptors for DC activation. For example, in GM-CSF-generated BMDCs, RIG-I expression was essential for the type I IFN response to Sendai virus [93]. RIG-I also mediated BMDC production of IL-6 and type I IFNs in response to Newcastle disease virus [94]. In BMDCs infected with influenza virus, RIG-I deficiency significantly reduced CD86 expression and priming of IFN γ ⁺ CD8⁺ T cells isolated from infected mice [95]. MDA5 expression was essential for type I IFN secretion by BMDCs stimulated with encephalomyocarditis virus, which caused significant mortality in MDA5- but not RIG-I-deficient mice, supporting the concept that RIG-I and MDA5 can recognise distinct RNA ligands [93]. BMDCs lacking MAVS exhibited reduced antigen presentation in response to OVA plus 5'-triphosphorylated RNA, as well as decreased secretion of IL-6, IL-12 and expression of CD86 [96]. This led to impaired DC priming of OVA-specific CD8⁺ T cells and their production of IFN γ [96]. Finally, siRNA-mediated knockdown of RIG-I, MDA5 or MAVS in human monocyte-derived DCs significantly reduced expression of IL-1 β , IL-6, TNF α , IL-12p35 and CD86 in response to Dengue virus [97]. These defects were associated with impaired polarisation of IFN γ ⁺ CD4⁺ T cells in *in vitro* co-cultures [97]. Thus, RLR signalling is important for DC activation at the level of cytokine production, co-stimulatory molecule expression and T cell priming.

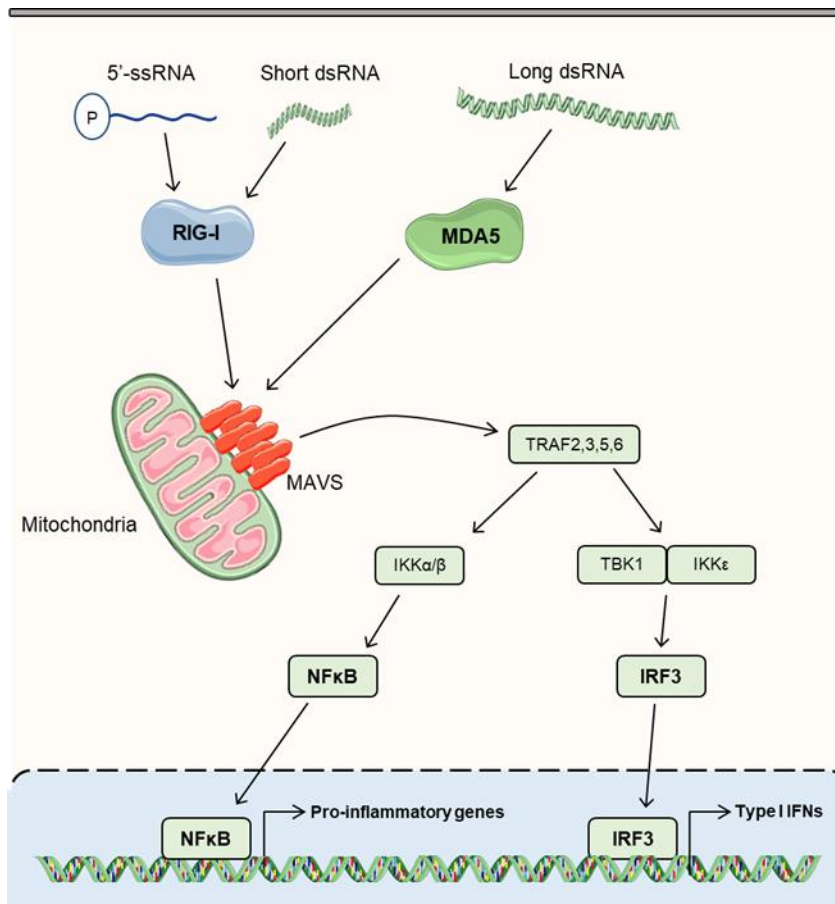


Fig 1.4: RLR Signalling for responses to RNA. In the cytosol, RIG-I binds to 5'-ssRNA or short dsRNA, while MDA5 binds to long dsRNA. After a conformational change, RIG-I and MDA5 interact with the adaptor MAVS, leading to its oligomerisation. MAVS recruits TRAF proteins, triggering activation of the IKK complex and NFκB, as well as a TBK1/IKKε complex that activates IRF3. NFκB upregulates pro-inflammatory genes, while IRF3 upregulates type I IFNs. Abbreviations: MAVS, mitochondrial antiviral signalling; MDA5, melanoma differentiation association gene 5; RIG-I, retinoic acid-inducible gene I.

1.3.4 Cytosolic DNA sensors

A key cytosolic DNA sensor is cyclic GMP-AMP synthase (cGAS). This enzyme was discovered to directly bind DNA in the cytosol [98]. Unlike other PRRs discussed, recognition is independent of the DNA sequence, creating the risk of aberrant inflammation in response to host DNA. However, under steady-state conditions, host DNA is sequestered from cGAS, through compartmentalisation in the nucleus and mitochondria, while host DNA escaping to the cytosol is constitutively digested by nucleases. Therefore, cGAS predominantly binds to microbial DNA that accumulates in the cytosol during infection [99]. cGAS recognition does depend on DNA length, as more than 16 bp are required for activation [100].

Structural studies found that DNA binding activates cGAS through key conformational changes at the catalytic site and subsequent cGAS dimerization [101]. When activated, cGAS synthesises the second messenger cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) from ATP and GTP [102]. In turn, cGAMP forms a complex with stimulator of interferon genes (STING), an adaptor protein that resides on the ER membrane [102, 103]. A dimer under steady-state conditions, cGAMP binding induces a conformational change that triggers formation of a STING tetramer [104]. Following this, STING dissociates from the ER retention protein stromal interaction molecule 1 (STIM1) and confocal microscopy studies found that activated STING migrates from the ER to ER-golgi intermediate compartments (ERGIC) [105, 106]. Here, STING interacts with and is phosphorylated by TBK1 [107]. This enables STING to recruit IRF3, which is also phosphorylated and activated by TBK1 [107]. In turn, IRF3 translocates to the nucleus and upregulates expression of type I IFNs [102, 103, 107, 108]. In addition to activating IRF3, STING-induced TBK1 was found to activate IKK β , leading to activation of NF κ B and induction of pro-inflammatory genes [92].

In addition to the cGAS-STING pathway, cytosolic DNA can be detected by absent in melanoma 2 (AIM2). After directly binding dsDNA, a number of studies have highlighted that AIM2 recruits the inflammasome adaptor protein ASC [109-111]. Similar to the NLRP3 pathway (discussed previously in section 1.3.2), AIM2 recruitment of ASC triggers activation of caspase-1, which cleaves pro-IL-1 β into its mature secretory form.

A number of studies highlight the role of DNA sensors in DC activation. In GM-CSF DCs, vaccinia virus triggered activation of TBK1 and IRF3 and secretion of type I IFNs, a response that was abrogated by cGAS or STING deficiency [112]. In addition, the vaccine adjuvant chitosan induced cGAS- and STING-dependent type I IFN secretion and maturation in GM-CSF DCs [113]. Furthermore, incubating GM-CSF, Flt3L-cultured or splenic DCs with cGAMP, in the absence of PRR stimulation, significantly increased IFN β secretion and expression of CD86 [114]. Moreover, infecting BMDCs with *Mycobacterium bovis* triggered a cGAS-STING-TBK1-IRF3-type I IFN pathway, leading to DC maturation and production of TNF α and IL-6, all of which were decreased by siRNA knockdown of cGAS [115]. The functional relevance of these pathways was established in *in vitro* co-cultures, where cGAMP enhanced BMDC induction of antigen-specific effector CD69⁺CD8⁺ T cells [114]. Intriguingly, in human DCs pre-primed with LPS, cGAMP enhanced IL-1 β secretion, suggesting formation of an inflammasome [116]. Indeed, in the murine JAWSII DC line, confocal microscopy found that fluorescently labelled cGAMP co-localised with AIM2, NLRP3 and ASC. This helped to explain cGAMP-induced IL-1 β secretion in DCs, which was subsequently found to depend on cGAS expression [116]. Overall, cytosolic DNA sensors play an important role in DC activation by inducing cytokine secretion and maturation.

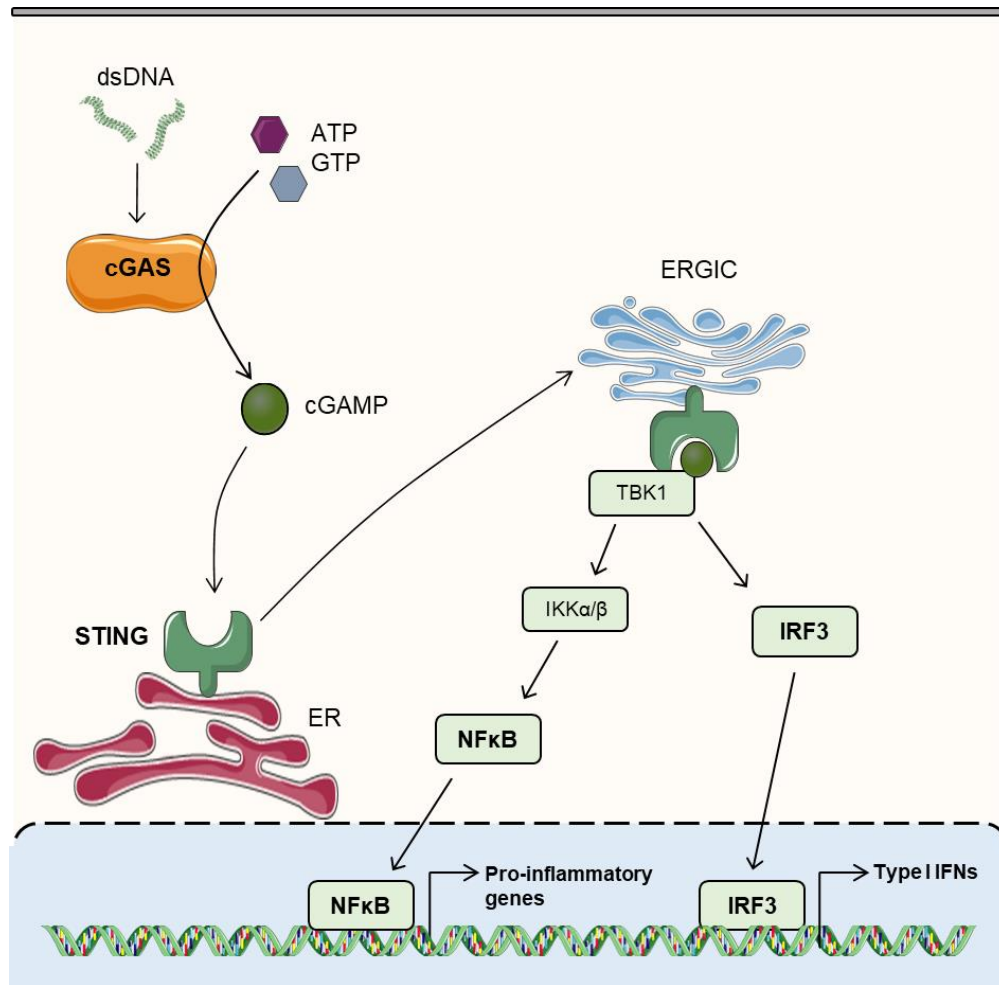


Fig 1.5: Cytosolic DNA sensor signalling. cGAS directly binds to dsDNA in the cytosol. Activated cGAS synthesises the second messenger cGAMP from ATP and GTP. cGAMP binds to STING on the ER membrane, leading to its migration to the ERGIC. Here, STING is phosphorylated by TBK1, allowing activation of IRF3. TBK1 also activates the IKK complex and NFκB. In turn, NFκB upregulates pro-inflammatory genes, while IRF3 induces type I IFNs. Abbreviations: cGAMP, Cyclic guanosine monophosphate–adenosine monophosphate; cGAS, cyclic GMP-AMP synthase; ER, endoplasmic reticulum; ERGIC, ER-golgi intermediate compartment; STING, stimulator of interferon genes.

1.3.4 CLRs

The C-type lectin superfamily includes more than one thousand proteins made up of secretory factors and transmembrane receptors. Soluble C-type lectins function as growth factors, opsonins and antimicrobial proteins, while many transmembrane CLRs trigger intracellular signalling pathways involved in innate immune responses, development and homeostasis (see [117] for detailed C-type lectin review). For the scope of this section, the best-characterised CLR, with PRR features that trigger DC activation is Dectin-1. This receptor contains an extracellular C-type lectin-like domain, which mediates ligand binding, as well as a transmembrane stalk and intracellular cytoplasmic tail that triggers intracellular signalling [118].

Dectin-1 was discovered to bind fungal yeasts, specifically zymosan, a fungal cell wall extract containing proteins and carbohydrates, notably β -1-3-linked glucans (“ β -glucans”) [119, 120]. Importantly, mice lacking Dectin-1 were significantly more susceptible to *Candida albicans* infection, highlighting that recognition of β -glucans is key to antifungal immunity [121]. The cells walls of *Candida albicans* and *Saccharomyces cerevisiae* are ~50% β -glucans by weight [122]. Intriguingly, yeasts readily colonise the mammalian gastrointestinal tract, co-existing with the host as commensals when confined to the gut lumen [123]. However, when the intestinal epithelial barrier breaks down during experimental colitis, dectin-1 was crucial in limiting disease severity, through recognition of β -glucans [124]. Yeasts predominantly express β -glucans beneath a layer of mannoprotein, reducing their exposure, an adaptation that may limit inflammatory antifungal responses by the host [125]. In support of this, active germinating *Aspergillus fumigatus* conidia exposed more surface β -glucans than resting conidia and, after intratracheal injection, induced higher innate immune cell influx to the lung [126]. Thus, dectin-1 enables the host to respond rapidly to yeasts that are potentially pathogenic due to germination or invasion of mucosal tissues. As such, pathogenic yeast

infections associated with systemic dissemination and immunopathology predominantly occur in hosts that are immunocompromised.

Although Dectin-1 can bind both particulate and soluble β -glucan, only the particulate form triggered intracellular signalling [127]. Initially, Dectin-1 binding leads to receptor clustering and recruitment of Src family kinases. These kinases phosphorylate tyrosine residues in the cytoplasmic tail of dectin-1, which contains a hemi-immunoreceptor tyrosine-based activation motif (hemi-ITAM) [127]. In turn, phospho-tyrosine residues in the hemi-ITAM recruit the SH2 domain of Syk family kinases [128]. Following this, Syk engages phospholipase C- γ (PLC- γ), which hydrolyses membrane-bound phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) [129]. IP₃ triggers an increase in intracellular Ca²⁺, which activates calcineurin, a phosphatase that activates the transcription factor NFAT [130, 131]. Meanwhile, DAG activates protein kinase C- δ (PKC- δ) and this was found to directly phosphorylate the adaptor protein Card9 [132]. This allows Card9 to associate with Bcl10 and Malt1 [131-133]. Downstream, the Card9-Bcl10-Malt1 complex recruits a TRAF6 and TAK1 complex, which, consistent with other PRR pathways, engages the IKK complex, leading to NF κ B activation [131, 132, 134].

A number of studies highlight the functional relevance of Dectin-1 signalling for DC activation. For example, Syk-deficient splenic DCs produced lower levels of IL-12 in response to Zymosan [128]. Studies using Zymosan and the alternative Dectin-1 agonist curdlan found that PLC γ -deficient BMDCs exhibited reduced maturation; defective production of IL-6, TNF α , IL-12 and IL-23; as well as impaired priming of OVA-specific TH1 and TH17 cells in *in vitro* co-cultures [129, 131]. Similarly, PKC- δ ^{-/-} BMDCs were defective in TNF α production when stimulated with Zymosan [132]. Furthermore, Card9^{-/-}, Bcl10^{-/-} and Malt1^{-/-} BMDCs infected with *Candida albicans* produced significantly less IL-6 and TNF α [133]. This pathway mediating DC activation is key for protecting against fungi *in vivo*, as mice lacking

Dectin-1, Card9 or PKC δ were more susceptible to *Candida albicans* infection, with greater fungal burden in the kidney, liver, lung, intestine and stomach [121, 132, 133].

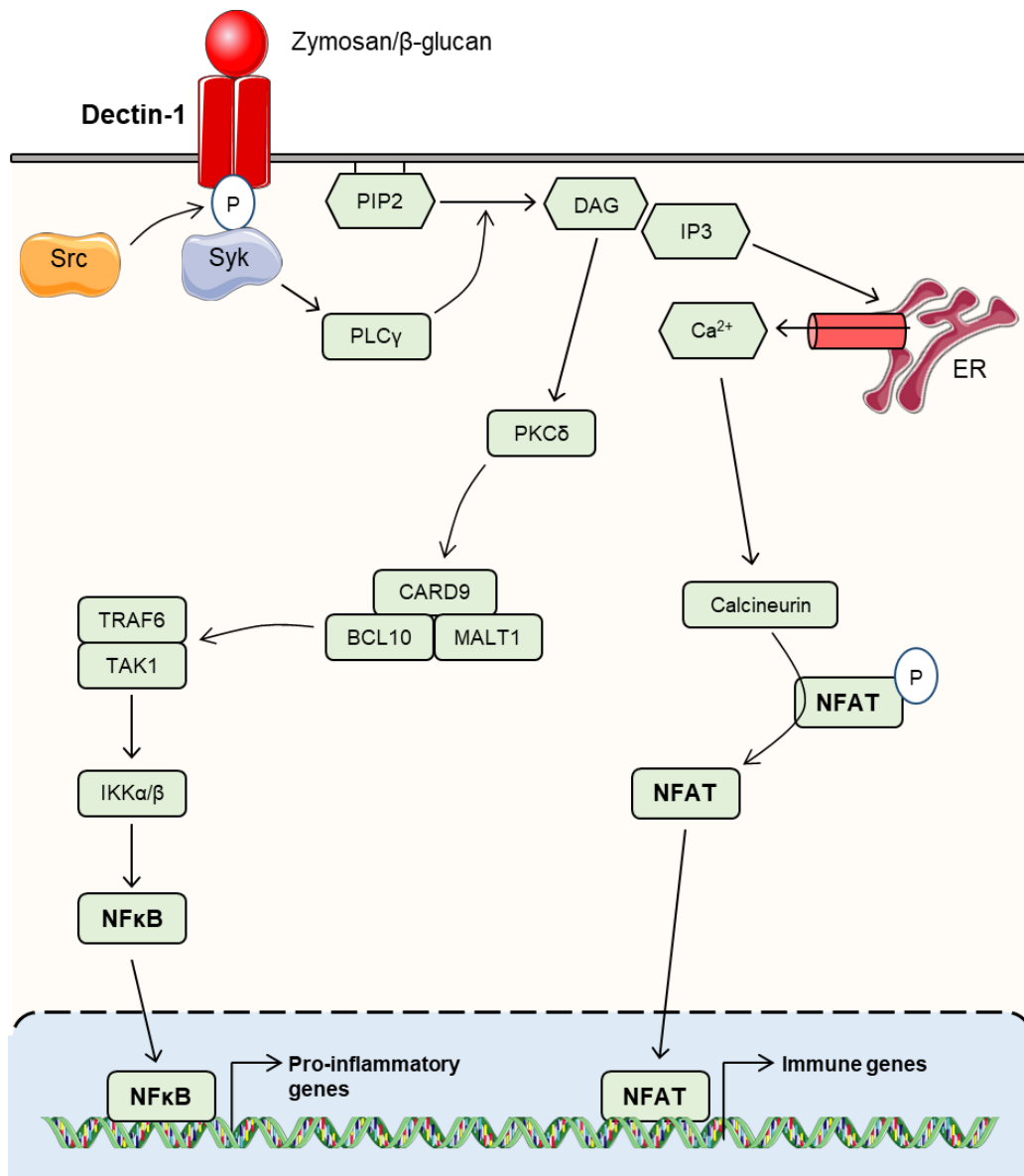


Fig 1.6: Dectin-1 signalling upregulates anti-fungal genes. Zymosan/β-glucans are a prominent fungal PAMP bound by Dectin-1 on the cell surface. Bound Dectin-1 recruits Src and Syk family kinases. Syk activates PLCγ, which converts PIP2 into IP3 and DAG. IP3 induces a Ca²⁺ influx from the ER, which activates calcineurin, a phosphatase that activates the transcription factor NFAT. Meanwhile, DAG activates PKCδ, which triggers assembly of the CARD9-BCL10-MALT1 complex. This engages TRAF6 and TAK1, activating IKK complex and NFκB. Abbreviations: Bcl10, B-cell lymphoma/leukemia 10; DAG, diacylglycerol; IP3, inositol-1,4,5-triphosphate; MALT1, Mucosa-associated lymphoid tissue lymphoma translocation protein 1; NFAT, nuclear factor of activated T cells; PIP2, phosphatidylinositol-4,5-bisphosphate; PKCδ, Protein kinase C-δ PLCγ, phospholipase C-γ.

1.4 The role of Type I IFNs in DC activation

Isaacs and Lindenmann discovered IFNs more than 60 years ago. In their experiments, incubating chick chorioallantoic membranes with inactivated influenza virus, followed by washing and live infection, was found to interfere with viral replication [135]. Since then, three classes (type I-III) of IFNs have been described and for the scope of this section only type I IFNs will be discussed. The type I IFN cytokine family comprises 13 homologous IFN α subtypes (14 in mice), IFN β and the less well-defined members IFN ϵ , IFN τ , IFN κ , IFN ω , IFN δ and IFN ζ [136]. A number of cell types secrete type I IFNs, including DCs, macrophages, fibroblasts and epithelial cells [137]. As discussed, DC production of type I IFNs can be induced by PRR signalling from the cell surface, cytosol or endosomal compartments.

After the initial phase of secretion, type I IFNs bind to their receptor, IFNAR, a heterodimer composed of the subunits IFNAR1 and IFNAR2, ultimately upregulating IFN stimulatory genes (ISGs) [137, 138]. Since IFNAR is expressed on most cell types, the type I IFN response is widespread and propagated through autocrine or paracrine signalling. Regarding how IFNAR induces ISGs, co-immunoprecipitation studies found that this receptor pre-associates with the kinases JAK1 and TYK2 [139, 140]. Ligand binding triggers IFNAR clustering and activation of JAK1 and TYK2, through close proximity and trans-phosphorylation. Following this, the activated kinases phosphorylate the intracellular chain of IFNAR [139-142]. Phosphorylated IFNAR residues provide docking sites for signal transducer and activator of transcription (STAT)1 and STAT2, which bind using their SH2 domains. Upon binding, STAT1 and STAT2 are phosphorylated by JAK1 and TYK2 and subsequently form a heterodimer, through reciprocal phosphotyrosine-SH2 domain interactions [137, 143]. Following this, STAT1/2 dimers dissociate from IFNAR and recruit IRF9, forming the ISGF3 complex. This complex translocates to the nucleus, binds IFN-stimulated response elements and

upregulates ISGs. [137, 143, 144]. Recently, one study suggested that IRF9 recruitment and ISGF3 assembly can occur within the nucleus, as opposed to the cytosol [145]. In addition to ISGF3, IFNAR signalling can generate STAT1 homodimers that preferentially bind gamma-activated sequences and upregulate pro-inflammatory genes [137]. By contrast, IFNAR-induced STAT3 homodimers bind to the co-repressor SIN3 transcription regulator homologue A (SIN3A) and suppress pro-inflammatory gene expression by histone deacetylation [137]. Finally, IFNAR can engage STAT3, STAT4 and STAT5, which are typically associated with other cytokine signalling pathways [136]. This broad activation of STATs may explain how type I IFN signalling impacts a wide range of genes [136]. In summary, type I IFN responses occur in two phases, an initial secretion phase induced by PRRs, followed by auto- or paracrine feedback to IFNAR and upregulation of ISGs, leading to an amplified secondary response.

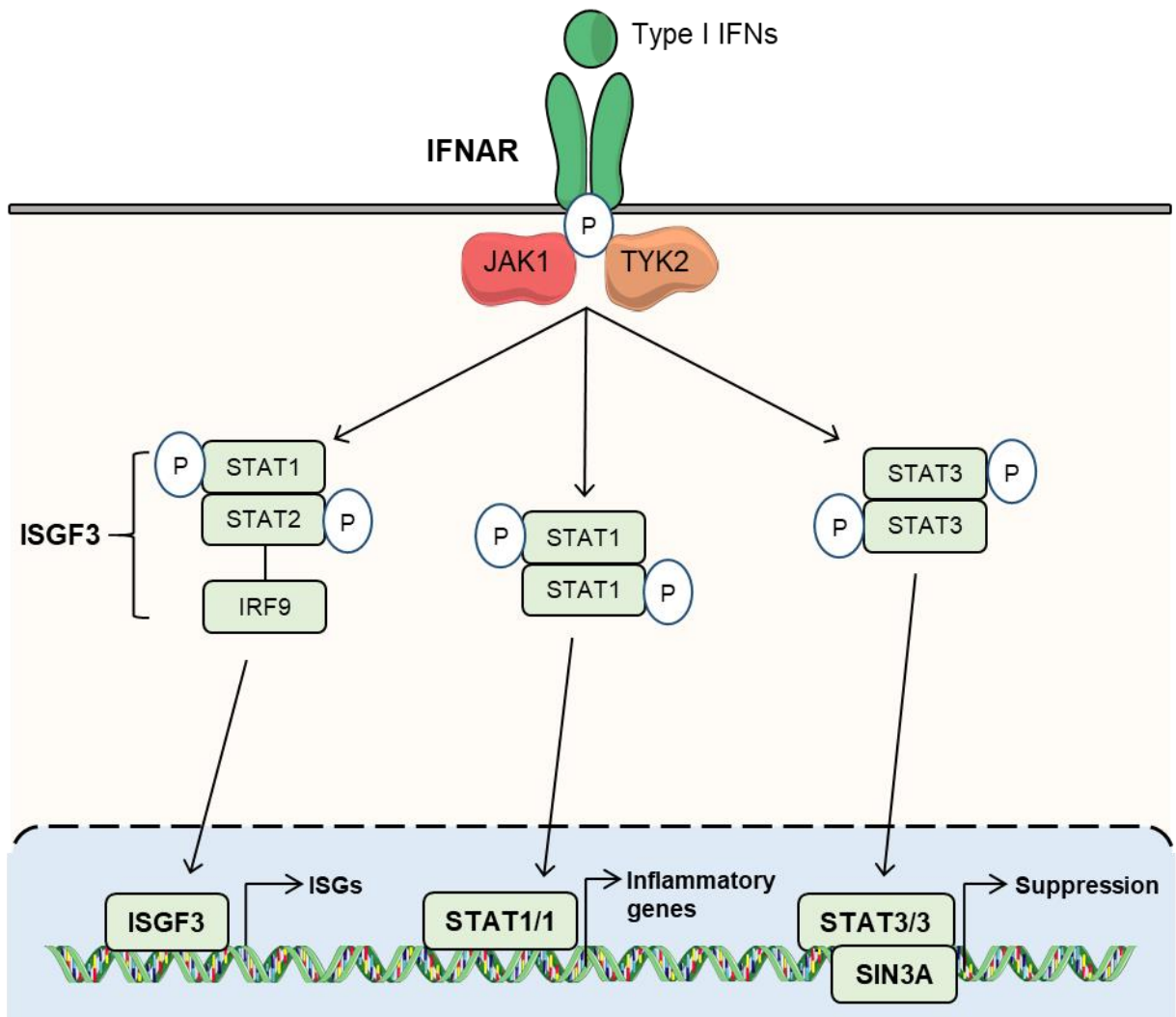


Fig 1.7: IFNAR signalling for enhanced DC activation. IFNAR associates with the kinases JAK1 and TYK2 and becomes phosphorylated upon ligand binding. This provides docking sites for STATs, which are phosphorylated by JAK1 and TYK2, leading to STAT dimerization. STAT1/2 associate with IRF9 to form the complex ISGF3, which upregulates ISGs associated with DC activation. STAT1 homodimers induce additional pro-inflammatory genes, while STAT3 dimers play a regulatory role upon binding to the co-repressor SIN3A. Abbreviations: ISG, interferon-stimulated gene; ISGF3, ISG factor 3; JAK1, Janus kinase 1; SIN3A, SIN3 transcription regulator homologue A; STAT, signal transducer and activator of transcription; TYK2, Tyrosine kinase 2.

Functionally, type I IFNs influence innate and adaptive immune responses during infection and are particularly important for activating DCs. Classical ISGs include Protein kinase R (PKR) and 2'-5' oligoadenylate synthetase-dependent ribonuclease (OAS), which restrict viral replication. Indeed, PKR phosphorylates eIF2a to inhibit host cell protein translation, while OAS generates RNA cleavage products from self-RNA that activate RIG-I and MDA5 and enhance IFN β production [146, 147]. Studies using IFNAR-deficient mice have proved that type I IFNs protect against a wide range of pathogens, including vesicular stomatitis virus, vaccinia virus, Semliki Forest virus, lymphocytic choriomeningitis virus, *Escherichia coli*, *Streptococcus pneumoniae*, *Candida albicans*, *Cryptococcus neoformans* and *Histoplasma capsulatum* [148-152].

While type I IFNs protect against pathogens by affecting a range of immune cells (see [136] for detailed review), a key mechanism is their activating effect on DCs. This has been demonstrated in a number of studies. For example, injection of IFN α/β increased splenic DC expression of CD40 and CD86 [153]. Consistently, IFNAR-deficient BMDCs exhibit reduced co-stimulatory molecule expression in response to Poly I:C, CpG, LPS and live Newcastle disease virus [55]. Highlighting the importance of type I IFN feedback to DCs, IFNAR deficiency or anti-IFN α/β treatment reduced DC capacity to prime T cell proliferation or IFN γ secretion after *in vitro* co-culture [153, 154]. Moreover, incubating isolated DCs with OVA and IFN α , before re-injection, led to heightened antigen-specific IFN γ production in re-stimulated splenocytes, while no response was induced by IFNAR^{-/-} DCs [155]. In DCs derived from human PBMCs, IFN α treatment delayed intracellular degradation of OVA and promoted its loading onto MHC class I molecules, thereby enhancing cross-presentation to CD8⁺ T cells [156]. Further highlighting the role of positive feedback, IFNAR expression was required for sustained BMDC expression of type I IFNs, in response to LPS, R848 or Poly I:C [157]. Regarding other cytokines, STAT1^{-/-} and IFNAR^{-/-} BMDCs secreted less IL-12p70 in response

to LPS, R848 or Poly I:C, while similar reductions are seen in human monocyte-derived DCs treated with anti-IFNAR [157]. Moreover, *IFNAR*^{-/-} BMDCs had defective TNF α , IL-6 and IL-12p70 responses to LPS and β -glucan [154]. In addition to maturation and cytokines, IFN-treated DCs exhibit greater CCR7 expression and migration to lymphoid tissue, as well as enhanced adhesion to and transmigration through endothelial cells [158, 159]. One study suggested a mechanism by which type I IFNs enhance DC activation, by demonstrating that Poly I:C-induced IFNAR signalling drove activation by upregulating HIF1 α [160]. This was necessary to shift DC metabolism from oxidative phosphorylation to glycolysis, thereby meeting the increased energy demands associated with activation [160]. Overall, type I IFNs are a key mediator of DC activation and this helps to explain their protective role against a range of pathogens.

1.5 DC activation leads to migration to draining lymph nodes

The detection of pathogens in peripheral tissues, such as the gut, lungs and skin, activates PRR signalling pathways and ultimately leads to DC maturation. As highlighted, mature DCs exhibit increased antigen presentation and co-stimulatory molecule expression and enhanced capacity to prime naïve T cells within lymphoid tissues. While DCs frequently acquire antigen within peripheral tissues, T cell priming elicited by these cells typically occurs within the draining lymph nodes. Thus, DC migration and entry into lymph nodes is vital for the induction of adaptive immunity, as it enables a physical interaction with naïve T cells. In parallel, lymph node-resident DC populations can acquire antigen that is drained into lymph node conduits [161].

DC migration is regulated by a number of molecules. In particular, a key mediator is CC-chemokine receptor 7 (CCR7), a surface molecule that was found to be upregulated by NF κ B

in response to PRR signalling [162, 163]. CCR7 is expressed by a number of other cell populations, including naïve T cells. Regarding DCs, CCR7 enables their migration along chemokine gradients formed by the soluble ligand CCL19, as well as CCL21, which can be expressed in either a soluble form or immobilised on the surface of interstitial lymphatic endothelial cells [164, 165]. Mechanistically, CCR7 appears to enable DC migration by signalling through PI3K. Indeed, in *in vitro* chemotaxis assays, DCs lacking PI3K demonstrated defective migration in response to CCL19 [166]. Moreover, PI3K-deficient DCs injected into the footpad of WT mice were defective in migrating to draining popliteal lymph nodes [166]. In addition, DC expression of the GPI-anchored protein semaphorin 7A – another marker of maturation – was vital for the formation of actin-based protrusions, reduced adhesion to the extracellular matrix and subsequent migration [167]. Similarly, the signalling adaptor Esp8 was necessary for DC formation of migratory protrusions and trafficking to skin draining lymph nodes in oxazolone-induced contact hypersensitivity assays [168]. There appears to be a functional opposition between DC migration and antigen processing, which is maintained at the DC cytoskeleton level. For example, DC migration and antigen processing appear to be governed by two distinct intracellular actin pools – a RhoA-Mdia1-dependent pool supporting motility and a CDC42-ARP2/3-dependent pool enabling phagocytosis [169]. In addition, the MHC class II-associated invariant chain (CD74) reduces DC migration – and concomitantly supports antigen processing – through its interaction with the actin-based motor protein myosin II [170].

After following chemokine gradients from interstitial tissue towards the lymphatic endothelium, DCs enter lymphatic vessels through pre-existing pores in the basement membrane [171]. While migration through collecting lymphatics is enhanced by the passive flow of lymph, DCs were observed to actively crawl along the initial lymphatic luminal surface, extending filopodia at their leading edges [172]. This was found to be driven by a

CCL21 gradient present in the lumen of lymphatic vessels, inducing CCR7-dependent DC motility [173]. Further insight was provided by oxazolone-induced contact hypersensitivity assays, in which DC migration within skin-draining lymphatic vessels was dependent on the Rho-associated protein kinase (ROCK), a protein that promotes contraction of actomyosin filaments [173].

Upon reaching the lymph node, imaging studies revealed that DCs enter the subcapsular sinus [174]. Following this, DCs migrate to the paracortex, also known as the T cell zone. This is aided by local fibroblastic reticular cells (FRCs), which were a dominant source of CCL19 and CCL21 compared to other stromal cell populations, thereby providing an important contribution to the chemokine gradient [174, 175]. In addition, FRCs express podoplanin, a glycoprotein that binds to CLEC-2 on the surface of mature DCs [176]. This interaction promotes DC actin polymerisation, formation of protrusions and migration along stromal networks present in the paracortex [176]. Overall, DC migration is mediated by a number of cooperating mediators. The CCL21/CCR7 axis is particularly key, as it drives migration towards lymphatic vessels, transmigration through the endothelium, intra-lymphatic crawling and transit towards the T cell zone within lymph nodes.

DC migration to lymph nodes enables these cells to prime adaptive T cell responses and a number of studies highlight the importance of this during infection. For example, a genome-wide association study revealed that pathological *Mycobacterium tuberculosis* infection was associated with reduced expression of *Asap1*, a key mediator of DC podosome formation and migration through the extracellular matrix [177]. Moreover, murine influenza studies demonstrated that antigen cross-presentation and priming of CD8⁺ T cells is dependent on migratory DCs trafficking flu antigen from the lungs to the draining lymph nodes [178-180]. DC migration after influenza infection was found to be rapid but transient, with DC levels in lung-draining lymph nodes increasing after only 6 hours, peaking at 18 hours and steadily

declining after 24 hours [181]. Intriguingly, the same study found that respiratory DCs were refractory to a second migration stimulus delivered 18 hours after the initial stimulus [181]. While this may prevent excessive inflammatory damage by regulating adaptive immune responses, it is thought that impaired DC trafficking may also delay adaptive immunity to secondary bacterial infections, a common complication of severe influenza viral infection [181]. Similarly, during respiratory syncytial virus infection, the priming of CD4⁺ and CD8⁺ T cell responses was dependent on antigen uptake by DCs in the lungs, migration to the draining lymph nodes and subsequent antigen presentation [182].

The importance of DC migration has also been demonstrated by studies using *CCR7*^{-/-} mice, in which migration is defective. Indeed, these mice failed to clear subcutaneous infection with *Leishmania major*, due to suboptimal priming of TH1 cell responses within draining lymph nodes [183]. Similarly, after infection with *Mycobacterium tuberculosis* by the aerosol route, *CCR7*^{-/-} mice had impaired trafficking of DCs from the lungs to mediastinal lymph nodes [184]. This led to delayed priming of antigen-specific CD4⁺ T cells, increased lung bacterial burden and reduced survival [184]. During oral *Salmonella typhimurium* infection, DCs were responsible for CCR7-dependent trafficking of bacteria to mesenteric lymph nodes and this was essential for protection against systemic infection [185]. A number of groups have suggested that CCR7 deficiency is more likely to increase mortality in murine models when a high infectious dose is applied [183, 184]. Regardless, even in studies where CCR7-deficient mice can survive infection, the priming of T cell responses remains significantly impaired. For example, during non-lethal influenza virus infection, CCR7 deficiency significantly reduced DC migration to lung-draining lymph nodes and the induction of IFN γ ⁺ TH1 and CD8⁺ T cell responses [186]. Consistently, although *CCR7*^{-/-} mice ultimately cleared *lymphocytic choriomeningitis virus* and *Listeria monocytogenes* infections, pathogen clearance was delayed and this was attributed to impaired priming of acute antigen-specific CD8⁺ T cell

responses [187, 188]. In summary, the above studies highlight that DC migration to the draining lymph nodes is a key mechanism of protection against infections in peripheral tissues, such as the gut, lungs and skin.

1.6 DC heterogeneity and subsets

Emerging research highlights that DCs are not homogeneous but comprise subsets that differ in localisation and functionality. Current nomenclature divides DCs into three categories – conventional DC1 (cDC1), conventional DC2 (cDC2) and plasmacytoid DC (pDC) [189]. These lineages are differentiated by transcription factors. For example, cDC1s, which include lymphoid-resident CD8 α ⁺ DCs and migratory CD103⁺ DCs, failed to develop in mice lacking the transcription factors IRF8 or BATF3 [190, 191]. Meanwhile, lymphoid- and tissue-resident cDC2s, which lack CD8 α and CD103 but highly express CD11b, were dependent on IRF4 [192]. Finally, pDCs, which are prominent in lymphoid tissue and distinguished by B220 expression, require the transcription factor E2-2 [193].

A number of recent studies compared how different DC subsets present antigen, produce cytokines and prime T cells. For example, splenic cDC1s and cDC2s showed equal proficiency in antigen (OVA) uptake and retention [194]. However, cDC1s and cDC2s had distinct cytokine profiles. Indeed, murine cDC1s isolated from the spleen or lymph nodes produced significantly higher levels of IL-12p70 and type I IFNs than cDC2s in response to CpG. In addition, lung-sorted cDC2s expressed higher IL-6, IL-23 and IL-1 β than cDC1s [16, 195, 196]. These distinct cytokine profiles may be explained in part by differential expression of PRRs. For example, cDC1s expressed higher levels of TLR3 but less TLR2, TLR1, TLR6 and TLR4 than cDC2s [197-199]. In contrast to both cDCs, pDCs are primarily known to produce extremely high levels of type I IFNs in response to viral nucleic acids [200]. In studies comparing induction of T cell responses, splenic cDC1s were superior in antigen cross-

presentation on MHC class I molecules, enabling more proficient priming of CD8⁺ T cells, while splenic cDC2s triggered more potent responses in CD4⁺ T cells [194].

Selective T cell priming by DC subsets is due to differential expression of MHC-associated proteins, as well as distinct mechanisms of antigen uptake and processing. Splenic cDC1s expressed greater levels of Tap1, Tap2, Tapasin, Erp57 and calreticulin – proteins that mediate peptide loading onto MHC class I molecules in the ER [201]. Meanwhile, cDC2s expressed more cathepsins and H2-DM, which degrade antigens and remove the invariant chain from the peptide-binding cleft of MHC class II molecules, ensuring peptide loading within endosomes [201, 202]. One study suggested that the transcription factors for each DC subset determine these differences in protein expression. For example, the cDC2 master regulator IRF4 was found to directly bind MHCII-associated genes encoding Cathepsin S and H2-DM in cDC2s [202].

In addition to MHC proteins, one study suggested that distinct pathways of antigen uptake could play a role. Indeed, uptake through the mannose receptor was found to target antigens to non-degradative endosomes, facilitating antigen escape and subsequent cross-presentation [203]. By contrast, uptake by pinocytosis led to degradation in lysosomes and poor cross-presentation [203]. Consistent with these findings, cDC1s were found to express particularly high levels of the mannose receptor [204]. Although mannose receptor expression was found to regulate cytotoxic CD8⁺ T cell responses in a viral infection model, it can be speculated that DC subsets favour MHC class I or II presentation in part through distinct pathways of antigen uptake [204].

Regardless of how antigens are engulfed, one crucial study found that DC subsets exhibit clear differences in antigen processing. Specifically, cDC1 endosomes are alkalinised, due to Rac2-dependent assembly of the NADPH oxidase NOX2, allowing generation of ROS that capture protons and reduce acidification [205]. This unique feature impairs pH-dependent activation

of lysosomal proteases. This is crucial for cross-presentation, as preserving antigens allows their escape into the cytosol and subsequent processing by the proteasome, before MHC I loading in the ER [205]. By contrast, reduced Rac2 expression in cDC2s leads to more acidic endosomes, favouring peptide degradation and loading onto endosomal MHCII molecules [205]. Collectively, DC subsets differ in T cell priming due to distinct patterns of cytokine production and antigen presentation, a division of labour that offers great potential to target each subset for differential effects on innate and adaptive immune responses.

1.7 CD103⁺ DCs

CD103⁺ DCs continually acquire antigens in peripheral tissues and migrate to the draining lymph nodes for presentation to T cells (Fig. 1.8). In addition, CD103⁺ DCs are crucial for establishing CXCL9, CXCL10 and CXCL11 chemokine gradients in peripheral tissues and priming expression of the cognate receptor CXCR3 on T cells in draining lymph nodes [206]. Crucially, this ensures recirculation of pro-inflammatory T cells but also Treg cells to affected peripheral tissues during infectious and inflammatory diseases [206]. CD103⁺ DCs are classified as cDC1, which is highlighted by their absence in mice lacking the essential cDC1 transcription factor BATF3 [191]. As with other DC subsets, CD103⁺ DC development requires the growth factor Flt3L [207]. CD103 itself is an integrin that forms a heterodimer with the $\beta 7$ chain. This dimer was found to bind E-cadherin, which is expressed on the basolateral membrane of epithelial cells, an interaction that facilitates antigen capture by CD103⁺ DCs in peripheral tissues [208, 209]. CD103 was found to control cellular shape and motility upon encounter with E-cadherin, enhancing close-contact with epithelial cells [210]. CD103⁺ and CD8 α ⁺ DCs are both cDC1 but occupy distinct locations, with CD8 α ⁺ DCs residing in lymphoid tissues – including the spleen and lymph nodes – while CD103⁺ DCs

survey peripheral tissues such as the gut, lungs and skin, before migrating to draining lymph nodes.

1.7.1 Intestinal CD103⁺ DCs

In the gut, CD103⁺ DCs ensure homeostasis by inducing regulatory T cell responses to the microbiota and dietary antigens. One study found that CD103⁺ DCs in the gut lamina propria (LP) may originate from $\text{lin}^{-}\text{c-kit}^{\text{hi}}\text{CD115}^{+}\text{Flt3}^{+}$ macrophage and DC precursors, the downstream $\text{Lin}^{-}\text{c-kit}^{\text{lo}}\text{CD115}^{+}\text{Flt3}^{+}$ DC precursor, as well as the further downstream $\text{CD11c}^{\text{int}}\text{Flt3}^{+}\text{SIRP}\alpha^{\text{int}}$ pre-conventional DCs [211]. Here, differentiation requires Flt3L and GM-CSF, as *Flt3l*^{-/-} and *Csf2r*^{-/-} mice showed depleted levels of CD103⁺ DCs in the LP and Peyer's patches respectively [211, 212]. Upon differentiation, CD103⁺ DCs acquire antigens from the intestinal lumen under steady-state or inflammatory conditions. The primary route of antigen uptake in the gut occurs through microfold (M) cells, which deliver antigen to DCs within Peyer's patches [213]. However, in the LP, CD103⁺ DCs engulf luminal antigens that are delivered through goblet cell passages [214]. In an alternative pathway, CX3CR1⁺ mononuclear phagocytes (MPs), which tightly adhere to the epithelium, directly sample antigens from the lumen using trans-epithelial dendrites. Following this, CX3CR1⁺ MPs transfer antigens to CD103⁺ DCs, which is facilitated by mutual expression of the tight junction protein connexin-43 [215]. Subsequently, antigen-loaded CD103⁺ DCs readily migrate to the mesenteric lymph nodes (MLNs) due to high expression of the chemokine receptor CCR7 [216, 217].

In MLNs, CD103⁺ DCs maintain intestinal homeostasis by inducing gut-homing Treg cell and IgA responses. This is aided by their production of cytokines, particularly TGFβ. For example, LPS stimulation induced significantly less production of TNFα and IL-6 in intestinal CD103⁺ DCs than CD103⁻ DCs [218]. In parallel experiments, these CD103⁺ DCs express significantly higher levels of TGFβ – an essential differentiation factor for Treg and IgA plasma cells – as

well as $\alpha_v\beta_8$, an integrin that cleaves TGF β to ensure its active secretion [218-220]. Furthermore, CD103⁺ DCs exclusively express indoleamine 2,3-dioxygenase (IDO), which enhances tolerance by metabolising tryptophan, as highlighted by its inhibition leading to reduced induction of Foxp3⁺ Treg cells and enhanced IFN γ ⁺ and IL-17⁺ T cell responses [221]. In addition to TGF β and IDO, intestinal CD103⁺ DCs produce high levels of the vitamin A metabolite retinoic acid (RA). This metabolite is formed by retinaldehyde dehydrogenases (RALDHs) that are more highly expressed in CD103⁺ DCs than CD103⁻ DCs [218]. Importantly, RA enhances Treg cell and IgA plasma cell differentiation and drives their expression of the gut-homing receptors CCR9 and $\alpha_4\beta_7$ [222, 223].

Intestinal CD103⁺ DCs are anti-inflammatory due to imprinting by local intestinal epithelial cells. In the steady-state, these cells secrete thymic stromal lymphopoietin (TSLP) and TGF β , which inhibit DC production of IL-12 and the induction of TH1 cells [224, 225]. In addition, epithelial cells produce RA, which conditions DCs to induce IL-10-producing Treg cells [226, 227]. During inflammation, DC conditioning is disrupted, causing these cells to amplify pathological T cell responses. For example, epithelial cells isolated from inflammatory bowel disease (IBD) patients expressed significantly reduced levels of TSLP, leading to increased production of IL-12 in DCs and potent TH1 cell responses [224]. In addition, CD103⁺ DCs isolated from mice with experimental colitis failed to induce Treg cells and favoured the priming of IFN γ ⁺ T cells [228]. Thus, maintaining a tolerogenic phenotype in CD103⁺ DCs is vital for intestinal homeostasis, making these cells a promising target for therapeutics aiming to reduce inflammation in the gut.

1.7.2 Lung CD103⁺ DCs

Lung CD103⁺ DCs acquire inhaled antigens derived from microbes, allergens and particulates. One study suggested that lung CD103⁺ DCs are derived from CD45⁺MHCII⁺CD11c⁻Flt3^{hi}SIRP α ⁻ precursor cells [229]. However, separate studies highlighted that circulating

Ly6C^{hi}CCR2⁺ monocytes also generated these cells [230]. CD103⁺ DCs are absent in the lungs of *Flt3l*^{-/-} and *Flt3*^{-/-} mice, suggesting that the differentiation of these cells is dependent on Flt3L [229].

Lung CD103⁺ DCs maintain homeostasis in the steady-state but are equipped to prime inflammatory responses during allergy or infection. Regarding the maintenance of homeostasis, treating OVA-tolerized wild-type and CD103⁺ DC-deficient *Batf3*^{-/-} mice with cholera toxin-adjuvanted OVA led to heightened inflammatory responses in *Batf3*^{-/-} mice upon re-challenge with OVA, whereas wild-type mice remained tolerant [231]. Mechanistically, lung CD103⁺ DCs induce tolerance through high expression of TGFβ and RALDH2, leading to potent induction of Foxp3⁺ Treg cells [231]. Despite this, lung CD103⁺ DCs readily adapt to environmental signals. For example, during *Aspergillus fumigatus* infection, these DCs induce protective TH17 cell responses through combined secretion of IL-2 and IL-23 [232]. In separate studies, CD103⁺ DCs were found to promote allergic T cell responses in the airway of mice that were pre-sensitized with OVA-LPS and subsequently challenged with OVA [233]. Treating lung CD103⁺ DCs with house dust mite allergens led to potent priming of TH2 cell responses *ex vivo* [233]. In mice infected with influenza virus, CD103⁺ DCs readily uptake viral antigen and migrate to the draining lymph nodes, which is assisted by high CCR7 expression. These cells induce greater CD8⁺ T cell responses than other DC subsets, due to more efficient cross-presentation of viral antigens on MHC class I molecules. This is mediated by higher expression of the peptide transporters TAP1 and TAP2 [178]. In summary, environmental signals in the lung determine whether CD103⁺ DCs prime pro- or anti-inflammatory T cell responses. Therefore, targeting this subset may enhance immune tolerance, while also predisposing to opportunistic infections in the lung.

1.7.3 Skin CD103⁺ DCs

In the skin, CD103⁺ DCs are confined to the dermis, despite their expression of langerin (CD207), an epidermal marker expressed by Langerhans cells [234, 235]. Previous studies suggested that dermal CD103⁺ DCs develop from blood-borne precursors [235, 236]. Differentiation is driven by Flt3L and GM-CSF, as CD103⁺ DCs were depleted in the dermis of *Flt3L*^{-/-}, *Flt3*^{-/-} and *Csf2*^{-/-} mice [229, 237]. In the steady-state, CD207⁺CD103⁺ DCs represent a minor population that corresponds to 3% of MHCII^{high} cells in the dermis [234]. However, this is due to frequent acquisition of antigens breaching the keratinocyte barrier and migration to skin-draining lymph nodes, which occurs in a CCR7-dependent manner [236]. Indeed, dermal CD103⁺ DCs constitute 50% of CD207⁺ DCs in the skin-draining lymph nodes [238].

Dermal CD103⁺ DCs prime potent TH1 and CD8⁺ T cell responses. For example, these cells triggered IL-12-dependent TH1 cell responses that were essential for clearing intradermal *Leishmania major* infection [239]. After subcutaneous injection of B16 or B78chOVA melanoma cells, CD103⁺ DCs were the main subset transporting tumour antigens to draining lymph nodes and inducing anti-tumour CD8⁺ T cell responses [240, 241]. In addition, skin-derived CD103⁺ DCs, which were isolated from mice cutaneously infected with herpes simplex virus-1 (HSV-1), were the most proficient subset in priming HSV-1 glycoprotein-specific CD8⁺ T cells *ex vivo* [242]. This was due to superior cross-presentation of viral antigens. Elegant studies by Henri *et al* sorted skin-derived DCs from the draining lymph nodes of K5.mOVA mice, which express OVA on the keratinocyte surface, before co-culture with OT-I CD8⁺ T cells. Importantly, only CD103⁺ DCs induced T cell proliferation in the absence of exogenous OVA, highlighting their exclusive ability to cross-present endogenous keratinocyte-derived antigens [243]. CD103⁺ DCs can acquire commensal bacterial antigens by reaching through skin appendages, which leads to migration to the draining lymph nodes

and priming of CD8⁺ T cells [244]. Interestingly, the commensal-specific T cells can cross-protect against fungi. Indeed, after migrating back to the skin, these T cells become activated by IL-1 produced by CD11b⁺ DCs and secrete IL-17 [244]. This induces keratinocytes to secrete antimicrobial peptides that protect against *Candida albicans* [244].

Compared to the gut and lung, dermal CD103⁺ DCs induce relatively weak Treg cell responses and may exacerbate inflammatory diseases. Depletion of epidermal Langerhans cells led to an exacerbated psoriasis phenotype, while depleting CD207⁺CD103⁺ dermal DCs had no impact, suggesting that these cells are less important for controlling inflammation in the skin [245]. During psoriasis, increased levels of CD207⁺CD103⁺ DCs were detected in the dermis and dermal DCs are thought to prime pathological T cell responses through production of IL-12 and IL-23 [245, 246]. Furthermore, using ALDEFLUOR assays, it was shown that dermal CD103⁺ DCs exhibit significantly less RALDH activity compared to CD103⁻ DCs, which was associated with less efficient priming of antigen-specific Foxp3⁺ Treg cells *ex vivo* [247]. Overall, suppressing CD103⁺ DCs in the skin may protect against immunopathology, while increasing susceptibility to opportunistic infections and cancers.

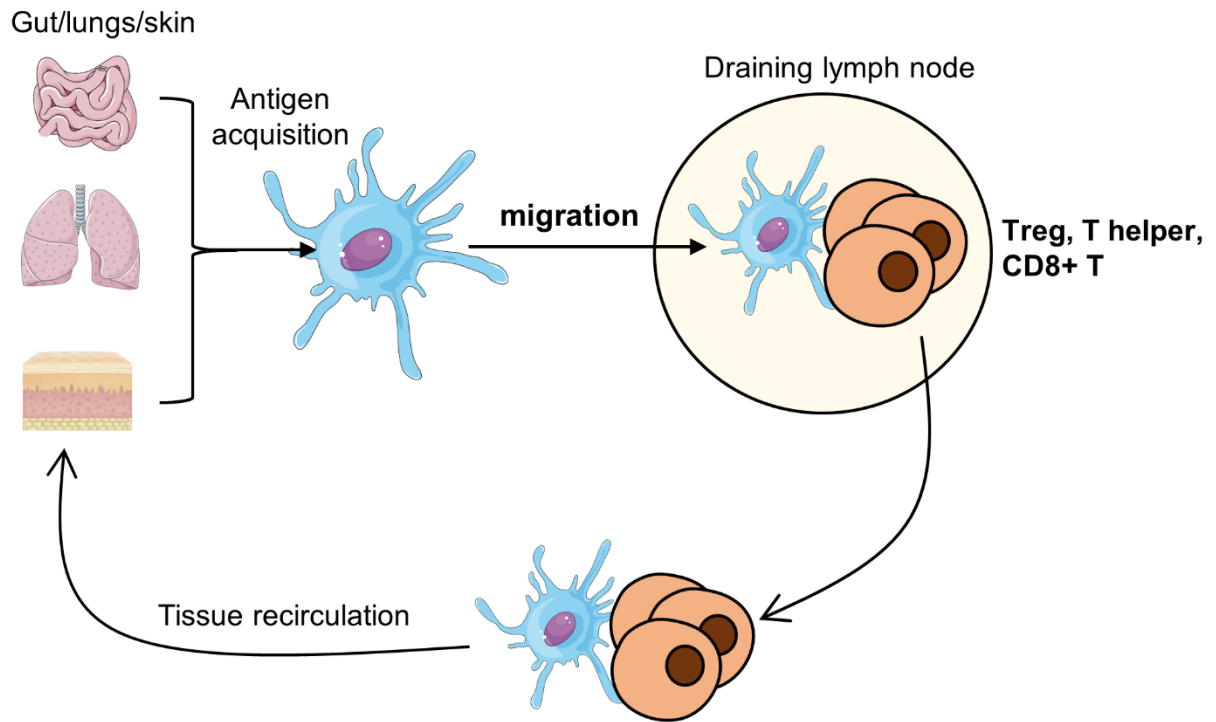


Fig. 1.8: CD103⁺ DCs are vital for homeostasis in peripheral tissues. CD103⁺ DCs acquire antigens from peripheral tissues including the gut, lungs and skin, under steady-state or inflammatory conditions. Following this, these cells readily migrate to the draining lymph nodes. Here, CD103⁺ DCs induce Treg, TH1 and TH17 cells, while priming CD8⁺ T cell responses by cross-presentation. Induced T cells and CD103⁺ DCs recirculate to peripheral tissues and mediate effector responses.

1.7.4 Assessing CD103⁺ DC function

CD103⁺ DCs are an attractive therapeutic target due to their efficiency in collecting antigen from peripheral tissues, migrating to the draining lymph nodes and priming anti-inflammatory or effector T cell responses (Fig. 1.8). During colitis, CD103⁺ DCs are dysfunctional and contribute to pathology [228], while increased numbers of CD103⁺ DCs infiltrate the dermis during psoriasis [245]. On the other hand, CD103⁺ DCs protect against a number of pathogenic infections [152, 178, 232, 248]. Thus, targeting CD103⁺ DCs may enable amelioration of inflammatory diseases but also increase the risk of opportunistic infection. Currently,

investigating CD103⁺ DC function and their potential as a therapeutic target is a challenging area of research, especially as processing murine tissues for isolation and sorting of primary cells leads to low and variable yields. Mayer *et al* addressed this issue, devising a method that efficiently generates CD103⁺ DCs *in vitro* by culturing murine bone marrow cells with GM-CSF and Flt3L [198]. These cells – termed induced-CD103 DCs (iCD103 DCs) – resembled cDC1s, as their CD11c⁺CD103⁺CD11b⁺B220⁻CD8α⁻ phenotype failed to develop from *Batf3*^{-/-} mouse bone marrow cells [198]. Analogous to CD103⁺ DCs *in vivo*, iCD103 DCs were more efficient than GM-CSF-generated BMDCs in antigen cross-presentation to OT-I T cells [198]. In addition, CpG-stimulated iCD103 DCs express significantly higher levels of CCR7 than BMDCs, explaining why subcutaneous injection of these cells led to more efficient migration to skin-draining lymph nodes [198]. In the steady-state, iCD103 DCs are less mature than BMDCs, suggesting that these cells may be more tolerogenic. *In vitro* co-cultures demonstrated that iCD103 DCs only induced marginally greater antigen-specific Treg cell proliferation than BMDCs [198]. However, after supplementation with TGFβ and RA, which are normally provided by local epithelial cells *in vivo*, iCD103 DCs induced markedly stronger Treg cell responses than supplemented BMDCs [198, 225, 227]. Thus, iCD103 DCs exhibit a number of key properties associated with CD103⁺ DCs *in vivo*, aiding investigation into how these cells respond to direct immunomodulation.

1.8 Antifungal innate and adaptive immunity

Fungi inhabit all humans at barrier surfaces, such as the oral cavity, gut, lungs and skin, forming what is known as a “mycobiota”. In addition, humans are constitutively exposed to fungi through inhalation, ingestion or skin trauma [249]. Of the estimated 5 million species of fungi, approximately 600 have been reported to infect humans [250]. Despite this, the majority of fungal infections are asymptomatic. This is largely due to colonisation at barrier surfaces,

which protect the host against dissemination, as well as homeostatic crosstalk with the immune system, a relatively unstudied phenomenon compared to the bacterial microbiota [251]. A significant proportion of symptomatic infections are superficial – affecting the skin, nails and hair – and are readily treated with antifungal drugs [250]. However, millions of people suffer from invasive fungal infections, which, for example, may manifest as severe pathology in the lungs followed by systemic dissemination to other organs such as the kidney, liver and brain [249]. Disease severity is dictated by the immune status of the host, as invasive disease is typically caused by opportunistic infections in patients that are immunocompromised [249]. Globally, invasive fungal infections are estimated to cause 1.5 million deaths per year [250, 252]. The majority of these are caused by *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*, all of which have a high mortality rate [250, 252]. Indeed, a large meta-analysis by Brown et al. estimated that the mortality rates for invasive infections with these fungi are 30-95%, 46-75% and 20-70% respectively [250].

Firstly, *Candida albicans* is a unicellular yeast that adopts a branched hyphal morphology during virulent infection [252]. Transcriptional profiling of *Candida albicans* found that Hyphal cells upregulate a number of genes promoting pathogenic candidiasis [253]. These include hyphal wall protein, which promotes adhesion to the host, as well as the mannosyltransferase PMT2 and secreted protease Sap6, proteins that were found to promote tissue invasion [253]. Secondly, *Aspergillus fumigatus* is a mould, which forms filamentous structures, ending with conidial heads that release spores to enhance airborne transmission [252]. Upon inhalation, this fungus was found to disrupt the integrity of the respiratory epithelium, which aids aspergillosis and systemic dissemination from the lungs [254]. Finally, *Cryptococcus neoformans* is an encapsulated yeast with a spherical cell morphology [252]. An intriguing feature of this fungus is the *in vivo* formation of large “titan” cells with a

diameter of $\sim 100\mu\text{m}$ [255]. This morphological change was found to be important for establishing infection, in particular by evading phagocytosis [255].

Regarding the host immune response, recognition of fungal PAMPs by PRRs is critical. CLRs are an established family of PRRs recognising fungal PAMPs and these include Dectin-1, a receptor for β -glucans that has been discussed in detail in section 1.3.4. Other CLRs include Dectin-3, which was found to form heterodimers with Dectin-2 and recognise α -mannans on the surface of fungi [256]. This recognition activated NF κ B and was necessary for protection against intravenous *Candida albicans* infection [256]. In addition, mass spectrometry and nuclear magnetic resonance studies found that the CLR Mincle recognised fungal glyceroglycolipids and mannosyl fatty acids, leading to secretion of TNF α by BMDCs [257]. Finally, the CLR CD209 (DC-SIGN) was found to bind galactomannans on *Aspergillus fumigatus*, an interaction that was important for monocyte-derived DC maturation [258]. The TLR family, discussed in section 1.3.1, is also known to interact with fungal PAMPs. Indeed, TLR2 can directly bind to Zymosan, a fungal cell wall extract primarily composed of β -glucans [259]. In addition, TLR4 was found to recognise O-linked mannosyl residues in the cell wall of *Candida albicans*, triggering IL-6 and TNF α production by mouse peritoneal macrophages [260]. *Aspergillus fumigatus* contains unmethylated CpG sequences, which can be recognised by TLR9, leading to secretion of TNF α , IL-12 and type I IFNs by DCs [261]. Moreover, in macrophages, phagocytosis of *Aspergillus fumigatus* conidia activated a TLR9-Btk-PLC γ -calcineurin-NFAT pathway, which enhanced TNF α secretion, neutrophil recruitment and fungal killing in the lungs [262]. In addition to the established PRRs discussed above, novel mediators of fungal recognition are emerging. For example, the CLR MelLec, which is expressed on endothelial and myeloid cells, was recently discovered to recognise melanin in *Aspergillus fumigatus* conidial spores, an interaction that protected against disseminated infection in mice [263]. Furthermore, DCs produce Pentraxin 3 (PTX3), a

secretory PRR that binds to *Aspergillus* conidial galactomannan, leading to opsonisation, through enhanced FcγR- and complement-dependent phagocytosis by neutrophils [264]. This recognition is crucial as *Ptx3*^{-/-} mice rapidly succumbed to invasive Aspergillosis [265].

While the aforementioned PAMPs and PRRs are associated with inflammatory responses, homeostatic fungal signals have also been described. Similar to mammalian cells, *Aspergillus fumigatus* express IDO enzymes, which metabolise tryptophan into kynurenine, a molecule that enhances tolerogenic immune responses [266]. In addition to direct signals from fungi, it must be noted that the bacterial microbiota can also contribute to protection. Indeed, *Lactobacilli* metabolise tryptophan into indole-3-aldehyde, an aryl hydrocarbon receptor ligand that promotes expression of IL-22 [267]. This cytokine maintains intestinal epithelial barrier integrity and a diverse microbiota, providing resistance to *Candida albicans*-induced inflammation [267]. In addition, *Bacteroides thetaiotamicron* and *Bacteroides producta* were found to increase host activation of HIF-1α in the gut, enabling expression of antimicrobial peptides that prevent the growth of *Candida albicans* [268]. Conversely, in some settings bacteria can worsen fungal infections. For instance, co-infecting the oral cavity with *Candida albicans* and *Streptococcus oralis* led to exacerbated inflammation in the mouth and oesophagus [269]. This was associated with excessive expression of IL-17, chemokines and neutrophil recruitment, culminating in greater systemic dissemination of the fungus [269].

Regarding the immune response downstream from classical PRRs, macrophages are a key line of defence against fungi. While less proficient than DCs, one role of these cells is antigen presentation. In addition, macrophages were found to contribute to the cytokine milieu, secreting the pro-TH17 cell cytokine IL-1β in response to *Candida albicans* [270]. During acute phases of infection, macrophages adopt a classically activated M1 phenotype and a prominent feature of this is increased production of ROS and RNS [271]. Indeed, infection with *Candida albicans* leads to internalisation into phagosomes by macrophages, followed by

acidification and production of nitric oxide that mediates fungal killing [272, 273]. Similarly, in mice infected with *Aspergillus fumigatus*, alveolar macrophages were found to phagocytose and kill the fungus by producing ROS, which include superoxide, H₂O₂ and HOCl [274, 275]. This rapid production of ROS is termed the respiratory burst and is driven by NADPH oxidase, superoxide dismutase and myeloperoxidase [274, 275]. Human PBMC-derived monocytes, which are blood borne precursors of macrophages, were also able to internalise and kill *Candida albicans ex vivo* [276]. Engulfing long hyphae in particular is a physical challenge for phagocytes. However, live imaging found that macrophages overcome this by forming podosomes, which exert a mechanical force, leading to hyphal folding that damages their structural integrity and facilitates internalisation [277]. Aside from direct fungal killing, colonic macrophages were found to maintain homeostasis in the presence of fungi, by inserting “balloon-like” protrusions into the epithelium to sample absorbed fluid for fungal metabolites. Since these metabolites can damage epithelial integrity, macrophages prevent fluid absorption upon detection [278].

Similar to macrophages, neutrophils are essential for antifungal immunity. Indeed, neutropenia is a significant risk factor for Candidiasis and Aspergillosis [279]. Neutrophils produce similar mediators to macrophages and the mobilisation of these cells during the acute phase of infection is crucial. Indeed, studies in NADPH oxidase- and CXCR2-deficient mice found that early recruitment of neutrophils and their generation of ROS were essential to protect against *Aspergillus fumigatus* infection [280]. In addition, neutrophils were found to produce ROS that were important for killing internalised *Candida albicans* [281]. While ROS may directly disrupt fungal integrity, studies in neutrophils found that ROS are generated to induce a K⁺ influx into the phagosome, increasing the pH [282]. This enables activation of elastase and cathepsin G, granule proteases that directly mediate microbial destruction [282]. The importance of ROS generation is further highlighted by patients with chronic

granulomatous disease, which is caused by genetic defects in NADPH oxidase, leading to increased susceptibility to fungal infection [283]. In addition to these mediators, neutrophils form NETs, which are extracellular chromatin fibres containing antimicrobial granular proteins [284]. Treating PBMC-derived neutrophils with *Candida albicans* led to the formation of NETs, which trapped and killed the fungus, a process that was dependent on granular proteins [285]. Proteomic analysis of NETs identified 24 proteins, including nuclear histones; granular elastase, cathepsin G, myeloperoxidase, lysozyme C and α -defensins; as well as the cytoplasmic protein complex calprotectin [286]. Intriguingly, calprotectin, which chelates essential metal ions including Zn^{2+} and Mn^{2+} , was essential for NET-mediated killing of *Candida albicans in vitro* [286]. In addition, mice deficient for calprotectin fail to control *Candida albicans* growth *in vivo*, leading to significantly reduced survival [286].

DCs are another key mediator of antifungal immunity. While these cells are capable of killing *Candida albicans*, their capacity is lower than macrophages, monocytes and neutrophils [287]. Instead, the primary roles of DCs during fungal infection are detection, internalisation and migration to draining lymphoid organs [288, 289]. This is followed by antigen presentation and priming of adaptive T cell responses, processes that have previously been discussed. In particular, DC responses begin with fungal detection during the early phase of infection, which is enabled by their expression of a number of the discussed antifungal PRRs. As a key example, DCs detect fungal cell wall β -glucans with Dectin-1, triggering activation of Syk and Card9, ultimately leading to upregulation of CD40, CD80 and CD86 and production of the cytokines IL-2, IL-6, TNF, IL-12 and IL-23 [290]. This enables DCs to prime antigen-specific TH1 and TH17 cell responses [290]. A number of studies have specifically demonstrated that DCs are required to clear fungal infections. Indeed, deletion of IRF4 in CD11c⁺ cells – to deplete cDC2s – significantly reduced the induction of TH17 cell responses during *Aspergillus fumigatus* infection, leading to enhanced fungal growth in the lungs [16]. During invasive

Aspergillosis, CD103⁺ DCs were a dominant source of IL-2 and deletion of IL-2 in DCs significantly impacted mouse survival during infection, due to dysregulated TH17 cell responses and excessive neutrophil recruitment to the lungs [232].

Appropriate priming of T cells is essential, as these cells will ultimately coordinate the clearance of fungal infections. TH17 cells, which are a dominant source of IL-17, are crucial for antifungal immunity. Studies using mice deficient for IL-23, IL-17 and the IL-17R, or mice treated with anti-IL-17, reported increased susceptibility to *Candida albicans* and *Pneumocystis carinii* infections [291-293]. In addition, patients with mutations in STAT3, fail to generate effective IL-17⁺ T cell responses and this predisposes to invasive infections with *Candida albicans* and *Aspergillus fumigatus* [294, 295]. TH1 cells, which are another important subset against fungi, have been established as a dominant source of IFN γ and this cytokine is elevated during fungal infection [296]. IFN γ ^{-/-} and IFN γ R^{-/-} mice failed to prevent systemic dissemination of *Candida albicans* and *Cryptococcus neoformans* respectively [297, 298]. Consistently, adoptive transfer of *Aspergillus fumigatus*-specific IFN γ ⁺ CD4⁺ T cells into naïve mice protected against subsequent primary infection [299]. A key role of T cells is to coordinate phagocyte recruitment to the site of infection. Indeed, antibody-mediated depletion of CD4⁺ or CD8⁺ T cells significantly reduced macrophage and neutrophil influx to the lung during respiratory challenge with *Cryptococcus neoformans* [300]. Moreover, IL-17R signalling was required for elevating blood neutrophil counts after intravenous challenge with *Candida albicans*, highlighting a role in granulopoiesis [291]. T cells enhance phagocyte recruitment during fungal infection by increasing the secretion of growth factors and chemokines, including G-CSF, CXCL1, CXCL5, RANTES, MIP1 α , MIP1 β and CXCL10 [292, 293]. In addition to recruitment, T cell cytokine secretion enhances phagocyte activation. For example, IFN γ produced by TH1 cells can enhance macrophage production of ROS and RNS and intracellular killing of fungi [272, 281, 301]. Moreover, IL-17RA deficiency

demonstrated the importance of TH17 cells for neutrophil activation, by causing a decrease in neutrophil myeloperoxidase activity during infection with *Candida albicans* [291].

While less-defined than CD4⁺ T cells, other cell types such as CD8⁺ T cells, NK cells, mast cells and $\gamma\delta$ T cells may also contribute to protection against fungi. Adoptive transfer of CD8⁺ T cells previously activated during *Pneumocystis carinii* infection protected against infection in naïve mice [302]. Moreover, antibody-mediated depletion of CD8⁺ T cells increased susceptibility to *Cryptococcus neoformans* [303]. Regarding the mechanisms of protection, CD8⁺ T cell production of IFN γ was found to limit the growth of *Cryptococcus neoformans* within lung macrophages [304]. CD8⁺ T cells have also demonstrated cytotoxicity to *Candida albicans* and *Cryptococcus neoformans* *in vitro* and this appears to depend on the production of granulysin [305, 306]. Similarly, NK cells were found to kill *Aspergillus fumigatus* and *Candida albicans* and this was associated with upregulation of IFN γ and perforin [307, 308]. In addition, adoptive transfer studies found that NK cells promoted clearance of *Cryptococcus neoformans* infection [309]. Interestingly, mast cells were found to kill *Candida albicans* in the extracellular space, potentially through secretion of antimicrobial mediators and formation of extracellular traps [310]. In the gut and vaginal tract, mast cells increased *Candida albicans* pathogenicity through excessive secretion of IL-9, which reduced epithelial barrier function [311, 312]. However, a healthy gut microbiota can enable gut stromal mast cell secretion of IL-9 at optimal levels, in conjunction with TGF β , leading to production of IDO, which, as discussed, can promote Treg cell-mediated tolerance of *Candida albicans* [311]. These studies highlight how the outcome of a fungal infection is highly context-dependent and influenced by the cross-talk between the host immune system and the microbiota. Regarding $\gamma\delta$ T cells, these cells were found to secrete IL-17, providing protection during oral and systemic candidiasis [313, 314]. By contrast, TCR $\delta^{-/-}$ mice had enhanced IFN γ production in the lungs after intratracheal *Cryptococcus neoformans* infection, suggesting a regulatory role for these

cells [315]. Thus, the role of $\gamma\delta$ T cells during fungal infection requires further study and may vary between fungal species and routes of infection.

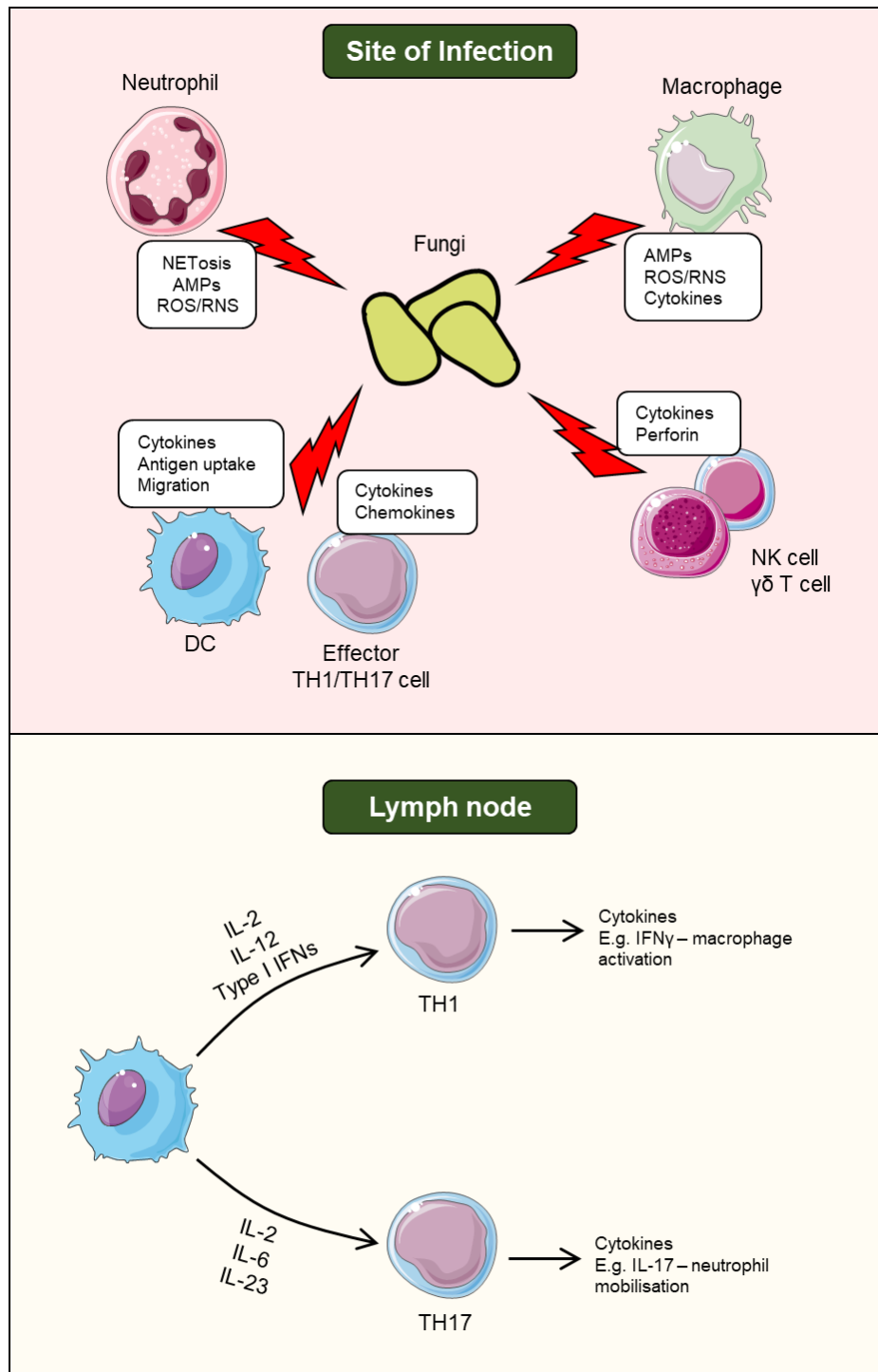


Fig 1.9: Overview of key antifungal immune responses. At the site of infection, DCs detect and uptake fungal antigen and migrate to the draining lymph nodes to prime naïve T cells. Through antigen presentation, co-stimulation and secretion of cytokines, such as IL-2, IL-12, Type I IFNs, IL-6 and IL-23, DCs induce TH1 and TH17 cells. Effector T cells recirculate to the infection site and aid fungal clearance through secretion of IFN γ and IL-17. IFN γ boosts fungicidal responses in macrophages, including AMPs, ROS and RNS. IL-17 ensures neutrophil recruitment and activation, which involves NETosis and production of AMPs and ROS. While less-defined, other cell types such as NK cells (production of IFN γ and perforin) and $\gamma\delta$ T cells (secretion of IL-17) can also play a role in antifungal immunity. AMP, antimicrobial protein.

1.9 Cyclosporine A and its impact on innate immunity

The discovery of Cyclosporine A (CsA), by researchers of Sandoz Pharmaceuticals, was a pivotal landmark in the field of immunosuppression [3]. Initially, CsA was purified from *Tolypocladium inflatum* Gaams, a fungus identified during a novel antibiotic screening programme. CsA performed poorly in animal trials for its intended use as an antibiotic, however, the drug exhibited low toxicity and weak non-specific cytostatic activity. This prompted testing as an immunosuppressive agent, where CsA was found to ameliorate inflammatory responses in Freund's adjuvant-induced arthritis, skin graft rejection and graft-versus-host disease [316]. Follow up studies showed that CsA inhibited Concanavalin A-induced lymphocyte proliferation [317, 318]. Moreover, in "nude" mice, which lack T cells due to a defective thymus, CsA failed to inhibit such proliferation, suggesting that T cells were the sole target of inhibition [317, 318]. This was supported by a lack of toxicity in myeloid cells [317]. Collectively, early CsA studies demonstrated T cell inhibition without widespread cytostatic activity, potentially indicating enhanced efficacy and an improved safety profile compared to pre-existing immunosuppressive drugs.

As initial findings were promising, attention turned to whether CsA could prevent T cell-mediated transplant rejection. Administration of CsA improved survival of heart grafts in pigs and kidney grafts in rabbits [319, 320]. Subsequently, in a multicentre cadaveric renal transplantation trial, CsA treatment led to a significantly greater one-year graft survival rate (72%) compared to a combination of azathioprine and steroids (52%) [321]. CsA also reduced cutaneous manifestations in patients with graft-versus-host disease caused by bone marrow transplantations [322]. The efficacy shown in human trials proved decisive in CsA receiving clinical approval in the early 1980s and becoming the primary therapeutic for transplant

patients. Soon after, mechanistic studies revealed that CsA dampens T cell proliferation by inhibiting the phosphatase calcineurin, preventing nuclear factor of activated T cell (NFAT) activation and expression of genes required for proliferation, including IL-2 and cluster of differentiation (CD)25 [323]. CsA has since been applied clinically to treat T cell-associated autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [324, 325].

Despite protecting against inflammation, CsA was found early on to cause nephrotoxicity and increased susceptibility to opportunistic infections [326]. While these issues can be controlled by applying tailored doses for each patient, understanding how CsA affects immune cell populations remains a priority. Although the capacity of CsA to suppress T cell activation has been known for decades, emerging evidence highlights that CsA also impacts innate immune cells, including dendritic cells (DCs), macrophages and neutrophils [3]. *In vivo*, these innate immune cells mediate direct protective roles against infection but also work co-operatively with the adaptive immune system, to mediate effector functions and adaptive memory responses against pathogens. This is particularly highlighted by the unique role of DCs in bridging innate and adaptive immunity. Likewise, in the context of inflammatory diseases, cells of both the innate and adaptive immune system can contribute to pathology and resolution. Thus, the outcome of therapy with CsA may reflect simultaneous targeting of DCs, macrophages and neutrophils, in addition to T cells [3]. Indeed, the calcineurin-NFAT pathway is active in innate immune cells and induces expression of both inflammatory and homeostatic genes, significantly affecting the outcome of immune responses [327]. In addition, CsA can prevent the release of danger-associated molecular patterns (DAMPs) from the mitochondria that trigger multiple innate immune signalling pathways. Thus, innate immune cells are now seen as potentially important targets of CsA [3]. This section addresses

the effects of CsA on innate immune cells and the potential impact of this on the induction of adaptive immune responses.

1.9.1 CsA Inhibits IL-2 Production in DCs

Expression of IL-2, which is critical for T cell proliferation, depends on the transcription factor NFAT. The T cell calcineurin-NFAT-IL-2 pathway and its inhibition by CsA has been reviewed in detail elsewhere [323, 328]. Briefly, engagement of the T cell receptor leads to activation of phospholipase C- γ (PLC- γ), which hydrolyses phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-triphosphate (IP₃) and diacylglycerol. These second messengers trigger an increase in intracellular calcium, leading to activation of calcineurin. Following this, calcineurin dephosphorylates NFAT, allowing NFAT to translocate into the nucleus and increase IL-2 gene expression. This process is inhibited by CsA, which binds to cyclophilin A in the cytosol. Following this, the CsA-cyclophilin A complex directly binds to calcineurin and inhibits its phosphatase activity, forcing NFAT to be retained in the cytosol and reducing T cell proliferation.

DCs are now regarded as a possible target for CsA, due to their ability to secrete IL-2 in a calcineurin-dependent manner [3]. Initially, microarrays showed that DCs upregulate IL-2 mRNA in response to *Escherichia coli* stimulation [329]. These findings are now supported by multiple *in vitro* and *in vivo* studies showing that DCs rapidly secrete IL-2 protein when stimulated with whole bacteria, toll-like receptor (TLR) ligands or, with particular interest to this research, dectin-1 agonists [128, 329, 330]. In parallel studies, bone marrow-derived macrophages (BMDMs) failed to produce IL-2 in response to these stimuli. This may be due to negative regulation of NFAT by leucine-rich repeat kinase 2 (LRRK2) and the non-coding RNA repressor of NFAT (NRON) complex [130, 329, 331, 332]. Thus, innate immune cell production of IL-2 appears to be most prominent in DCs. Treating Langerhans cells or GM-CSF-generated BMDCs with CsA before stimulation with lipopolysaccharide (LPS) was

found to inhibit IL-2 production, confirming that CsA can modulate cytokine production by DCs [330, 333] (Fig 1.10A).

The mechanisms underlying the induction and inhibition of IL-2 in DCs have parallels with those in T cells (Fig. 1.11A) [3]. Activated bone marrow-derived DCs (BMDCs) upregulate calcineurin and NFAT activity [130, 334]. Specifically, the stimulation of DCs with LPS or zymosan activated Src- and Syk- family kinases respectively, which engage PLC- γ [128, 131, 334, 335]. In conjunction with inositol-Trisphosphate 3-Kinase B (ITPKB), PLC- γ triggers an influx of extracellular calcium and calcineurin-mediated dephosphorylation of NFAT [128, 131, 334, 335]. Following this, NFAT translocates to the nucleus and binds the *Il2* promoter, boosting IL-2 expression [232, 336]. Accordingly, studies using BMDCs, Langerhans cells and “D1” DCs, which are generated from mouse splenocytes by culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and fibroblast-conditioned medium, found that CsA can effectively suppress both NFAT activity and IL-2 secretion [130, 330, 334, 336, 337].

While the ability of LPS and zymosan to trigger IL-2 production by DCs has been established, other stimuli that can trigger IL-2 responses are emerging [3, 332]. A recent study demonstrated that particulates such as the vaccine adjuvant alum, monosodium urate crystals and silicon dioxide enhanced IL-2 production in DCs pre-primed with LPS or CpG [338]. This synergy was abrogated by CsA, as particulate-induced IL-2 requires engagement of the calcineurin-NFAT pathway [338] (Fig. 1.11A). Furthermore, despite the structural differences between smooth and rough LPS, both can trigger calcineurin-dependent IL-2 production, with the TLR4 accessory protein CD14 being selectively essential for smooth LPS-induced IL-2 [339]. Recent studies showed that calcineurin-NFAT signalling is active in DCs resident in mucosal tissues and the spleen. Indeed, both CD11c⁺MHCII⁺CD11b⁺ and CD11c⁺MHCII⁺CD11b⁻ DCs directly isolated from the murine spleen, mesenteric lymph

node, Peyer's patches and colonic lamina propria express NFAT at comparable levels to splenic T cells [336]. In addition, CD11c^{high}MHC⁺CD11b⁺ and CD11c^{high}MHC⁺CD11b⁻ DCs from the murine colonic lamina propria express comparable levels of calcineurin to mesenteric lymph node T cells [336]. In the intestine, it appears that CD103⁺ DCs are particularly proficient in IL-2 production under steady-state conditions [336]. In addition, lung CD103⁺ DCs stimulated with *Aspergillus fumigatus* exhibited increased Ca²⁺ flux and IL-2 production. In the same study, *Aspergillus*-induced IL-2 was found to be inhibited by CsA in the D1 dendritic cell line [232]. It is plausible that CsA will inhibit IL-2 production in a number of different settings, due to the clear dependency on calcineurin-NFAT signalling in multiple dendritic cell types. Overall, DCs actively produce IL-2 under numerous conditions in a calcineurin-dependent manner, making these cells a significant target for CsA-mediated immunomodulation.

1.9.2 Inhibition of IL-2 production in DCs can impact adaptive immune responses

Following stimulation with PAMPs or particulates, DCs rapidly secrete IL-2. In turn, DC-derived IL-2 modulates T cell proliferation, Treg cells, NK cell activation and vaccine-induced T cell responses. Thus, direct targeting of DC-derived IL-2 by CsA can influence a number of downstream adaptive immune processes.

DC-derived IL-2 provides an initial signal to promote T cell proliferation, which is propagated at later time points, when T cells gain the ability to self-produce IL-2 [3]. *Il2*^{-/-} BMDCs were found to be deficient in promoting proliferation of CD4⁺ and CD8⁺ T cells, using both carboxyfluorescein succinimidyl ester (CFSE) staining and tritiated thymidine incorporation assays [329]. Furthermore, mice lacking *Il2* exclusively in DCs triggered poor antigen-specific CD4⁺ T cell proliferation after immunisation with alum-adjuvanted ovalbumin (OVA) [338]. Imaging studies showed that DC-produced IL-2 localises at the synapse between DCs and T

cells [330]. Moreover, human DCs express the IL-2 receptor subunit CD25, which is used to trans-present DC-derived IL-2 to T cells present at the synapse, facilitating proliferation of T cells that typically lack CD25 during the initial hours of proliferation [340]. *Il2*^{-/-} DCs were found to be competent in key DC functions such as antigen uptake and maturation [338]. When taken together, the evidence indicates that DCs provide an early source of IL-2 that is key for effective T cell expansion.

Importantly, CsA elicits a similar effect to genetic depletion of IL-2 [3]. Indeed, differentiating DCs in the presence of CsA, before removing residual CsA in culture medium, significantly reduces their T cell priming capacity (Fig. 1.10A) [341-343]. To a lesser extent, CsA also reduces the ability of DCs to prime anti-inflammatory Treg cells [333]. Since IL-2 originating from DCs is known to be important for Treg cell growth and effector function, CsA may reduce Treg responses by suppressing DC IL-2 secretion [344].

Although DCs are known to contribute to autoimmunity by producing the TH17- and TH1-polarising cytokines IL-23 and IL-12 (see [5] for detailed review), upstream inhibition of IL-2 – specifically in DCs – may underly an overlooked mechanism by which CsA decreases inflammatory T cell responses [3]. At the same time, CsA may impair DC-induced Treg cell responses that are key in preventing autoimmunity. This is supported by studies using DCs over-expressing IL-2, which elicit enhanced Treg cell responses, providing greater protection against mouse type I diabetes [345]. Despite this, it is important to note that in contrast to its effects on T cells, CsA reduces but does not abrogate DC-derived IL-2, allowing low levels of secretion to be maintained. In addition, Treg cells exhibit significantly enhanced IL-2 binding and responsiveness compared to other T cell subsets, due to increased expression of the IL-2 receptor subunit CD25. Accordingly, both high and low doses of IL-2 decisively favour Treg cell expansion over other subsets [346]. Therefore, CsA treatment of DCs may preferentially

impair inflammatory T cell proliferation, while leaving Treg cell responses relatively intact, although this remains to be addressed in established *in vivo* models.

Recently, it was demonstrated that DC calcineurin signalling is crucial for intestinal homeostasis under steady state conditions [336]. Mice genetically lacking calcineurin or IL-2, exclusively in DCs, have an inflamed gut phenotype characterised by enlarged mesenteric lymph nodes and increased intestinal permeability [336]. This is associated with impaired Treg cell induction and subsequent increased IL-17- and interferon- γ (IFN γ)-producing CD4⁺ T cells [336]. Accordingly, a lack of IL-2 in DCs leads to exacerbated colitis [336]. Collectively, this study implies that an absence of IL-2 production in DCs causes unfavourable loss of intestinal anti-inflammatory Treg cells and an increase in gut inflammation. This is intriguing, as CsA is already used to treat refractory colitis in the clinic [347]. However, unlike genetic deletion, CsA maintains low levels of IL-2 production in DCs, which, as discussed, may reduce inflammatory T cells but preserve sufficient Treg cell responses.

DCs have long been known to activate NK cells, which provide a key source of IFN γ , facilitating TH1 cell polarisation. This has mainly been attributed to DC production of type I interferons and IL-12 [3]. However, DC-derived IL-2 also directly enhances IFN γ secretion by NK cells [348]. *In vivo*, DCs are the primary source of IL-2 that triggers NK cells to produce IFN γ in the early phase of *Escherichia coli* infection [348]. Moreover, induction of IFN γ in NK cells – specifically by DC-derived IL-2 – is necessary for potent phagocytic and antitumoral responses against intravenously injected *Escherichia coli* and B16 melanoma cells respectively [348]. Since IFN γ secretion by NK cells facilitates TH1 cell polarisation, CsA-mediated inhibition of DC IL-2 production may block a positive feedback loop, whereby DC-derived IL-2 triggers NK cell production of IFN γ , facilitating TH1 cell differentiation [349]. In support of this, *in vivo* priming of T cells by DCs lacking IL-2 leads to reduced T cell production of IFN γ *ex vivo* [350]. Novel strategies that enable targeted delivery of CsA to DCs

will determine whether CsA can reduce TH1 cell responses by inhibiting DC production of IL-2 in an *in vivo* setting.

1.9.3 CsA directly modulates DC production of pro- and anti-inflammatory cytokines

In addition to inhibiting IL-2 secretion by DCs, there is evidence that CsA can also modulate the production of other cytokines (Fig. 1.10B) [3]. For example, CsA inhibited LPS-induced secretion of tumour necrosis factor (TNF) α and IL-12 by BMDCs and human monocyte-derived DCs, which were generated from monocytes by culture with GM-CSF and IL-4 [333, 343, 351, 352]. CsA also inhibited LPS-induced IL-12 secretion in human blood-derived CD11c⁺lin⁻MHCII⁺ DCs and steady-state production of TNF α in murine Langerhans cells [353, 354]. Although CsA reduced LPS-induced IL-12 production in BMDCs, there was no effect on secretion of IL-6, indicating that inflammatory cytokine inhibition is selective [355]. One study, which performed NFAT chromatin immunoprecipitation-sequencing in D1 DCs, found that NFAT binds to the promoter regions of a number of genes encoding pro-inflammatory cytokines such as TNF α , IL-12 and IL-23. Using microarray analysis on curdlan-stimulated BMDCs, in the presence or absence of the NFAT inhibitor tacrolimus, it was found that NFAT is important for upregulating gene expression of these cytokines. Collectively, this may help to explain the mechanism by which CsA modulates production of IL-12 and TNF α in DCs [356] (Fig 1.11A).

In striking contrast to the inhibitory effect of CsA on TNF α and IL-12 secretion, parallel experiments found that CsA, at concentrations ranging from 0.5-1 μ g/ml, can enhance LPS-induced production of anti-inflammatory IL-10 in BMDCs and human blood-derived CD11c⁺lin⁻MHCII⁺ DCs [3, 333, 343, 353] (Fig 1.10B). This effect was found in BMDCs pre-treated with CsA for 5 days, or at the same time as LPS, while human blood CD11c⁺lin⁻MHCII⁺ DCs produced more IL-10 when simultaneously treated with CsA and LPS [333, 343,

353]. Thus, the evidence suggests that CsA increases IL-10 in a number of DC culture models, independently of the treatment duration. By inhibiting IL-12 and inducing IL-10 production, CsA appears to impart an anti-inflammatory phenotype on these DCs that may lead to differential regulation of effector T cell subsets. Tajima *et al* found that human blood-derived CD11c⁺lin⁻MHCII⁺ DCs exposed to 500ng/ml CsA, in the presence of LPS for 72h, were significantly less effective in priming IFN γ ⁺ T cells, while induction of IL-10⁺ T cells remained unchanged [353]. In contrast, another study found that BMDCs differentiated for 5 days in the presence of 750ng/ml CsA, followed by 4h LPS stimulation, elicit reduced mouse Treg cell proliferation *in vitro* [333]. However, this co-culture combined BMDCs with purified CD4⁺CD25^{hi}Foxp3⁺ Treg cells, as opposed to naïve T cells and excluded markers of Treg cell effector function [333]. Thus, this study does not negate the possibility that CsA differentially modulates DC induction of functional TH1 and Treg cells from naïve T cells.

1.9.4 CsA reduces DC expression of maturation markers

A number of studies indicate that CsA can inhibit DC maturation, although the outcome depends on the DC under study and the stimulus used [3]. Incubation of unstimulated GM-CSF-cultured BMDCs with 1 μ g/ml CsA, for 48 or 72 hours, reduced expression of CD40, CD80 and CD86 [355, 357]. In addition, unstimulated Langerhans cells exhibited reduced expression of CD40 and CD80 when cultured with 0.1-1 μ M CsA for 12 hours [358]. Despite these findings, it is less clear whether CsA can inhibit DC maturation in response to a strong stimulus. For example, in BMDCs, pre-treatment with 1 μ g/ml CsA for 48 hours did not significantly reduce LPS-induced expression of CD80, CD86 or CD40 [343]. In addition, pre-treating BMDCs with 10 μ M CsA for 40 minutes failed to significantly decrease CD40 or CD86 expression induced by CpG [113]. In contrast, in human monocyte-derived DCs, 1 μ g/ml CsA reduced expression of CD80 and CD86 in response to TNF α , when added 6 days before or at the same time as TNF α [359]. In the same DCs, 1 μ g/ml CsA also reduced CD80

and CD86 expression when added with LPS, although this reduction was not observed with Poly (I:C) [359]. Moreover, 500ng/ml CsA reduced LPS-induced expression of CD80 and CD86 in CD11c⁺MHCII⁺ DCs isolated from human blood when added in conjunction with LPS for 72 hours [353].

The mechanism by which CsA affects DC maturation may also vary depending on the type of DC [3]. For example, 72h incubation with 50ng/ml CsA reduced the initial uptake of fluorescein isothiocyanate (FITC)-labelled dextran in human blood-derived CD11c⁺MHCII⁺ DCs [353]. However, human monocyte-derived DCs exhibit normal FITC-dextran uptake after culture with 1µg/ml CsA for 5 days [359]. In BMDCs, CsA-reduced maturation is associated with impaired nuclear translocation of NFκB, an important transcription factor for co-stimulatory molecule expression [357, 360] (Fig. 1.11A). Finally, in the “DC2.4” immortalised DC line, 1µM CsA inhibited MHC presentation of unprocessed OVA but not purified “SIINFEKL” (OVA residues 257-264) peptide, indicating direct inhibition of intracellular antigen processing, rather than presentation [361].

In the same studies that demonstrated CsA can reduce DC maturation, CsA was also found to inhibit T cell proliferation through direct targeting of DCs (Fig. 1.10A) [3]. Indeed, CsA reduced T cell proliferation mediated by GM-CSF-generated BMDCs, human DCs propagated from monocytes and CD11c⁺MHCII⁺ DCs derived from human blood [343, 353, 357, 359, 361]. These studies used extensive DC washing, to remove CsA from culture medium before co-culture with T cells, negating the possibility of direct T cell inhibition. It can be speculated that CsA can reduce DC priming of T cells by targeting a number of DC functions such as antigen presentation, expression of maturation markers and cytokine secretion (Fig. 1.10) [343, 353, 357, 359, 361]. Aside from inhibition of calcineurin-dependent IL-2, the mechanisms by which CsA affects these responses in DCs remain to be clearly defined.

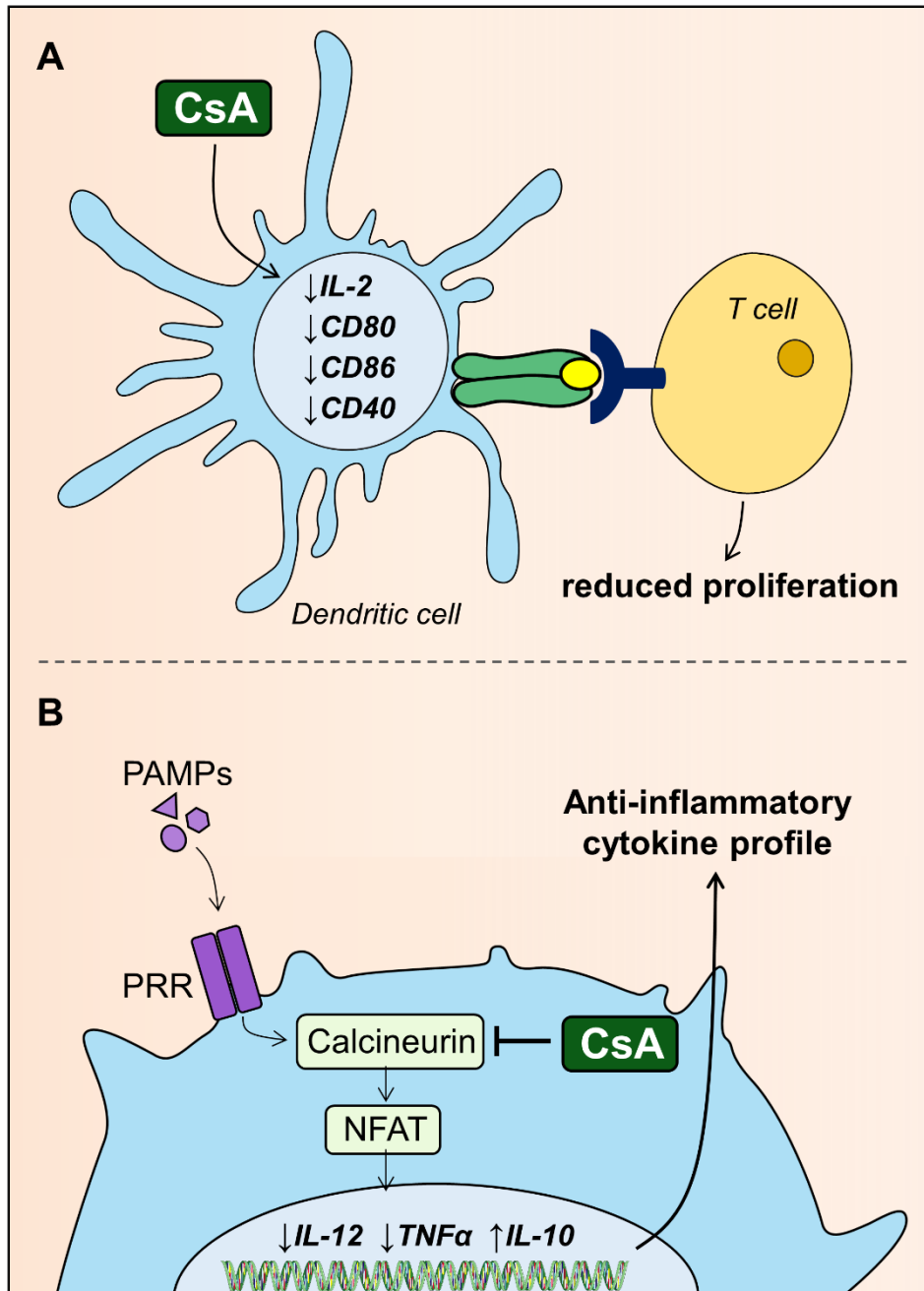


Fig. 1.10: Cyclosporine A (CsA) modulates dendritic cell (DC) priming of T cell responses. (A) CsA inhibits DC expression of IL-2 and the maturation markers CD80, CD86 and CD40, leading to reduced T cell proliferation. (B) CsA imparts an anti-inflammatory cytokine profile on DCs by inhibiting NFAT-dependent production of IL-12 and TNF α , while increasing secretion of IL-10. Abbreviations: CD, cluster of differentiation; PAMPs, pathogen-associated molecular patterns; PRR, pattern-recognition receptor; NFAT, nuclear factor of activated T cells. Taken from Liddicoat A.M. and E.C. Lavelle *Biochem Pharmacol*, 2019. 163: p. 472-480 [3].

1.9.5 CsA reduces inflammatory mediators in macrophages and neutrophils

Macrophages and neutrophils are key mediators of innate anti-microbial immunity, due to their ability to detect, phagocytose and destroy pathogens through numerous toxic mediators. Briefly, upon internalisation by these phagocytes, microbes are enclosed in microbicidal compartments known as phagolysosomes. Here, phagocytes create a highly toxic environment by means of acidification, hydrolytic enzymes, reactive oxygen species (ROS), reactive nitrogen species (RNS) and antimicrobial peptides (see for [275] detailed review). In addition, neutrophil extracellular traps (NETs) – extruded DNA backbones decorated with histones and antimicrobial granular proteins – can capture and kill pathogens in the extracellular space. These mediators can also damage host tissues, particularly when their production is dysregulated. Furthermore, aberrant NET formation may provide a source of self-antigens that can trigger autoimmune responses [362]. Together, hyperactivated macrophages and neutrophils are considered to perpetuate a number of autoimmune diseases, including RA and SLE, highlighting the potential of therapeutically targeting these cells [362, 363]. Importantly, evidence highlights that CsA can directly impact the production of tissue-damaging factors and pro-inflammatory cytokines in these cell types, suggesting additional mechanisms by which CsA induces immunosuppression, as well as potentiating opportunistic infections [3].

CsA inhibits the production of RNS, inflammatory cytokines and prostaglandins in macrophages, potentially enhancing protection against tissue damage [3]. For example, in BMDMs, NFAT-promoter interactions are required for the expression of inducible nitric oxide synthase (iNOS), an essential enzyme for RNS generation (Fig. 1.11A). Accordingly, CsA restricts NFAT from binding to the iNOS promoter, leading to significantly reduced iNOS expression and nitrite production [364]. Therefore, despite reducing bactericidal activity, CsA can potentially ameliorate tissue damage during autoimmune responses by directly regulating

RNS production in macrophages. Similarly to DCs, CsA significantly reduces TNF α and IL-12 production by mouse peritoneal macrophages, while IL-10 secretion was intact [354]. Mechanistically, IL-12p40 expression in RAW264.7 macrophages was dependent on NFAT binding to the p40 promoter, an interaction that was abrogated by CsA [365] (Fig. 1.11A). Thus, *in vivo*, the capacity of CsA to inhibit IL-12 secretion may impair TH1 cell differentiation, conferring therapeutic benefits in conditions where excessive production of IFN γ contributes to pathology. Macrophages produce significantly higher levels of inflammatory cytokines when deficient for LRRK2 – a negative regulator of NFAT in myeloid cells – suggesting that inhibition of NFAT activity in macrophages can inhibit inflammatory responses [331]. In support of this, CsA reverts the exacerbated colitis observed in *Lrrk2*^{-/-} mice [331]. Hence, CsA may reduce inflammatory cytokine responses during autoimmunity by directly inhibiting NFAT activity in macrophages [331]. Finally, in RAW264.7 macrophages, CsA downregulates the enzyme cyclooxygenase-2 (COX-2), which is essential for metabolism of arachidonic acid into prostaglandin E₂ (PGE₂), an inflammatory mediator that increases vascular permeability to facilitate immune cell recruitment [130]. CsA significantly reduced both COX-2 expression and PGE₂ production in RAW264.7 macrophages, potentially indicating that CsA can impair macrophage-induced leukocyte infiltration [130]. Collectively, the evidence suggests that CsA may directly reduce macrophage-mediated inflammation by inhibiting production of noxious RNS, inflammatory cytokines and prostaglandins.

CsA can inhibit multiple neutrophil processes such as ROS generation and the formation of NETs (Fig. 1.11B) [3]. *Rag2*^{-/-} mice, which lack lymphocytes, exhibit normal survival against *Candida albicans* but rapidly succumb to infection when treated with CsA, highlighting that CsA can impair anti-fungal functions in innate immune cells [366]. More specifically, this fungal susceptibility is also observed in mice lacking calcineurin in neutrophils alone,

suggesting that CsA may directly target neutrophil killing mechanisms [366]. Indeed, *ex vivo* studies found that CsA inhibits primary mouse neutrophil killing of *Candida albicans* [366]. Similar studies showed that CsA impairs human neutrophil clearance of *Aspergillus fumigatus* [367, 368]. Strikingly, one study demonstrated an inverse correlation between the concentration of CsA detected in patient blood and the ability of isolated patient neutrophils to inhibit *Aspergillus fumigatus* growth *ex vivo* [368]. Regarding the mechanism of inhibition, CsA appears to target multiple neutrophil mediators. For example, CsA was shown to reduce production of ROS in isolated human neutrophils [366, 367]. In addition, CsA directly inhibited formation of human NETs, in response to phorbol-12-myristate-13-acetate (PMA), ionomycin or IL-8 [369]. Thus, CsA may partly induce immunosuppression and protect against tissue damage through specific blockade of ROS and NET generation in neutrophils.

When taken together, these findings further challenge the classical view that CsA only affects adaptive immunity. Indeed, it is plausible that CsA also suppresses inflammation through direct modulation of macrophages and neutrophils. Overall, this may help to explain the immunosuppressive potency of CsA and how the drug can protect against autoimmune diseases but also confer susceptibility to opportunistic infections [3].

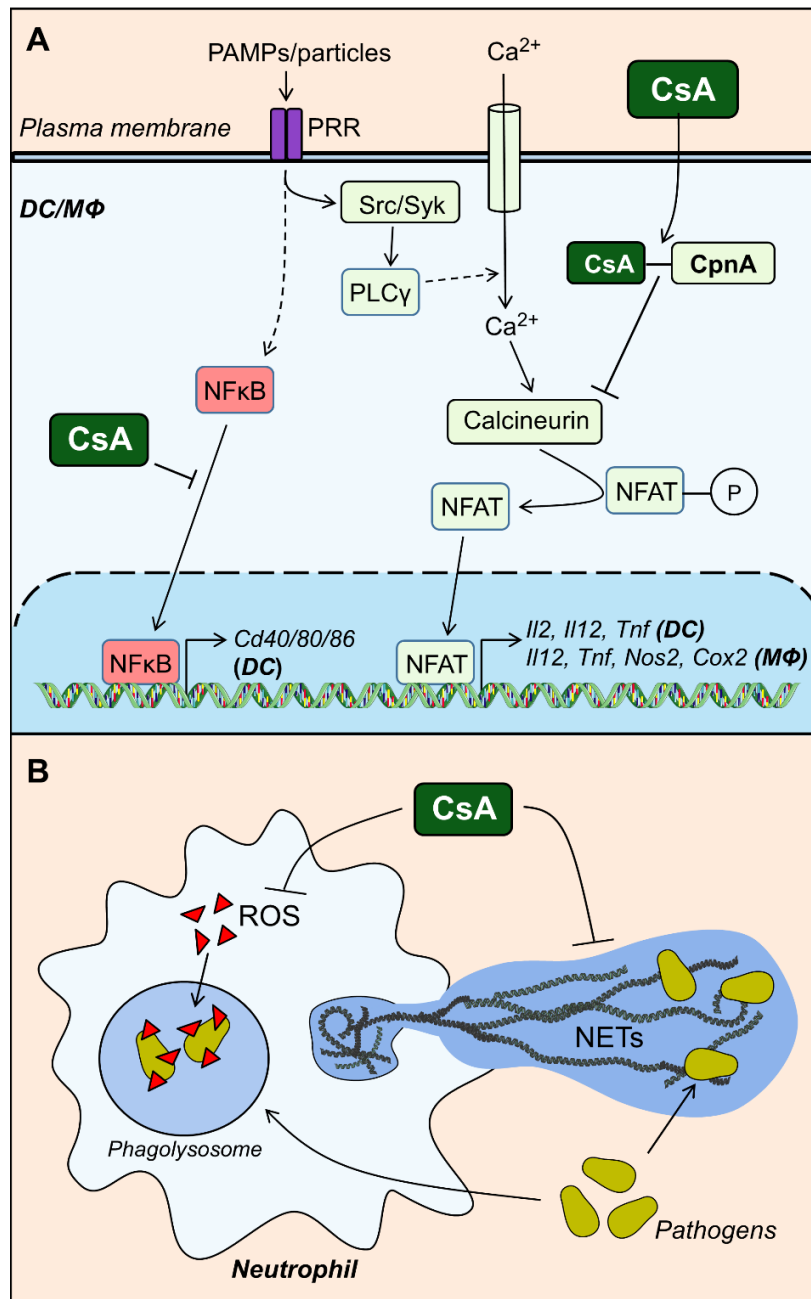


Fig. 1.11: Proposed mechanisms by which Cyclosporine A (CsA) modulates responses in dendritic cells (DC), macrophages (MΦ) and neutrophils. (A) CsA inhibits DC expression of CD40, CD80 and CD86, potentially by reducing nuclear translocation of NFκB. PRR signalling engages Src and Syk family kinases, which activate PLCγ, leading to extracellular Ca²⁺ influx and calcineurin activation. Calcineurin dephosphorylates NFAT, which translocates to the nucleus and aids transcription of inflammatory genes. These include *Il2* (IL-2), *Il12* (IL-12) and *Tnf* (TNFα) in DCs, as well as *Il12* (IL-12), *TNF* (TNFα), *Nos2* (iNOS) and *Cox2* (COX-2) in MΦs. CsA inhibits NFAT-dependent gene expression by forming a complex with cyclophilin A. This complex directly binds to calcineurin and inhibits its phosphatase activity, retaining NFAT in the cytosol. (B) CsA reduces ROS production and NET formation in neutrophils, leading to impaired antimicrobial killing responses. Abbreviations: CpnA, cyclophilin A; MΦ, macrophage; NETs, neutrophil extracellular traps; PLCγ, phospholipase-c-γ; PRR, pattern recognition receptor; ROS, reactive oxygen species; Src, Src family kinases; Syk, Syk family kinases; Zym, zymosan. Taken from Liddicoat A.M. and E.C. Lavelle *Biochem Pharmacol*, 2019. 163: p. 472-480 [3].

1.9.6 CsA reduces inflammatory responses in innate immune cells by inhibiting the release of mitochondrial DAMPs

Mitochondria evolved in eukaryotic cells from bacteria that were internalised and retained in the cytosol [370]. Mitochondria retain bacterial features, such as circular DNA genomes containing non-methylated CpG motifs and membranes containing the prokaryotic phospholipid cardiolipin. Consistent with their bacterial origin, mitochondrial factors such as DNA, ROS and phospholipids – collectively termed DAMPs – can activate innate immune cell PRRs that are classically known to detect bacteria [370]. Under homeostatic conditions, DAMPs are retained in the mitochondria. However, cellular stress, which can be caused by dysregulated ROS production, ATP release and elevated intracellular calcium levels, induces opening of the mitochondrial permeability transition (MPT) pore, a multi-protein complex that transports DAMPs from the mitochondria to the cytosol [371, 372].

After mitochondrial egress, DAMPs activate innate immune cell PRRs, culminating in inflammatory cytokine secretion (Fig. 1.12) [3]. For example, mitochondrial DNA significantly enhanced TLR9 activation in human neutrophils and promoted severe tissue inflammation in rats upon intravenous injection [373]. Studies using THP-1 and J774A.1 monocytes, BMDMs and RAW264.7 macrophages demonstrated that mitochondrial ROS, cardiolipin and DNA can drive mature IL-1 β secretion by activating the NLRP3 inflammasome [73, 74, 374]. In BMDMs, TLR signalling increased the synthesis of new mitochondrial DNA, thereby amplifying NLRP3 ligation [375]. Mitochondrial DNA also increases production of type I interferons (IFNs) by engaging the DNA sensor cyclic GMP-AMP synthase (cGAS) [376]. Finally, mitochondrial ROS can drive mitochondrial antiviral signalling protein (MAVS) oligomerisation – independently of PRR signalling – in human peripheral blood mononuclear cells (PBMCs), inducing type I IFN production and NF κ B activation [377]. Thus, mitochondrial DAMPs are key inflammatory mediators in innate

immune cells. Although DAMPs can enhance anti-microbial responses, dysregulated DAMP release potentiates inflammatory diseases such as SLE and RA, implicating the MPT pore as a potential therapeutic target [378].

CsA potently inhibits MPT pore formation, preventing DAMP release and inflammatory cytokine secretion in innate immune cells (Fig. 1.12) [3]. Although the precise structure of the MPT pore is currently unclear (see [379] for detailed review), multiple studies support the key role of mitochondrial cyclophilin D in pore assembly [379-382]. Independently of cytosolic cyclophilin A and calcineurin, CsA inhibits MPT pore formation by directly binding to mitochondrial cyclophilin D [379-381, 383, 384]. This prevents the release of mitochondrial DAMPs. Indeed, in purified mitochondria, 1 μ M CsA reduced the release of DNA in response to cellular stress signals such as H₂O₂, Ca²⁺ and inorganic phosphate [371, 372]. In innate immune cells, inhibition of the MPT pore by CsA can reduce inflammatory responses. For example, in BMDMs, 10 μ M CsA potently inhibited the release of mitochondrial DNA in response to LPS and ATP [385]. In parallel experiments, CsA significantly inhibited secretion of IL-1 β but not TNF α by BMDMs stimulated with LPS and ATP [73, 385]. In addition, pre-treating LPS-primed J774A.1 monocytes with 20 μ M CsA led to significantly reduced secretion of IL-1 β but not TNF α in response to silica and nigericin [374]. Together, these studies suggest that CsA can inhibit inflammasome activity in macrophages and monocytes by preventing DAMP release through the MPT pore, thereby reducing inflammatory cytokine secretion [73, 374, 385]. In BMDCs, the vaccine adjuvant chitosan induces mitochondrial ROS production and DNA release, resulting in cGAS activation and subsequent type I IFN secretion [113]. However, pre-treating BMDCs with 20 μ M CsA abrogated chitosan-induced IFN β secretion, while only a marginal decrease was observed when BMDCs were stimulated with CpG. Thus, in response to certain stimuli, CsA may inhibit type I IFN responses in DCs by blocking the MPT pore [113]. Collectively, the evidence suggests that CsA can reduce

inflammatory responses in both DCs and macrophages by inhibiting mitochondria-triggered PRR signalling, potentially underlying an additional mechanism of immunosuppression.

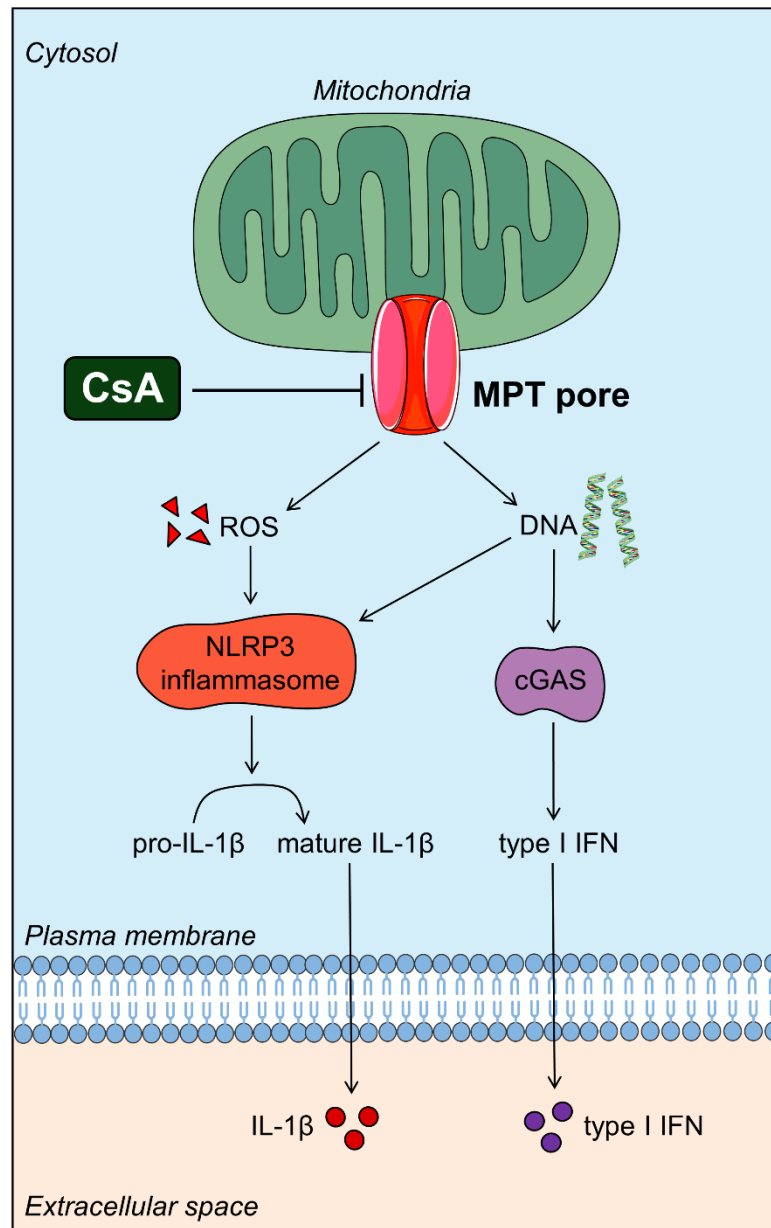


Fig. 1.12: Cyclosporine A (CsA) inhibits the mitochondria permeability transition (MPT) pore, resulting in reduced activation of innate immune cells. Mitochondrial DAMPs, including DNA and ROS, are transported through the MPT pore and into the cytosol in response to cellular stress. Here, DAMPs activate the NLRP3 inflammasome and cGAS, leading to upregulation of inflammatory IL-1 β and type I IFNs respectively. In innate immune cells, CsA can reduce secretion of these cytokines by inhibiting DAMP release through the MPT pore. Abbreviations: cGAS, cyclic GMP-AMP synthase; DAMPs, danger-associated molecular patterns; ROS, reactive oxygen species. Taken from Liddicoat A.M. and E.C. Lavelle *Biochem Pharmacol*, 2019. 163: p. 472-480 [3].

1.10 Susceptibility to invasive fungal infection and the risks imposed by CsA

Healthy individuals typically remain tolerant to fungi, maintaining a commensal relationship. However, opportunistic infections occur when the host response is impacted by external factors such as immunosuppressive treatments. Due to its immunosuppressive potency, CsA has shown great efficacy against a range of autoimmune disorders, including RA, SLE, psoriasis and ulcerative colitis [324, 325, 386, 387]. However, a drawback of this drug is the reported increase in risk of cancers and opportunistic infections [324, 325, 388-392]. In particular, a number of animal and human studies have demonstrated that CsA treatment increases the risk and severity of fungal infections, including *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Candida albicans* [366, 368, 391, 393-398]. One patient received oral CsA treatment for steroid-refractory colitis and, although symptoms rapidly improved, later developed significant pneumonia and lung pathology due to invasive *Aspergillus fumigatus* infection [399]. Similarly, intravenous CsA treatment of a steroid-refractory Crohn's disease patient led to fatal Aspergillosis [400]. As such, CsA has long been considered a risk factor for invasive fungal diseases, by the European Organisation for Research and Treatment of Cancer and The Mycoses Study Group [401]. Moreover, the patient information leaflet for the current CsA formulation Neoral®, which is a systemically-absorbed oral solution, states that the drug predisposes patients to opportunistic infections:

(<https://www.medicines.org.uk/emc/files/pil.5300.pdf>).

As previously stated, invasive fungal infections have a high mortality rate and are estimated to cause >1.5 million deaths per year [250]. Systemic treatment can be achieved by antifungal drugs, which include polyenes, triazoles, echinocandins and pyrimidine analogues. Polyenes include the gold-standard amphotericin B, which penetrates the fungal cell membrane, leading

to pore formation and efflux of essential ions K^+ , Mg^{2+} , Ca^{2+} and Cl^- and ultimately fungal death [402]. Triazoles are fungistatic by inhibiting enzymatic demethylation of fungal lanosterol, preventing synthesis of the key cell membrane component ergosterol [403]. Echinocandins target β -glucan synthases, inhibiting production of the major cell wall component β -glucan [404]. Pyrimidine analogues include flucytosine, which is converted into fluorouridine triphosphate, leading to incorporation into fungal RNA and disruption of protein synthesis [405]. Despite the range of treatments, antifungals have significant drawbacks. In immunocompromised patients, which would include those receiving CsA, systemic antifungal treatment may be required for long periods and this is associated with renal, gastrointestinal, cutaneous, cardiac and hepatic toxicities [406]. Moreover, the emergence of antifungal resistance represents a major clinical challenge. This can occur through fungal genetic changes that are more likely to occur during long-term treatment courses. Key examples include *Candida* species developing resistance to amphotericin B and echinocandins, due to mutations in *Erg2* and the β -glucan synthase subunit *Fks1p* respectively [407, 408]. Moreover, *Aspergillus fumigatus* mutations in the target gene *Cyp51A* confer resistance to triazoles [409].

In addition to the challenges posed by antifungal resistance, fungi possess immune-evasive and virulence factors that are a significant threat to immunocompromised patients. Indeed, *Candida albicans* can evade immune recognition by masking β -glucans beneath outer cell wall proteins [410]. *Cryptococcus neoformans* and *Histoplasma capsulatum* reduce β -glucans exposure by shielding with polysaccharide capsules and α -glucans respectively [411, 412]. Using *Aspergillus fumigatus* mutants with defective RodA, it was found that this protein reduces Dectin-1- and Dectin-2-mediated recognition of conidia. This led to impaired production of chemokines and $TNF\alpha$ by macrophages, reduced neutrophil recruitment and delayed clearance of infection in mice [413]. If internalised, fungi may evade killing mechanisms within phagocytes. *Aspergillus fumigatus* melanin impairs acidification of the

phagosome in macrophages and neutrophils [414]. Moreover, ROS quenching can be achieved by superoxide dismutase in *Candida albicans* and *Cryptococcus neoformans*, while gliotoxin in *Aspergillus fumigatus* was found to inhibit NADPH-oxidase-dependent synthesis of superoxide anion in neutrophils [415-417]. Intriguingly, *Candida albicans* was found to inhibit IL-17 production by PBMCs and this was due to increased host cell production of the metabolite 5-hydroxytryptophan [418]. These findings suggested that in immunosuppressed hosts, where *Candida albicans* are not rapidly phagocytosed and killed, the fungus may further dampen host defence by impairing TH17 cell responses [418]. In conjunction with evasion, fungi may actively promote virulence. For example, *Candida albicans* expresses the secretory toxin candidalysin, which is required for mucosal pathogenesis due to its ability to damage epithelial cell membranes [419]. These fungal adaptations are typically overcome by immunocompetent hosts, through sufficient production of inflammatory mediators such as cytokines, to induce robust activation of fungal killing in phagocytes. By contrast, in immunosuppressed patients receiving CsA, the risk of fungal survival and dissemination is significantly increased.

Adding to these challenges, there are currently no licenced fungal vaccines, despite there being a number of promising candidates. Indeed, subcutaneous immunisation of mice with heat-killed *Saccharomyces cerevisiae* (HKSC) induced T cell clonal expansion and antigen-specific production of IFN γ and IL-17 in the spleen and lymph nodes [420]. Intriguingly, HKSC-induced immunity in mice was cross-protective against Candidiasis, Aspergillosis and Cryptococcosis, due to common epitopes within fungal cell walls that raise the possibility of a “pan-fungal” vaccine for immunocompromised patients [421-423]. Another potential approach is the use of subunit vaccines. Immunisation with NDV-3, which contains recombinant *Candida albicans* invasin agglutinin-like sequence 3 protein (Als3p) and the adjuvant alum, protected mice against candidiasis and primed antigen-specific IgG, IgA, IFN γ

and IL-17 responses in humans [424, 425]. Similarly, PEV7, which is a virosome incorporating recombinant Sap2 protein – a key determinant of candidiasis – protected against candidal vaginitis in rats through generation of local and systemic anti-Sap2 IgA and IgG [426]. Other candidates include conjugate vaccines, which involve linkage of polysaccharide antigens to protein, to increase immunogenicity. This is a practical approach as fungal cell walls are rich in polysaccharide epitopes. Immunising mice with glucuronoxylomannan – a capsular polysaccharide of *Cryptococcus neoformans* – conjugated to tetanus toxoid increased survival after intravenous infection [427]. Consistently, a *laminaria digitata* glucans and diphtheria toxoid conjugate promoted antibody-mediated cross-protection against systemic *Candida albicans* and *Aspergillus fumigatus* [428]. While pre-clinical findings are promising, relatively few candidates have reached late-stage clinical trials and none have obtained approval for use in humans. Pressing concerns include the structural homology between commensal and pathogenic fungal species, creating the risk of inadvertently disrupting beneficial relationships between fungi and the host by activating latent infections [429]. While new antifungal interventions are in the pipeline (see [430, 431] for detailed reviews), invasive fungal infections remain a significant challenge. Therefore, it is vital to improve current understanding of the mechanisms by which CsA increases susceptibility, to minimise the incidence of severe disease.

As expected, the inhibition of T cell proliferation by CsA will impair TH1 and TH17 cell responses, increasing the risk of fungal infection in patients [432]. However, recent studies suggested that CsA can also directly target key antifungal functions in innate immune cells. For example, *ex vivo* assays found that CsA inhibited murine neutrophil killing of *Candida albicans*, while human neutrophil killing of *Aspergillus fumigatus* was also impaired [366, 368]. This reduced killing may be due to CsA inhibiting neutrophil production of antimicrobial NETs and ROS [367, 369]. In macrophages, phagocytosis of *Aspergillus fumigatus* conidia

triggers a TLR9-Btk-PLC γ -calcineurin-NFAT pathway, which is essential for TNF α secretion and subsequent neutrophil recruitment and fungal killing in the lungs [262]. In DCs, calcineurin signalling was also necessary for DC production of pentraxin-3 (ptx3), a secretory PRR that opsonises *Aspergillus fumigatus* conidia through direct binding, aiding innate immune cell phagocytosis and protecting against invasive aspergillosis [265, 433]. In addition, CsA treatment of human monocytes was found to inhibit Zymosan-induced expression of Ptx3, as well as the pro-inflammatory cytokine TNF α [434]. Moreover, CsA reduced zymosan-induced secretion of IL-2 and IL-12p70 in GM-CSF-generated BMDCs [130].

In a key study by Zelante *et al*, calcineurin deletion in CD11c⁺ cells, which are primarily composed of DCs, significantly increased susceptibility to invasive *Aspergillus fumigatus* [433]. This suggested that the direct action of CsA on DCs is a key risk factor for fungal infections in patients. However, the specific subsets of DC required for protection – those with high clinical relevance – were not addressed in this study [433]. As discussed, CD103⁺ DCs are vital for immune responses to pathogens but also homeostasis in peripheral tissues such as the lungs, gut and skin – all key sites of fungal infection. Indeed, these cells continually acquire antigens *in situ*, before migrating to the draining lymph nodes and priming T cell expansion and recirculation to affected tissues [206, 238]. Crucially, in recent studies, CD103⁺ DCs have emerged as an important mediator of antifungal immunity. Indeed, although *Batf3*^{-/-} mice – which lack CD103⁺ DCs – were protected against epicutaneous *Candida albicans* challenge, these animals were significantly more susceptible to systemic infection [248]. Moreover, CD103⁺ DCs prime CD8⁺ T cells specific for commensal bacteria in the skin that are cross-protective against fungi. Indeed, these T cells are activated in the epidermis by IL-1 and secrete IL-17, inducing keratinocytes to produce antimicrobial peptides that prevent colonisation with *Candida albicans* [244]. In addition, during invasive pulmonary aspergillosis, CD103⁺ DCs were a key source of IL-2 and IL-23, which were necessary for priming an optimal TH17 cell

response that protected against disease without triggering severe immunopathology [232]. Intriguingly, one study found that compared to other lung DCs subsets, monocytes and alveolar macrophages, CD103⁺ DCs were the strongest producers of type I IFNs after intranasal infection with the fungus *Histoplasma capsulatum* [152]. This is a key finding when considering that the type I IFN-IFNAR pathway is now a recognised mechanism of protection against fungi. Indeed, IFNAR was crucial for mice survival against *Histoplasma capsulatum*, *Cryptococcus neoformans* and *Candida albicans* [150-152, 435]. Moreover, PBMCs isolated from patients suffering chronic candidiasis exhibited defective expression of type I IFN pathway genes [436]. It is important to note that the source of type I IFNs may vary depending on the fungal species. For example, after intratracheal challenge with *Aspergillus fumigatus*, CCR2⁺ monocytes produced the highest levels of type I IFNs in the lungs [437]. The secretion of type I IFNs during fungal infection is dependent on Dectin-1 and, importantly, type I IFN treatment prevented the increased susceptibility of Dectin-1-deficient mice to *Aspergillus fumigatus* [438]. This was due to type I IFNs restoring cytokine and chemokine secretion and neutrophil production of ROS in the lungs to WT levels [438]. Mechanistically, type I IFN production has also been shown to balance antifungal IL-17⁺ T cell responses with enhanced IL-12-dependent IFN γ ⁺ T cells, ensuring optimal protection [152, 436]. Moreover, IFNAR signalling was found to directly enhance intracellular fungal killing in DCs, by increasing ROS production [150, 439]. In addition to these discussed roles, CD103⁺ DCs likely contribute to antifungal immunity through classical DC functions such as antigen presentation, co-stimulation, cytokine production and subsequent priming of adaptive immune responses [4].

1.11 Research objectives

CD103⁺ DCs are essential for inducing T cell responses in the gut, lungs and skin and have been reported to protect against invasive fungal infections caused by *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Candida albicans* [152, 232, 248]. The emerging role of NFAT in DCs has encouraged research into the capacity of CsA to modulate DC activation induced by a number of different PAMPs (section 1.10). However, previous studies have failed to address whether CsA impacts antifungal responses in CD103⁺ DCs present in mucosal tissues. Importantly, this may help to explain why patients are more susceptible to fungal infections, which will assist in reducing the incidence of severe disease. It is difficult to assess direct modulation of CD103⁺ DCs by CsA *in vivo*. Indeed, clinically relevant treatments such as Neoral® reach the systemic circulation, enabling inhibition of T cell responses, which in turn may indirectly impact CD103⁺ DC activation. This would raise doubt over whether CsA modulates these DCs directly. To overcome this, *in vivo* assessment of antifungal responses by CD103⁺ DCs could focus on early timepoints, ideally <24h post-challenge. This allows direct activation of DCs before T cell responses are fully induced. In addition to this issue, determining the mechanisms of CD103⁺ DC modulation by CsA are particularly challenging, as primary cells are difficult to isolate in high numbers. This can be overcome using the iCD103 DC culture method developed by Mayer *et al* ([198]), a practical solution that enables direct treatment with CsA and fungal PAMPs and analysis of cytokine and maturation responses. Regarding fungal PAMPs, DCs primarily respond to fungi through Dectin-1 binding to β -glucans, which make up 50% of the fungal cell wall mass of key species such as *Candida albicans* [122, 130, 440]. As such, these studies use Zymosan, a crude fungal cell wall extract primarily containing β -glucans that bind to Dectin-1, along with other cell wall and nucleic acids PAMPs that engage PRRs such as TLR2 and TLR9 [120, 259, 435]. Therefore, Zymosan is useful in that it triggers a wide range of antifungal responses in DCs,

making it suitable for both *in vivo* studies and iCD103 DC cultures in this research. The hypothesis of this work is that CsA can impair antifungal responses in CD103⁺ DCs.

To test this, the project addresses the following 4 objectives:

1. To assess whether Neoral® treatment modulates Zymosan-induced CD103⁺ DC responses in the lungs and draining lymph nodes.
2. To optimise the iCD103 DC culture model in the lab.
3. To characterise antifungal responses in iCD103 DCs and assess for inhibition by CsA.
4. To address novel mechanisms by which CsA inhibits antifungal responses in CD103⁺ DCs.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Mice

C57BL/6 mice were bred in-house by the Comparative Medicine Unit (TCD) and were used at age 8-15 weeks. *Ifnar*^{-/-} 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). 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).

2.1.2 Lung *in vivo* studies

Neoral® (Novartis): Systemically-absorbed oral CsA solution. Diluted to 4mg/ml in PBS for oral gavage into mice (200µL).

Isoflurin®: Isoflurane liquid vapourised for anaesthesia by inhalation.

Zymosan (Invivogen): Fungal PRR agonist. Intranasally delivered at 10mg/ml, 20µL per nostril.

Perfusion buffer: 10mM EDTA (Gibco) in PBS, 4ml injected into the right ventricle of the heart.

Digestion mix: 20mg/ml collagenase type I (Gibco) and 100µg/ml DNase I from Bovine pancreas (Sigma) diluted in RPMI.

Filter rinsing buffer: 5mM EDTA in PBS.

2.1.3 Cell culture

Chinese hamster ovary (CHO) cell RPMI: Roswell Park Memorial Institute (RPMI) medium was supplemented with 8%, 4% or 2% (v/v) heat-inactivated, filter-sterilised fetal calf serum (FCS) (Biosera).

Complete RPMI (cRPMI): RPMI medium was supplemented with 50 units/ml (U/ml) penicillin (Gibco), 50µg/ml streptomycin (Gibco) and 8% (v/v) heat-inactivated, filter-sterilised FCS (Biosera).

iCD103 dendritic cell RPMI (CD103-RPMI): RPMI medium was supplemented with 50 units/ml (U/ml) penicillin (Gibco); 50µg/ml streptomycin (Gibco); 8% (v/v) heat-inactivated, filter-sterilised FCS (Biosera) and 45.5 µM β-mercaptoethanol (Gibco).

0.88% Ammonium Chloride: 8.8g ammonium chloride (ACl) dissolved in 1 litre endotoxin-free water (Baxter) and filter-sterilised.

Table 2.1: Cell culture treatments

Treatment	Target/function	Supplier
CpG ODN 1826	TLR9	Oligos etc
Zymosan	Dectin-1, TLR2, other	Invivogen
LPS from <i>Escherichia coli</i> , Serotype R515	TLR4	Enzo Life Sciences
Pam3CSK4	TLR2	Invivogen
R837	TLR7	Invivogen
R848	TLR8	Invivogen
Poly I:C	TLR3	Invivogen
Heat-killed <i>Escherichia coli</i> , strain BL21	PRR agonist	N/A
Heat-killed <i>Streptococcus pneumoniae</i>	PRR agonist	N/A
Cyclosporine A	Calcineurin, MPT pore inhibitor	Invivogen
CCL19	Chemokine, CCR7 ligand	R&D Systems
11R-VIVIT	NFAT inhibitor	R&D Systems
MitoTempo	Mitochondrial ROS scavenger	Merck

2.1.4 Enzyme-linked immunosorbent assay (ELISA)

10x PBS: 400g Sodium Chloride (Sigma-Aldrich), 58g Na_2HPO_4 (Sigma-Aldrich), 10g KH_2PO_4 (Sigma-Aldrich) and 10g Potassium Chloride (Sigma-Aldrich) dissolved in 5L of millipore water and adjusted to pH 7.2.

1x PBS: 100ml 10x PBS, 900ml millipore water.

Sodium Carbonate Buffer (SCB): 8.4mg NaHCO_3 (Sigma-Aldrich) and 3.56g Na_2CO_3 (Sigma-Aldrich) dissolved in 5L of millipore water and adjusted to pH 9.5.

Wash Buffer (0.05% PBS-Tween): 8.995L millipore water, 1L 10x PBS and 5ml Tween 20 (Sigma-Aldrich).

1% BSA: 1g Bovine serum albumin (BSA, Fisher Scientific) dissolved in 100ml 1x PBS and 0.2 μm filtered.

Tris-buffered saline (TBS)-BSA-Tween: 24.8g Tris base (Sigma-Aldrich), 87.6g NaCl (Sigma-Aldrich), 1g BSA and 0.5ml Tween-20 made to final volume of 1L with millipore water, adjusted to pH 7.2 and 0.2 μm filtered.

Phosphate citrate buffer: 10.19g $\text{C}_6\text{H}_8\text{O}_7$ (Sigma-Aldrich) and 14.6 g Na_2HPO_4 (Sigma-Aldrich) dissolved in 1L of millipore water and adjusted to pH 5.

OPD Substrate Solution: o-phenylenediamine dihydrochloride (OPD) tablets (Sigma-Aldrich) dissolved in phosphate citrate buffer to yield a final concentration of 0.4mg/ml and supplemented with 4 μL /10mls buffer of hydrogen peroxide (Sigma Aldrich).

Tetramethylbenzidine (TMB) Substrate Solution (BD Biosciences): TMB substrate reagent A combined 1:1 with TMB substrate reagent B.

Stop Solution (1M H_2SO_4): 1L millipore water, 54.3ml Sulfuric acid (Sigma-Aldrich).

Table 2.2: Cytokine ELISA kits

ELISA Kit	Supplier
Mouse Flt3L DuoSet ELISA	R&D Systems
Mouse TNF α DuoSet ELISA	R&D Systems
Mouse IL-2 DuoSet ELISA	R&D Systems
Mouse IL-12p70 DuoSet ELISA	R&D Systems
Mouse IL-23 DuoSet ELISA	R&D Systems
Mouse IL-1 β DuoSet ELISA	R&D Systems
Mouse IL-6 ELISA MAX TM	Biolegend

Table 2.3: IFN β ELISA reagents

Reagent	Supplier
Rat anti-mouse IFN β capture antibody	Santa Cruz Biotechnology
IFN β Standard	Interferome
Polyclonal rabbit anti-mouse IFN β detection antibody	Stratech
Anti-rabbit horse radish peroxidase	Sigma-Aldrich

2.1.5 Flow Cyometry

Fluorescent-activated cell sorting (FACS) buffer: 1x PBS supplemented with 2% (v/v) heat-inactivated, filter-sterilised FCS (Biosera).

Fixation buffer: 4% Paraformaldehyde (PFA) (Santa Cruz Biotechnology), diluted 1:2 to 2% PFA in 1x PBS.

Compensation controls: Two droplets of OneComp eBeads™ (Invitrogen™) added to 1ml PBS, 200µL of this mix added to each “single-stain” compensation tube.

Table 2.3: Panel for characterising iCD103 DC phenotype

	Fluorochrome	Clone	Supplier	Volume per sample
CD11c	PE-Cy7	HL3	BD Pharmingen™	0.4µL
MHC class II	eFluor450	M5/114.15.2	eBioscience	0.3µL
F4/80	AF700	Cl:A3-1	Bio-Rad	0.2µL
B220	BV711	RA3-6B2	BD Biosciences	0.2µL
CD11b	APC-eFluor780	M1/70	eBioscience	0.075µL
CD103	PE	2E7	eBioscience	0.4µL
CD8α	APC	53-6.7	BD Biosciences	0.2µL
Live/Dead (Aqua)	BV510	N/A	BD Biosciences	0.5µL

Table 2.4: Panel for assessing DC viability and maturation

	Fluorochrome	Clone	Supplier	Volume per sample
CD11c	PE-Cy7	HL3	BD Pharmingen™	0.4µL
MHC class II	eFluor450	M5/114.15.2	eBioscience	0.3µL
CD40	APC	1C10	eBioscience	0.3µL
CD80	FITC	16-10A1	BD Pharmingen™	0.2µL
CD86	PE	GL1	eBioscience	0.1µL
CCR7	PerCP/Cy5.5	4B12	Biolegend	0.7µL
Live/Dead (Aqua)	BV510	N/A	BD Biosciences	0.5µL

Table 2.5: Panel for assessing lung and mediastinal lymph node DCs

Target	Fluorochrome	Clone	Supplier	Volume per sample
CD3	V500	500A2	BD Biosciences	0.3µL
B220	V500	RA3-6B2	BD Biosciences	0.3µL
Gr1	APC-Cy7	RB6-8C5	BD Biosciences	0.15µL
F4/80	AF700	Cl:A3-1	Bio-Rad Laboratories	0.2µL
CD11c	PE-Cy7	HL3	BD Pharmingen™	0.45µL
MHC class II	BV711	M5/114.15.2	BD Biosciences	0.25µL
CD103	PE	2E7	eBioscience	0.4µL
CD11b	BV650	M1/70	BD Biosciences	0.23µL
CD40	APC	1C10	eBioscience	0.3µL
CD80	FITC	16-10A1	BD Pharmingen™	0.2µL
CD86	Pacific Blue	GL1	Biolegend	0.35µL
Live/Dead (Aqua)	BV510	N/A	BD Biosciences	0.5µL

2.1.6 Western blot

Lysis buffer: 2x Laemmli buffer (Sigma-Aldrich) diluted 1:2 in Baxter water and containing protease and phosphatase inhibitors (both Thermo Fisher) diluted 1:100.

1.5M Tris, pH 8.8: 18.15g Tris base, 80ml Millipore water. Brought to pH 8.8 and made up to final volume of 100ml with Millipore water.

1M Tris, pH 6.8: 12.1g Tris base, 80ml Millipore water. Brought to pH 6.8 and made up to final volume of 100ml.

10% Sodium dodecyl sulfate (SDS): 5g SDS, 50ml Millipore water.

10x SDS running buffer: 144g glycine, 30g Tris base and 10g SDS. Made up to final volume of 1L with Millipore water.

Transfer buffer: 10.5g glycine, 2.25g Tris base, 1g SDS and 200ml Methanol. Made up to final volume of 1L with Millipore water.

10x Tris-buffered saline containing Tween 20 (TBS-T): 80g NaCl, 2g KCl, 30g Tris base and 10ml Tween-20. Made up to final volume of 1L with Millipore water and brought to pH 7.4.

Wash buffer: 1x TBS-T.

Blocking buffer and secondary antibody diluent: 5% milk powder (w/v) in 1x TBS-T.

Primary antibody diluent: 5% BSA (w/v) in 1x TBS-T.

Table 2.6: Resolving gel compositions (per two gels)

	8% (70-100KDa)	12% (50-70KDa)	15% (<50KDa)	16% (<20KDa)
H ₂ O	9.3ml	6.6ml	4.6ml	4.2ml
30% Acrylamide/ Bis-acrylamide	5.3ml	8ml	10ml	10.6ml
1.5M Tris pH 8.8	5ml	5ml	5ml	5ml
10% SDS	200µL	200µL	200µL	200µL
10% Ammonium persulfate	200µL	200µL	200µL	100µL
Tetramethylethylenediamine (TEMED)	12µL	8µL	8µL	10µL

Table 2.7: Stacking gel composition (per two gels)

Reagent	Volume
H ₂ O	5.5ml
30% Acrylamide/ Bis-acrylamide	1.3ml
1M Tris pH 6.8	1ml
10% SDS	80µL
10% Ammonium persulfate	80µL
Tetramethylethylenediamine (TEMED)	8µL

Table 2.8: Antibodies for western blot

Antibody	Supplier	Dilution	Diluent
Mouse anti-mouse β -actin	Cell signalling	1:1500	5% BSA in TBS-T
Rabbit anti-mouse STAT1	Cell signalling	1:1000	5% BSA in TBS-T
Rabbit anti-mouse pSTAT1 (pY701)	Cell signalling	1:1000	5% BSA in TBS-T
Secondary IRDye anti-mouse IgG (700nm)	Odyssey	1:10,000	5% milk in TBS-T
Secondary IRDye Anti-rabbit IgG (800nm)	Odyssey	1:20,000	5% milk in TBS-T

2.2 Methods

2.2.1 Mouse Neoral® and intranasal lung studies

Studies assessing the impact of CsA *in vivo* used Neoral® (Novartis), an oral CsA solution that is systemically absorbed. A summary of the treatment schedule is depicted in figure 2.1 (see next page). Briefly, Neoral® was pre-diluted in PBS to 4mg/ml CsA and C57BL/6J mice received 200 μ L by oral gavage daily for 4 days (Day 0-3). Control mice were gavaged with PBS. On Day 3, 6 hours after the final gavage, mice were anaesthetised with isoflurane and intranasally administered PBS or Zymosan, with each nostril receiving 200 μ g in a volume of 20 μ L. The same intranasal procedure was followed for studies comparing responses between WT and *Ifnar*^{-/-} mice. After 12 hours, mice were euthanised and lungs were perfused by injecting 4ml of 10mM EDTA in PBS into the right ventricle of the heart. Lung tissue and mediastinal lymph nodes were collected and minced with a sterile scissors in a 24-well plate. Following this, samples were treated with digestion mix containing 20mg/ml collagenase type

I and 100µg/ml DNase in RPMI, 1ml for each lung sample and 500µL per lymph node sample. Samples were incubated at 37⁰c with 5% CO₂ and circular rotation for 20 min. Following this, samples were disrupted by pipetting up and down for 20 seconds. This yielded a suspension that was filtered through a 100µm sterile strainer. The filter was mashed with a syringe plunger to break up remaining debris and washed with 15ml of 5mM EDTA in PBS. Filtered cells were centrifuged at 400g for 5 mins, resuspended in 5ml RPMI and lung cells counted as described in section 2.2.1.5. After counting, 5x10⁶ lung cells and all node cells were transferred to FACS tubes. Cells were analysed for surface marker expression (panel in table 2.5) by flow cytometry.

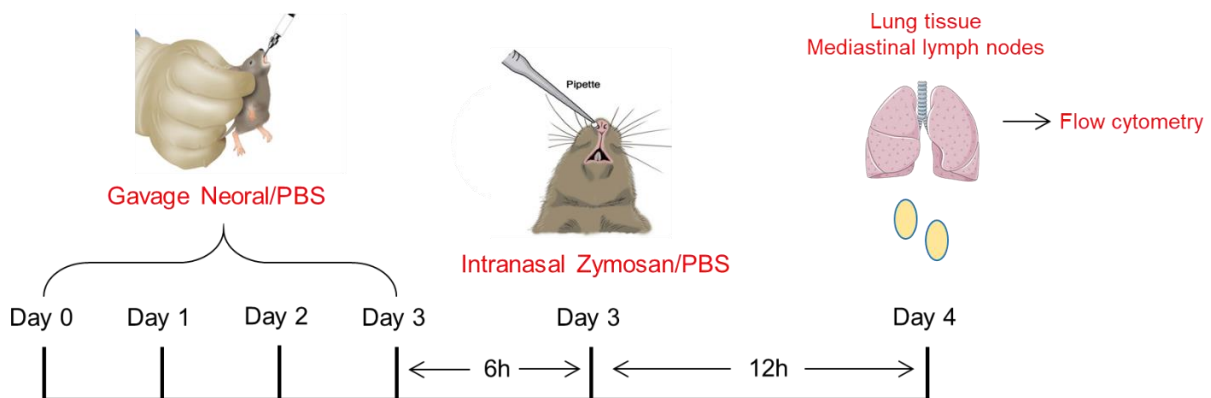


Fig. 2.1: Treatment schedule for lung DC *in vivo* studies.

2.2.1 Cell culture

2.2.1.1 CHO cell line expressing Flt3L

Flt3L was required for the culture of iCD103 DCs and obtained using a CHO cell line that had been transfected with the murine gene for Flt3L. These cells were generously provided by the Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, Medical School Hannover and the Helmholtz. For each passage described in the general protocol below, flask supernatants were removed and cells incubated briefly with trypsin-EDTA (Gibco) at 37°C until cells were detached (determined by light microscopy). Trypsin-EDTA was quenched with RPMI and detached cells were centrifuged (125 g, 10 minutes) and re-suspended in fresh RPMI before culturing in new flasks. The volumes used for each size of tissue culture flask are outlined in table 2.9 below.

Table 2.9: Volumes for CHO-Flt3L culture

Flask	Final volume of cells in RPMI	Trypsin-EDTA volume
T25	8mls	3mls
T75	25mls	8mls
T175	60mls	N/A

General CHO cell protocol:

Cells were seeded into two T25 flasks containing pre-equilibrated RPMI with 8% FCS and incubated at 37°C with 5% CO₂. After reaching ~75% confluence (~48-72 hours), cells from each T25 flask were passaged (1:3) into a T75 flask. After 48 hours, one T75 flask was used to freeze new stock vials of CHO-Flt3L cells, while the other was passaged (1:4) into two fresh T75 flasks containing RPMI with 4% FCS. When cells reached full confluence, each T75 flask was passaged (1:4) into two T75 flasks containing RPMI with 2% FCS. Next, fully

confluent T75 flasks were each passaged (1:3) into three T175 flasks containing RPMI with 2% FCS, yielding twelve T175 flasks. Cells were incubated for 7 days, until culture medium turned pale orange but remained translucent. T175 flask supernatants were removed, transferred to 50ml tubes and centrifuged at 125g for 10 minutes to pellet carryover cells. Flt3L-containing supernatants were filter-sterilised using a stericup (Millipore). The concentration of Flt3L was determined by ELISA (see section 2.2.3) and supernatants were stored at -20°C until further use.

2.2.1.2 Bone marrow-derived dendritic cells (BMDCs)

Dendritic cells were cultured from the bone marrow of C57BL/6 mice femurs and tibiae using the following method:

Muscle and fat were cut away from the bones using a sterile scissor. After separating the femurs and tibiae, the ends of each bone were excised. A 10ml syringe containing cRPMI was attached to a 27G needle and used to flush the bone marrow into a sterile petri dish. To break up aggregates, cells were passed through a 10ml syringe attached to a 19G needle. Suspended cells were then transferred through a 40µm filter into a 50ml tube and centrifuged at 400g for 5 minutes. Supernatants were decanted and re-suspended in 1ml of 0.88% ammonium chloride (ACI) for 2 minutes to lyse erythrocytes. To prevent excess lysis, 25mls cRPMI was added to the cells and the tube was mixed by inversion. Following this, cells were centrifuged as before, supernatants discarded and the pellet re-suspended in 8mls of cRPMI. Next, cells were counted as described in section 2.2.1.5, using a 1:10 trypan blue dilution. After calculating cell concentration, cells were diluted with cRPMI to a final concentration of 4.25×10^5 cells/ml and supplemented with 20ng/ml GM-CSF (Peprotech). Next, 30mls of cells were seeded into T175 flasks and incubated at 37°C with 5% CO₂.

On day 3, cells were fed by addition of 30mls of cRPMI (pre-heated to 37°C) supplemented with 20ng/ml GM-CSF.

On day 6, supernatants were gently removed to discard non-adherent cells and 30mls of fresh cRPMI (pre-heated to 37°C) supplemented with 20 ng/ml GM-CSF was added to each flask.

On day 7, cells were fed by adding 30mls of cRPMI (pre-heated to 37°C) supplemented with 20ng/ml GM-CSF.

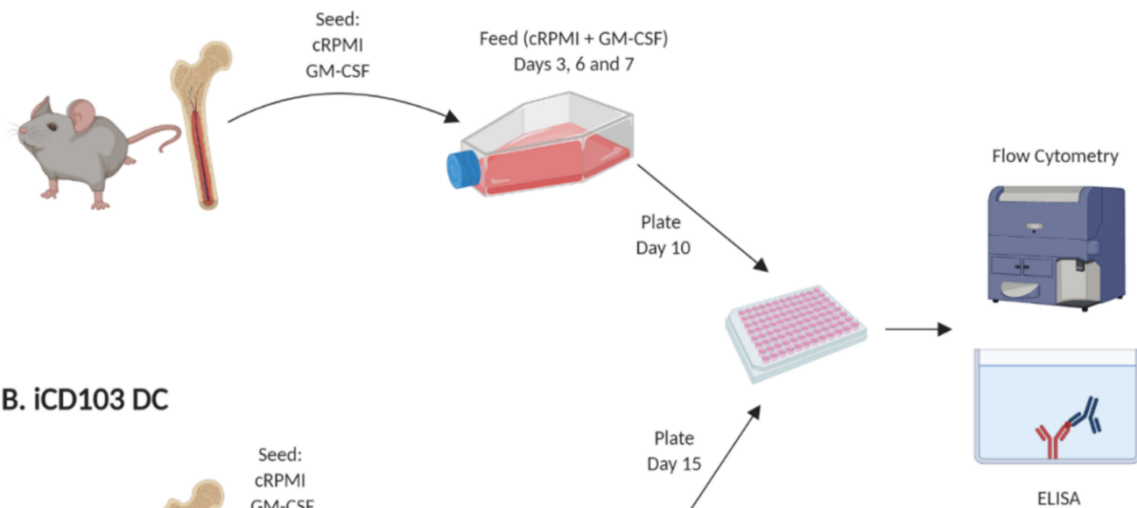
On day 10, loosely adherent cells were removed by gentle repeat pipetting of the medium and transferred to a 50ml tube. Following this, cells were centrifuged at 400g for 5 minutes, supernatants were decanted and pellets from each tube pooled in 10mls of cRPMI. Pooled cells were counted as before and adjusted to a final concentration of 6.25×10^5 cells/ml (ELISA) or 7.5×10^5 cells/ml (FACS) in cRPMI (pre-heated to 37°C) supplemented with 10ng/ml GM-CSF. 200 μ L of cells were added to each well of a 96-well plate for ELISA. 400 μ L were added to 48-well plates for FACS. After plating, cells were returned to the incubator for 2 hours and stimulated as described in figure legends. Hereafter, these cells will be referred to GM-CSF DCs.

2.2.1.3 Induced CD103 dendritic cells (iCD103 DCs)

On day 0, bone marrow cells were isolated from C57BL/6 mice as described in section 2.2.1.2. Following this, cells were adjusted to a final concentration of 1×10^6 cells/ml in CD103-RPMI containing 52.5ng/ml Flt3L (obtained from CHO-Flt3L cells) and 4ng/ml GM-CSF. Cells were seeded at a final volume of 10mls in sterile petri dishes and incubated at 37°C with 5% CO₂. On day 5 and day 7, cells were fed with 6mls CD103-RPMI. On day 9, cells were removed by gentle repeat pipetting of the medium and transferred to a 50ml tube. Following this, cells were centrifuged at 400g for 5 minutes, supernatants discarded and pellets re-suspended in 1ml CD103-RPMI per 50ml tube. Re-suspended cells from each tube were

pooled into a final volume of 5mls and counted as described in section 2.2.1.5. Cells were adjusted to 0.4×10^6 cells/ml in CD103-RPMI and supplemented with 52.5ng/ml Flt3L and 4ng/ml GM-CSF. Cells were added to fresh sterile petri dishes at a final volume of 10mls and returned to the incubator. On day 13, cells were fed with 2.5mls CD103-RPMI. On day 15, cells were harvested by gentle repeat pipetting of the medium and transferred to a 50ml tube. Immediately after, cells were centrifuged as before, supernatants discarded and pellets re-suspended and pooled as described on day 9. Pooled cells were counted as described in section 2.2.1.5 and adjusted to 6.25×10^5 cells/ml (ELISA) or 7.5×10^5 cells/ml (FACS and western blot) in CD103-RPMI. All media was supplemented with 52.5ng/ml Flt3L and 4ng/ml GM-CSF. 200 μ L of cells were added to each well of a 96-well plate for ELISA, while 400 μ L were added to 48-well plates for FACS and western blot. After plating, cells were allowed to settle for 2 hours in the incubator. Cells were stimulated as described in data figure legends. See figure 2.2 for illustrative summary of DC protocols.

A. GM-CSF DC



B. iCD103 DC

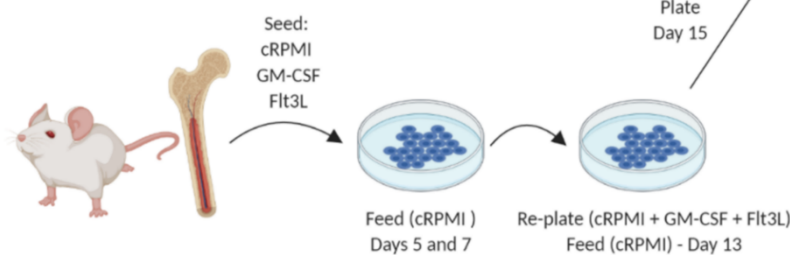


Figure 2.2: GM-CSF DC and iCD103 DC culture protocols.

2.2.1.4 Transwell migration assay

1x10⁶ iCD103 DCs were seeded into a 24-well plate in a volume of 800µL. Cells were pre-treated with 100µL CsA or medium for 1h. Following this, cells were stimulated with 100µL medium or Zymosan overnight, yielding a final volume of 1ml and cell concentration of 1x10⁶ cells/ml. Supernatants from each well were transferred into a 15ml tube. Each well was rinsed with 1ml medium containing 2.5mM EDTA and placed on ice for 2mins to facilitate detachment. After gentle repeat pipetting, cells were added into the corresponding 15ml tube. Cells were centrifuged at 400g for 5mins, supernatants discarded and cells resuspended with the leftover volume in the tube. Specialised 24-well plates and transwell inserts (both Corning®) were used to assess migration. CCL19 (R&D Systems) was diluted to a concentration of 200ng/ml in medium and 600µL was added to each well. Transwells were inserted and re-suspended cells were pipetted onto the top of the transwell. A spontaneous migration control was included, where only medium (no CCL19) was added below the transwell, along with medium-treated cells above the transwell. Cells were incubated for 3 hours at 37°C. Transwells were removed and light micrographs were obtained. 4µL of stock EDTA (500mM) was added to each well, yielding a final concentration of ~3.33mM and cells placed on ice for 3mins to facilitate detachment. After gentle repeat pipetting, cells were transferred into new 15ml tubes. Using a 1:2 dilution in trypan blue (100µL cells, 100µL trypan blue), cells were counted as described in section 2.2.1.5, yielding a value for cells/ml. The number of migrated cells from each sample was calculated by multiplying the cells/ml value by 0.6 (600µL in the lower chamber), followed by subtraction of the spontaneous control count. See figure 2.3 below for illustrative summary of assay.

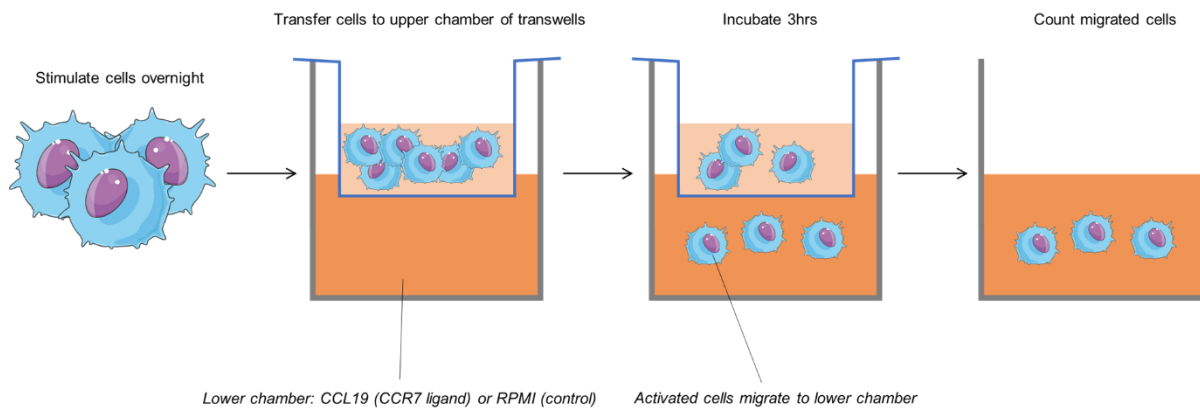
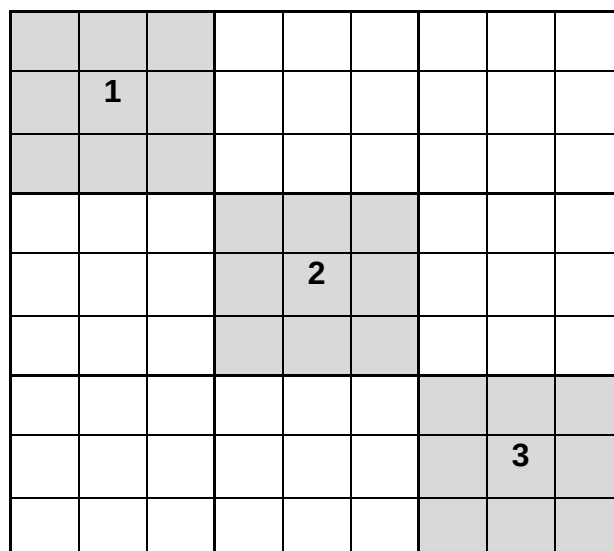


Figure 2.3: CCL19 transwell migration assay.

2.2.1.5 Cell counting

Cells were diluted in Trypan Blue (Sigma-Aldrich). 10µl of trypan blue-stained cells were transferred to a haemocytometer and viewed under the x10 objective lens of a light microscope. Cell viability was indicated by dye exclusion. As depicted in figure 2.4 below, three live cell counts were obtained and the average count used to calculate cell concentration (cells/ml).



$$\text{Cells/ml} = \frac{\text{Cell count}}{3} \times \text{dilution factor} \times 10^4$$

Figure 2.4: Cell counting on haemocytometer and formula for calculating concentration.

2.2.2 Lactate dehydrogenase (LDH) assay

Following *in vitro* stimulation of DCs, cell death was measured using a Pierce LDH cytotoxicity assay kit (Thermo Scientific). During cell death, cells release LDH, which forms NADH by reduction of NAD⁺ derived from the kit reaction mixture. In turn, this mixture converts NADH to red formazan, allowing absorbance values to be obtained for each sample at 492nm. These values are proportional to LDH release and hence cytotoxicity.

LDH assay protocol:

After iCD103 DCs were plated, spontaneous cell death controls were prepared by adding 10µL of sterile baxter water to a triplicate of wells containing unstimulated cells. Cells were then incubated at 37°C with 5% CO₂ for 24 hours. Following this, the maximum cell death controls were prepared by adding 25µL of lysis buffer from the kit to triplicate wells. Cells were returned to the incubator for 45 minutes. Next, plates were centrifuged at 400 g for 5 minutes and 50µL of supernatants were transferred to a 96-well medium-binding ELISA plate. Immediately after, 50µL of the kit reaction mixture was added to each sample and plates were incubated at room temperature for 30 minutes in darkness. Following this, 50µL of stop solution was added to each well and plates were read as described in section 2.2.4. The % cytotoxicity was calculated for each iCD103 DC sample, by inserting absorbance values into the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{Sample} - \text{Spontaneous control}}{\text{Maximum control} - \text{Spontaneous control}} \times 100$$

2.2.3 ELISA

Following stimulation, DC cytokine secretion was measured using the ELISA kits outlined in table 2.2. ELISA kit reagents and dilutions are described in tables 2.10 (Biolegend), 2.11 (R&D Systems) and 2.11 (IFN β) below.

Table 2.10: Biolegend ELISA reagents and dilutions

	IL-6
Capture Antibody	1:200, SCB
Block Buffer	1% BSA
Assay Diluent	1% BSA
Top Standard	500 pg/ml
Detection Antibody	1:200, assay diluent
Avidin-HRP	1:1000, assay diluent
Substrate Solution	OPD
Stop solution	1M H ₂ SO ₄

Table 2.11: R&D ELISA reagents and dilutions

	TNFα	Flt3L	IL-2	IL-12p70	IL-23	IL-1β
Capture and Detection Antibodies	Lot-specific, 1x PBS (capture) or assay diluent (detection)					
Block Buffer	1% BSA	1% BSA	1% BSA	1% BSA	1% BSA	1% BSA
Assay Diluent	1% BSA	1% BSA	TBS-BSA-Tween	1% BSA	1% BSA	1% BSA
Top Standard	2000 pg/ml	800 pg/ml	1000 pg/ml	2500 pg/ml	2500 pg/ml	1000 pg/ml
Avidin-HRP	1:40	1:40	1:40	1:40	1:40	1:40
Substrate Solution	TMB	TMB	TMB	TMB	TMB	OPD
Stop solution	1M H ₂ SO ₄	1M H ₂ SO ₄	1M H ₂ SO ₄	1M H ₂ SO ₄	1M H ₂ SO ₄	1M H ₂ SO ₄

Table 2.12: IFN β ELISA reagents and dilutions

Reagent	Dilution
Capture Antibody	1:1000, SCB
Block Buffer, assay diluent	10% FCS in PBS
Standard	Top concentration 10U/ml
Detection Antibody	1:3000, assay diluent
Streptavidin-HRP	1:2000, assay diluent
Substrate Solution	TMB
Stop solution	1M H ₂ SO ₄

ELISA protocol:

96-well high binding ELISA plates were coated with 50 μ l per well of diluted capture antibody and incubated overnight at 4°C (Biolegend and IFN β) or room temperature (R&D systems). Plates were washed 4 times in 0.05% PBS-Tween and blotted dry on clean paper towels. Following this, 185 μ l of blocking buffer was added to each well to prevent non-specific binding and plates were incubated for 90 minutes at room temperature (Biolegend and R&D). For IFN β , 100 μ l of block buffer was added and plates were incubated for 2h at 37°C. Plates were washed and dried as before. Next, 50 μ l of samples (neat or pre-diluted) were added to the desired wells. Meanwhile, a standard curve was obtained by performing a duplicate serial dilution on recombinant cytokines of known concentrations. In addition, a blank control containing assay diluent was used. After addition of samples and standards, plates were incubated for 2 hours at room temperature (Biolegend and R&D) or overnight at 4°C (IFN β). Plates were washed and dried as before and 50 μ l of diluted detection antibody was added to each well. Plates were incubated for 1 hour (Biolegend), 90 minutes (IFN β) or 2 hours (R&D) at room temperature. Plates were washed and dried as before and 50 μ l of diluted Avidin-HRP solution was added to each well. Plates were incubated away from direct light for 20 minutes

(R&D), 30 minutes (Biolegend) or 90 minutes (IFN β) at room temperature. Following this, plates were washed 5 times. For the final 2 washes, plates were submerged in wash buffer for 30 seconds and then dried as before. Substrate solutions were prepared as described in section 2.1.4 and 50 μ l was added to each well. Plates were left to develop at room temperature away from direct light. Reactions were stopped by adding 50 μ l of 1M H₂SO₄ and plates were read as described in section 2.2.4.

2.2.4 ELISA and LDH assay plate reading

Plates were read on a Multiskan FC plate-reader (Thermo Scientific) using Skanit Software 1.5.1. Absorbance values were determined at 492nm for OPD substrate and LDH assays and 450nm for TMB substrate. For ELISA, sample concentrations were determined by comparing optical density values with the standard curve.

2.2.5. Flow Cytometry

Spectral overlap was eliminated using compensation beads (Invitrogen™) stained with a single antibody and control cells incubated in live/dead stain. Fluorescence-minus-one (FMOs) controls were used to distinguish positive and negative populations. Samples were acquired using FACS Diva and analysed on FlowJo software. During analysis, debris was distinguished as low forward scatter area (FSC-A) and side scatter area (SSC-A) dot plots. Single cells were distinguished by plotting FSC-A versus forward scatter width (FSC-W). Next, live cells were identified by analysing FSC-A versus live/dead stain fluorescence and analysed for expression of surface markers.

2.2.5.1 DC viability and maturation staining

Following DC stimulation, plates were centrifuged (400g, 5 minutes) and supernatants discarded. Cells were re-suspended with cold PBS and incubated on ice for 5 minutes to facilitate cell detachment. Next, cells were transferred into FACS tubes, washed with 2mls cold PBS and centrifuged as before. After discarding supernatants, cells were re-suspended by brief vortex and stained with 250µL PBS containing Aqua live/dead (0.5µL/sample). Cells were incubated on ice for 30 minutes in darkness. Next, cells were washed in 2mls PBS, centrifuged (400g, 5 minutes) and re-suspended in 100µL FACS buffer containing purified Fc block (0.5µL/sample), to prevent non-specific antibody binding. After a 10-minute incubation on ice in the dark, cells were stained with fluorochrome-labelled antibodies targeting CD11c and the maturation markers CD40, CD80, CD86, MHC class II and CCR7 (see table 2.4). Cells were incubated on ice for 30 minutes in darkness. Following this, cells were washed with 2mls PBS and centrifuged (400 g, 5 minutes). Supernatants were decanted and cells were re-suspended by brief vortex and fixed by addition of 200µL 2% PFA in PBS. Cells were incubated on ice for 20 minutes in darkness. Following this, cells were centrifuged as before, supernatants removed and cells re-suspended in 250µL FACS buffer. Samples were analysed for immunofluorescence using a BD FACS Canto II.

2.2.5.2 Lung tissue and mediastinal lymph node staining

Samples were processed into single-cell suspensions and transferred into FACS tubes as described in section 2.2.1. Cells were washed with 2mls cold PBS and centrifuged at 400g for 5 mins. After discarding supernatants, cells were re-suspended by brief vortex and stained with 250µL PBS containing Aqua live/dead (0.5µL/sample). Cells were incubated on ice for 30 minutes in darkness. Next, cells were washed in 2mls PBS, centrifuged (400g, 5 minutes) and re-suspended in 50µL FACS buffer (with 5mM EDTA) containing purified Fc block (0.5µL/sample), to prevent non-specific antibody binding. After a 10-minute incubation on ice

in the dark, cells were stained with fluorochrome-labelled antibodies targeting CD3, B220, Gr1, F4/80, CD11c, MHCII, CD103, CD11b, CD40, CD80 and CD86 (see table 2.5). Cells were incubated on ice for 30 minutes in darkness. Following this, cells were washed with 2mls PBS and centrifuged (400 g, 5 minutes). Supernatants were decanted and cells were re-suspended by brief vortex and fixed by addition of 200 μ L of 2% PFA. Cells were again washed as before and resuspended in 250 μ L of FACS buffer. Samples were analysed for immunofluorescence using a BD LSR Fortessa.

2.2.5.3 Mitosox staining for mitochondrial superoxide

Stimulated DCs were transferred from plates to FACS tubes as described in section 2.2.5.1. Cells were washed with 2ml cold PBS and centrifuged (400g, 5 minutes). After discarding supernatants, cells were re-suspended by brief vortex and co-stained with 250 μ L PBS containing Aqua live/dead (0.5 μ L/sample) and 250 μ L PBS containing Mitosox Red (0.1 μ L/sample). Cells were incubated at room temperature for 20 minutes in darkness. Following this, cells were washed with 2ml PBS, centrifuged (400g, 5 mins) and supernatants were discarded. After brief vortex, this wash was repeated and cells were re-suspended in 200 μ L FACS buffer. Without fixing, samples were immediately analysed for immunofluorescence using a BD FACS Canto II.

2.2.6. Western Blot

iCD103 DCs (7.5×10^5 cells/ml) were plated in 400 μ L cRPMI supplemented with 4ng/ml GM-CSF and 52.5ng/ml Flt3L in a 48-well round bottom tissue culture plate. After 2-hour incubation, cells were stimulated as described in figure legends. Cells were centrifuged at 400g for 5 minutes, supernatants removed and cells were lysed with 80 μ L Laemmli buffer pre-diluted 1:2 in sterile water and containing protease and phosphatase inhibitors (both Thermo

Fisher) diluted 1:100. After 5 minutes and a thorough vortex, lysates were transferred to Eppendorf tubes, boiled at 95°C for 5 minutes and stored at -20°C until further use.

Resolving and stacking gels were prepared in glass plates (Bio-Rad Laboratories) as described in tables 2.6 and 2.7. Once set, gels were wrapped in tissue pre-soaked with running buffer, sealed with cling film and stored at 4°C until further use. Gels were assembled in an SDS cassette (Bio-Rad Laboratories). SDS running buffer was poured into the central column, combs were removed and 5µL PageRuler™ pre-stained protein ladder (Thermo Scientific) was loaded into the left lane. 30µL of samples were loaded to each lane and gels were run at 80 volts, on a PowerStation 200 powerpack, until the dye front reached the end of the stacking gel. Following this, the remainder of the gel was run at 100 volts.

Proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane (Immobilon®) using a semi-dry transfer apparatus (Biometra). Initially, gels were removed from the glass plates and submerged in transfer buffer for 10 minutes. The PVDF membrane was activated in methanol by submerging for 40 seconds, washed in sterile water for 10 seconds and submerged in transfer buffer for 5 minutes. The PVDF membrane was placed onto a filter pad (Thermo Fisher), which had been pre-soaked in transfer buffer and the gel was carefully placed over the PVDF membrane. A second soaked filter pad was placed on top of the gel and air bubbles were removed gently with a roller. Proteins were transferred at 90mA per gel for 90 minutes.

After the transfer, the PVDF membrane was placed into a 50ml tube. Next, the membrane was incubated with 10ml blocking buffer (5% milk in TBS-T) for 1 hour at room temperature, while rotating on a tube roller. Blocking buffer was removed and membranes washed once with 20ml wash buffer for 2 minutes. The membrane was incubated on a tube roller in 3ml 5% BSA in TBS-T containing primary antibody overnight at 4°C. For β-actin primary antibody, the incubation was 1h at room temperature. Membranes were washed 3 times as

before for 5 minutes and incubated with 3ml dye-conjugated (700nm or 800nm) secondary antibody pre-diluted in blocking buffer at room temperature for 1 hour with rotation. The membrane was washed 3 times as before and target protein detected using a Licor Odyssey Infrared imaging system. Finally, blots were analysed using Image Studio Lite Version 5.2.

2.2.7. Statistical Analysis

Data were analysed using GraphPad Prism software. Mean values for two groups were compared by unpaired student's t-test. One-way ANOVA and Tukey multiple comparison tests were performed to compare the means of three or more groups. Bars graphs are depicted as mean $\pm$ standard deviation (SD).

Chapter 3: Results (i)

CsA Impairs Antifungal Responses in CD103⁺ Dendritic cells

3.1 Introduction, hypothesis and aims

CsA is a highly effective immunosuppressant with clinical applications for transplantation and inflammatory diseases including RA, SLE, colitis and psoriasis [321, 322, 324, 325, 441]. As discussed, a significant drawback of this drug is increased susceptibility to severe fungal infections [366, 368, 391, 393-401]. CsA was initially found to target T cells by inhibiting the phosphatase calcineurin, which activates NFAT, a transcription factor that upregulates proliferative genes such as IL-2 [323, 328]. Therefore, CsA inhibiting IL-2 expression reduces the overall magnitude of T cell responses. These include Th1 and Th17 cells, which contribute to antifungal immunity by secreting IFN γ and IL-17 respectively. Indeed, these cytokines are key for the mobilisation and activation of neutrophils, as well as activation of macrophages and DCs. During inflammatory disorders, this will allow CsA to effectively reduce tissue damage [4, 442]. However, as discussed in section 1.8, Th1 and Th17 cell responses are essential for antifungal immunity, highlighting that inhibition of T cell proliferation by CsA will increase the risk of severe fungal infections.

In addition to T cells, CsA can directly target DCs. These cells are well-known to protect against fungal infections, by migrating to draining lymph nodes, presenting antigen to T cells, secreting cytokines and upregulating co-stimulatory molecules [301]. DC stimulation with PAMPs, such as Zymosan and LPS, triggers the calcineurin-NFAT pathway, which is known to influence the expression of antifungal genes in DCs, such as IL-2 and IL-12 [130, 327, 334, 356]. In addition, CsA can prevent the release of danger-associated molecular patterns (DAMPs) from the mitochondria that trigger multiple signalling pathways in innate immune cells [3]. Furthermore, one study suggested that CsA impairs nuclear translocation of NF κ B in GM-CSF-generated BMDCs [357]. Importantly, targeting both innate and adaptive immune cells, through multiple mechanisms, helps to explain why CsA is highly effective against inflammatory diseases but also increases the risk of opportunistic fungal infections.

Despite the emerging impact of CsA on innate immunity, previous studies have failed to address whether CsA can directly modulate CD103⁺ DCs. As discussed, these cells have recently been highlighted as key mediators of protection against fungal infections [152, 232, 244, 248]. Therefore, CsA modulation of antifungal responses in this DC subset may underly a novel mechanism of susceptibility to fungal infection. As such, it will be essential to characterise the impact of CsA on antifungal CD103⁺ DC responses and, subsequently, investigate new mechanisms by which this occurs. This will provide further insight into why CsA patients are more susceptible to severe fungal infections than healthy individuals. The overall hypothesis underlying this research is that CsA inhibits Zymosan-induced maturation in CD103⁺ DCs, as well as cytokine responses, such as IL-2, IL-12, TNF α , IL-6, IL-23 and IL-1 β secretion. Therefore, this chapter will address the following aims:

1. Investigate whether Neoral® impacts lung CD103⁺ DC responses to intranasal Zymosan challenge.
2. Optimise an iCD103 DC culture model in the lab.
3. Determine the impact of CsA on Zymosan-induced iCD103 DC cytokine production and maturation marker expression.

3.2 Oral CsA (Neoral®) treatment does not significantly impact neutrophil recruitment or DC activation in the lungs after intranasal Zymosan challenge

Initially, it was important to assess whether CsA impacted CD103⁺ DC reactivity to fungal PAMPs in an *in vivo* model. Immune responses in the lungs, a key site of fungal infection were assessed, upon intranasal administration of Zymosan. These studies also analysed lung-draining mediastinal lymph nodes, a key site of T cell priming during respiratory infection. These studies used Neoral®, an orally-administered solution that leads to systemic absorption of CsA into the circulation (<https://www.medicines.ie/medicines/neoral-100mg-ml-concentrate-for-oral-solution-33001/spc>). Key parameters for these studies included assessment of the total number of CD103⁺ DCs in lung tissue and draining lymph nodes, which provides an indication of DC trafficking. In addition, cells were assessed for expression of the key co-stimulatory molecules CD40, CD80 and CD86.

Neoral® was diluted to 4mg/ml CsA in PBS and C57BL/6J mice received 0.8mg by oral gavage, in a volume of 200µL. This treatment was repeated daily for 4 days to allow CsA to accumulate in systemic organs. In addition, unpublished work in the Lavelle lab found that CsA peaks in mice serum 6h after treating with Neoral®. Therefore, 6h after the last treatment, mice were anaesthetised and treated intranasally with 400µg Zymosan in a total volume of 40µL (20µL per nostril). This relatively high volume enables the dose to reach the lower respiratory tract [443]. After 12h, lung and mediastinal lymph nodes were isolated and analysed by flow cytometry.

As seen in Fig. 3.1, single cells were gated to exclude dead cells (Aqua-BV510), T cells (CD3) and B cells and plasmacytoid DCs (both B220). Following this, neutrophils were gated as Gr1⁺F4/80⁻. In addition, DCs were gated as F4/80⁻Gr1⁻CD11c⁺MHCII^{hi}. Next, DCs were

divided into CD103⁺ and CD11b⁺ subsets. Finally, total DCs and each DC subset were gated for CD40, CD80 and CD86 expression (Fig. 3.1).

There was no significant impact of Zymosan or Neoral® treatment on total lung cell numbers (Fig 3.2 A). Neoral® marginally reduced the number of neutrophils in the lungs but did not significantly impact the number of DCs, CD103⁺ DCs or CD11b⁺ DCs in Zymosan-treated mice. (Fig. 3.2 B - Fig. 3.3 A-C). Neoral® increased the number of total DCs positive for the co-stimulatory molecules CD40, CD80 and CD86 compared to untreated mice, however, the MFI values for these markers were not significantly affected (Fig. 3.4 A-B). In mice treated with Zymosan or Neoral® plus Zymosan, there was no significant impact on total DCs numbers expressing CD40, CD80 or CD86 or MFI values (Fig. 3.4 A-B). Similarly, Neoral® increased the number of CD103⁺ DCs expressing CD80 and CD86 compared to the steady-state, while MFI values were unchanged (Fig 3.5 A-B). In Zymosan-treated mice, Neoral® reduced the number of CD103⁺ DCs expressing CD40, CD80 and CD86 (Fig. 3.5 A) and this was associated with a marginal reduction in MFI values for CD80 and CD86 (Fig. 3.5 B). For CD11b⁺ DCs, Neoral® increased the number of cells positive for CD40 and CD86 compared to the steady-state (Fig. 3.6 A), while the MFI values for CD40 were reduced (Fig. 3.6 B). In Zymosan-treated mice, Neoral® marginally reduced the number of CD11b⁺ DCs expressing CD80 and CD86, while no significant reduction in MFI values were observed (Fig. 3.6 A-B).

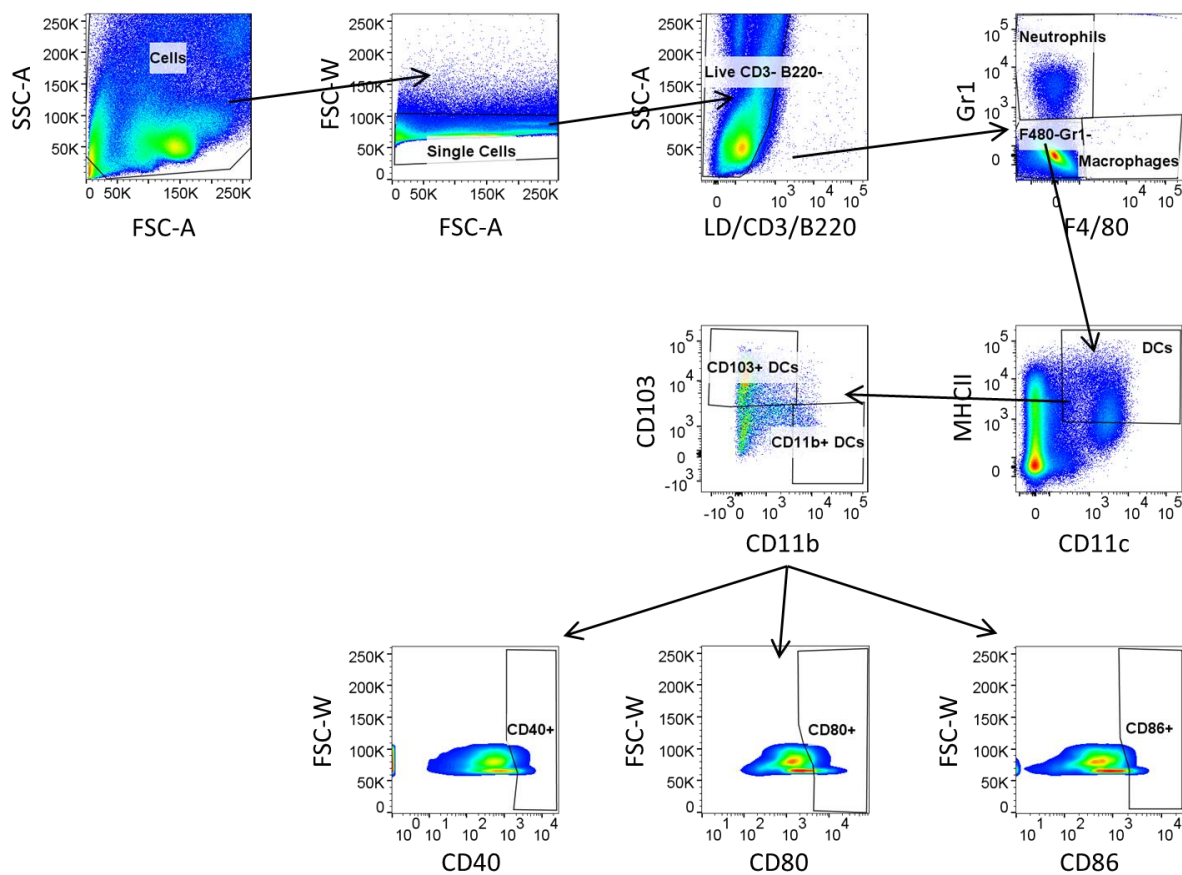
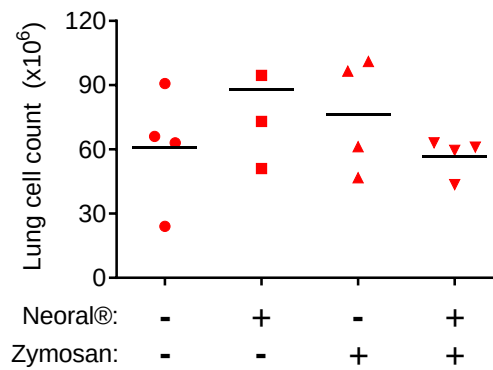


Fig 3.1: Gating strategy for immune cells in the lungs and mediastinal lymph nodes. Single-cell suspensions from C57BL/6J mouse lung tissue and mediastinal lymph nodes were prepared following Neoral® and Zymosan treatment. Cells were stained with Aqua-BV510 viability dye and antibodies for CD3, B220, Gr1, F4/80, CD11c, MHCII, CD103, CD11b, CD40, CD80 and CD86. Cells were acquired on a BD LSRFortessa cytometer.

(A)



(B)

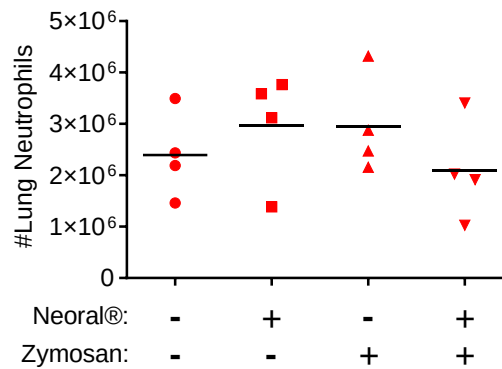


Fig 3.2: Neoral® does not significantly impact Zymosan-induced cellular influx to the lungs. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. (A) Trypan blue staining and light microscopy were used to obtain total cell counts. Following this, 5x10⁶ cells were stained for flow cytometry. (B) Neutrophils were gated as live CD3⁻B220⁻Gr1⁺F4/80⁺. For each sample, the number of neutrophils was divided by the number of single cells and subsequently multiplied by the corresponding total lung cell count *, p<0.05; One-way ANOVA, Tukey's multiple comparison test.

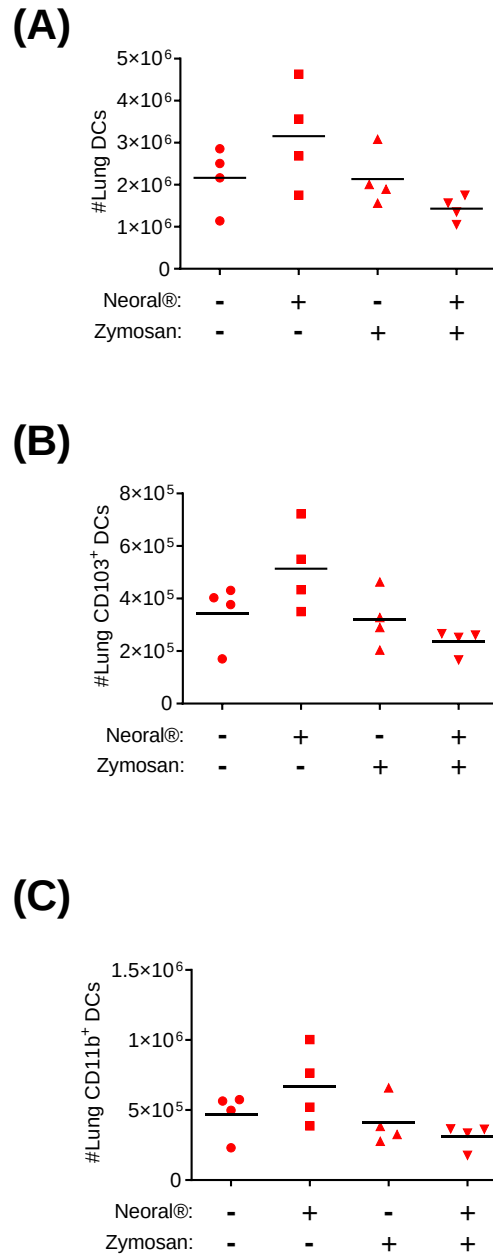
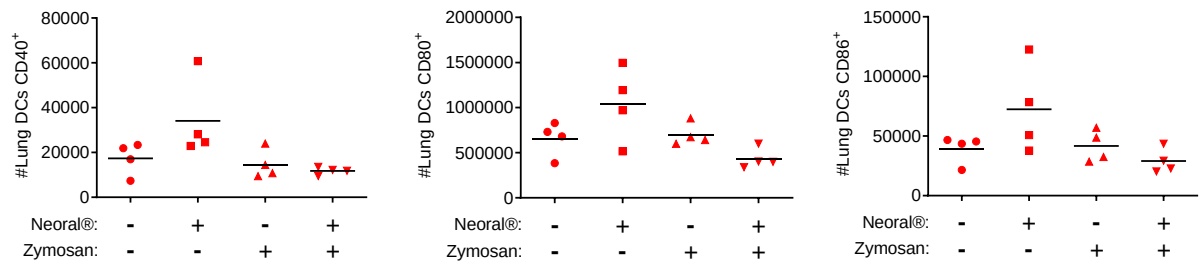


Fig 3.3: Lung DC numbers in Zymosan-treated mice are not significantly affected by Neoral®.

Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5×10^6 cells were stained for flow cytometry. (A) Total DCs were gated as live $CD3^- B220^- Gr1^+ F4/80^- CD11c^+ MHCII^{hi}$. (B) $CD103^+$ DCs were gated as live $CD3^- B220^- Gr1^+ F4/80^- CD11c^+ MHCII^{hi} CD103^+$. (C) $CD11b^+$ DCs were gated as live $CD3^- B220^- Gr1^+ F4/80^- CD11c^+ MHCII^{hi} CD11b^+$. For each sample, the number of total DCs (A), $CD103^+$ DCs (B) or $CD11b^+$ DCs (C) were divided by the number of single cells and subsequently multiplied by the corresponding total lung cell count (Fig. 3.2). *, $p < 0.05$; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group (n=4).

(A)



(B)

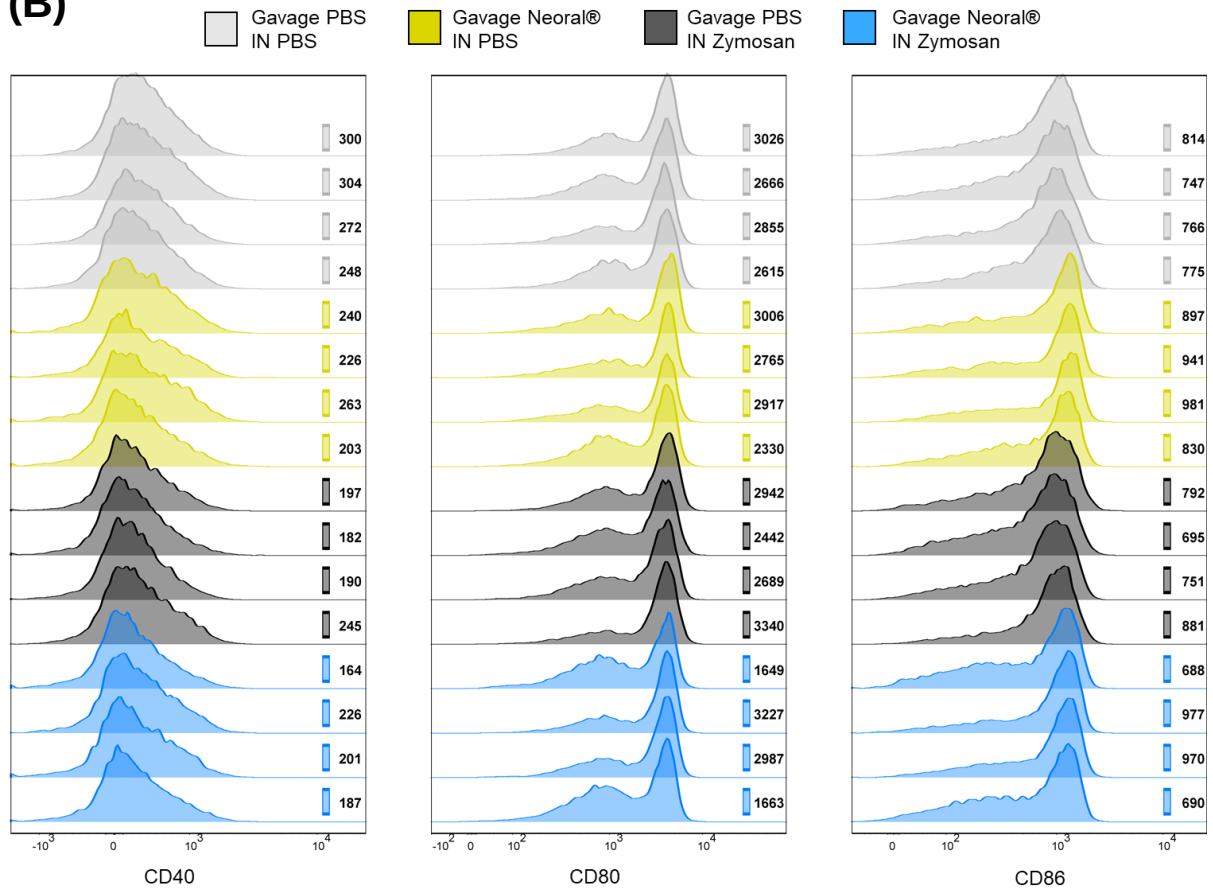
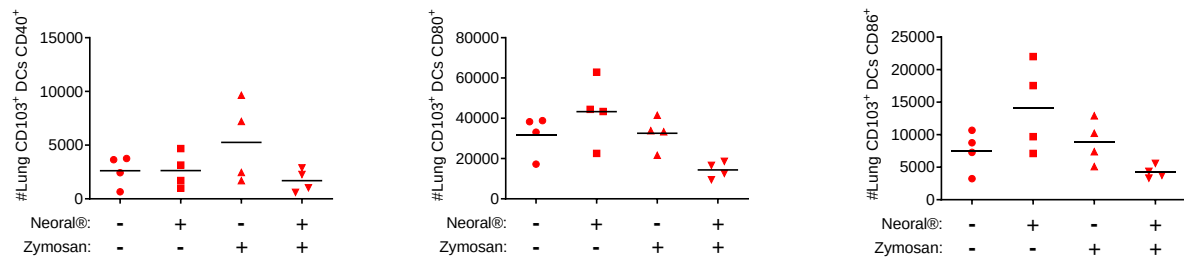


Fig 3.4: Neoral® did not significantly reduce total DC expression of CD40, CD80 and CD86 in the lungs of Zymosan-treated mice. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5×10^6 cells were stained for flow cytometry analysis. DCs were gated as live CD3-B220-Gr1-F4/80-CD11c⁺MHCII^{hi}. (A) Number of DCs positive for CD40, CD80 and CD86 were divided by the corresponding number of single cells and multiplied by total lung cell counts. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). Horizontal bars are mean for each group (n=4).

(A)



(B)

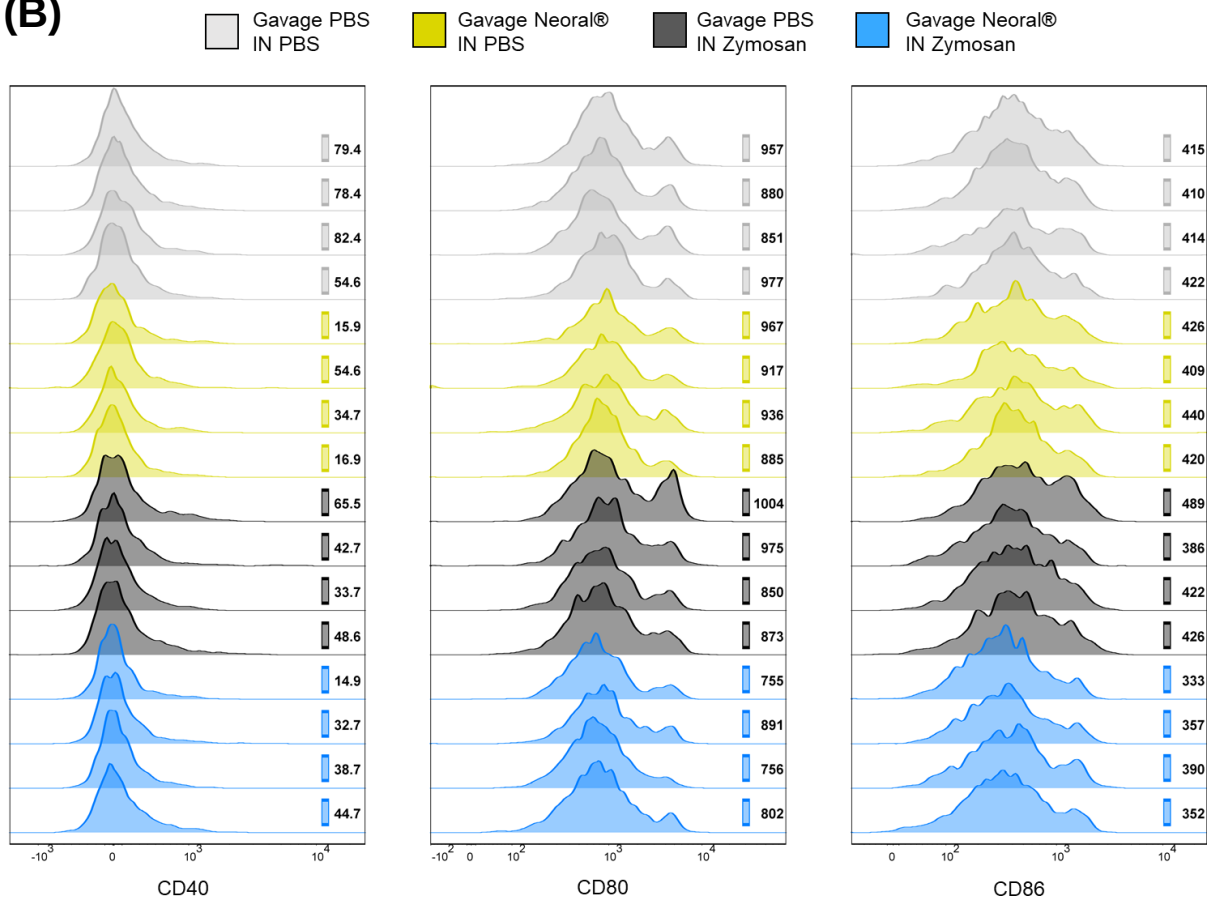
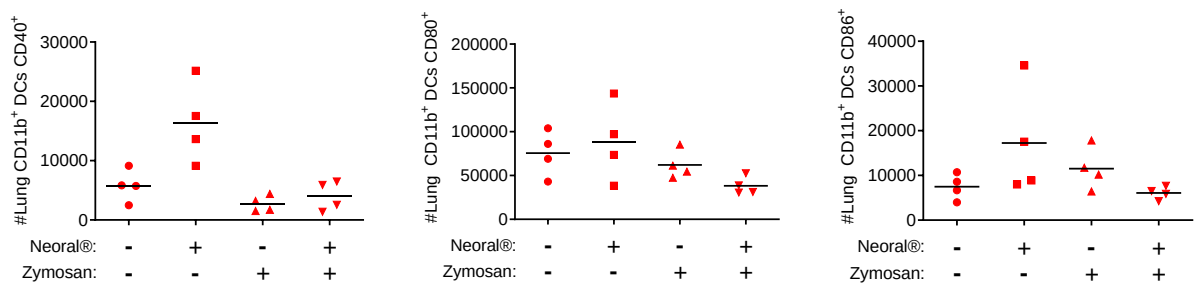


Fig 3.5: Neoral® has no significant impact on CD103⁺ DC expression of CD40, CD80 and CD86 in the lungs of Zymosan-treated mice. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5×10^6 cells were stained for flow cytometry analysis. CD103⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺. (A) Number of CD103⁺ DCs positive for CD40, CD80 and CD86 were divided by the corresponding number of single cells and multiplied by total lung cell counts. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). Horizontal bars are mean for each group (n=4).

(A)



(B)

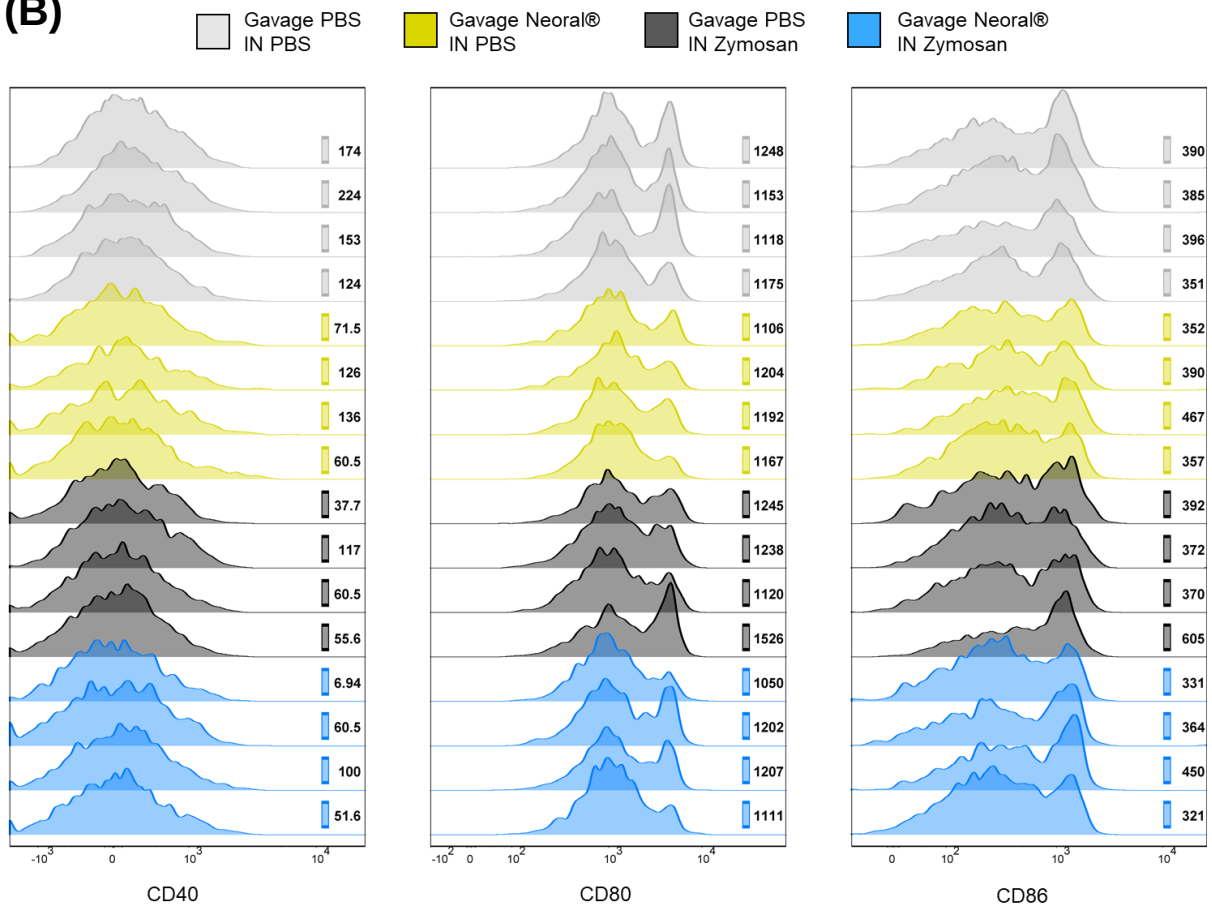


Fig 3.6: Neoral® treatment does not significantly affect CD11b⁺ DC expression of CD40, CD80 and CD86 in the lungs of Zymosan-treated mice. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5×10^6 cells were stained for flow cytometry analysis. CD11b⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. (A) Number of CD11b⁺ DCs positive for CD40, CD80 and CD86 were divided by the corresponding number of single cells and multiplied by total lung cell counts. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). Horizontal bars are mean for each group (n=4).

3.3 Neoral® treatment impairs DC trafficking to lung-draining lymph nodes after intranasal Zymosan challenge

In addition to the lungs, it was important to analyse the impact of Neoral® on immune cell populations in local lung-draining mediastinal lymph nodes, as these are a key site of T cell priming during respiratory infections. Indeed, during intranasal infection, for example with *Cryptococcus neoformans*, DCs uptake fungal spores, become activated in the lungs and subsequently complete the key process of migrating to the mediastinal lymph nodes [444]. Intriguingly, DCs migrate from the lungs to the draining lymph nodes in the steady-state, while activation accelerates this process [445]. One study found that DCs labelled in the lungs rapidly migrate to the draining lymph nodes within 6h, peak at 24h and wane over the following 4 days [446]. Therefore, lung DC migration is dynamic and localisation is constantly in flux. To account for this, the same lung *in vivo* studies described in the previous section also included isolation of mediastinal lymph nodes 12h after intranasal Zymosan challenge, for analysis by flow cytometry.

Interestingly, Zymosan treatment significantly increased the total number of DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}) in the nodes and this was significantly decreased by treatment with Neoral® (Fig. 3.7 A-B). Crucially, there were significantly higher numbers of CD103⁺ DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺) in the nodes of Zymosan-treated mice but this was decreased towards baseline levels by Neoral® (Fig. 3.8 A, C). Regarding CD11b⁺ DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺), lower cell number were detected compared to CD103⁺ DCs, however, Zymosan treatment triggered a marked increase in the mediastinal lymph nodes and this was markedly reduced by Neoral® (Fig. 3.8 B-C).

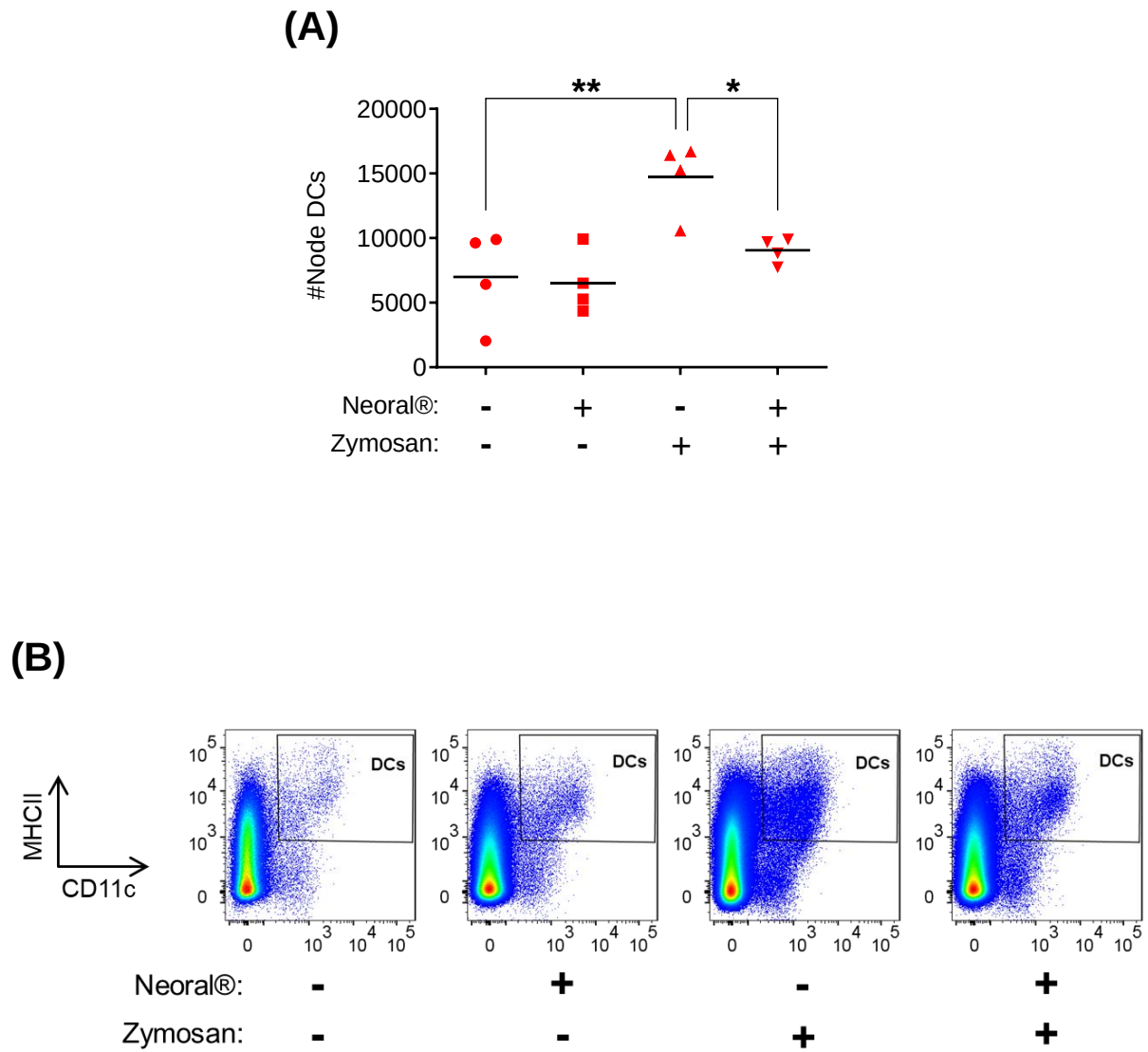


Fig 3.7: Neoral® significantly impairs DC trafficking to mediastinal lymph nodes during intranasal Zymosan challenge. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}. (A) Total lymph node DC counts. (B) Representative DC dot plots. *, $p < 0.05$; **, $p < 0.01$; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group ($n=4$).

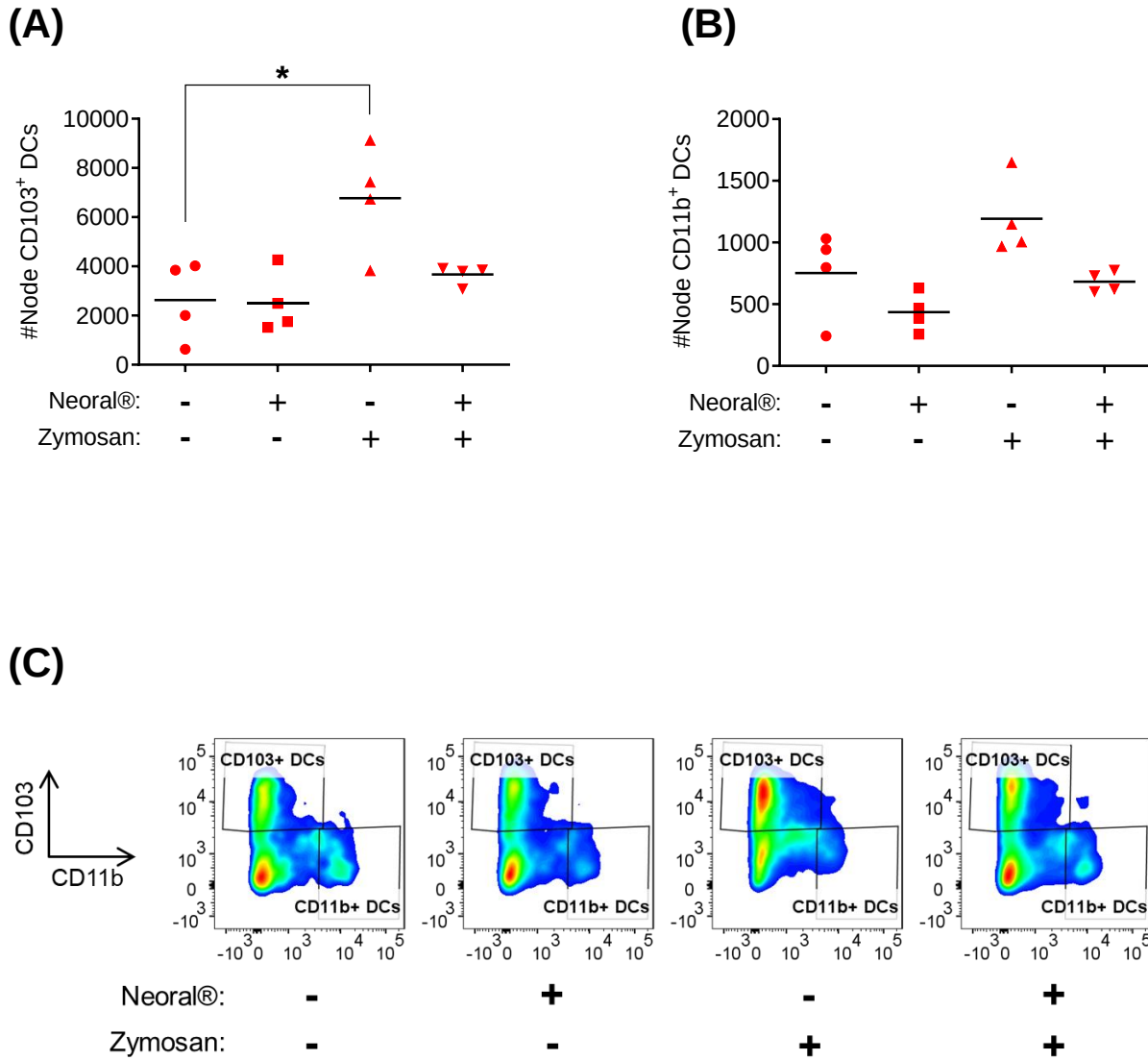


Fig 3.8: Neoral® reduces CD103⁺ DC trafficking to mediastinal lymph nodes during intranasal Zymosan challenge. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. CD103⁺ DCs (A, C) were gated as live CD3⁺B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺ cells, while CD11b⁺ DCs (B, C) were gated as live CD3⁺B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. (A) Total lymph node CD103⁺ DC counts. (B) Total lymph node CD11b⁺ DC counts. (C) Representative CD103⁺ and CD11b⁺ DC dot plots. *, $p < 0.05$; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group (n=4).

3.4 Oral CsA (Neoral®) treatment reduces the expression of CD40, CD80 and CD86 on DCs in mediastinal lymph nodes after intranasal Zymosan challenge

As previously stated, DC depletion studies highlight that these cells are essential for priming protective T cell responses during fungal infection [16]. Upon recognition of fungal PAMPs, an essential mechanism of T cell priming is the upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 on DCs, which directly interact with T cell co-stimulatory receptors, including CD40L and CD28 [4, 290]. Therefore, it was important to determine whether Neoral® was modulating the expression of co-stimulatory molecules on DCs trafficking to the mediastinal lymph nodes during intranasal Zymosan challenge. Indeed, such a finding would be highly suggestive of impaired antifungal immune responses and susceptibility to infection. Thus, DC populations in the mediastinal lymph nodes were analysed for surface expression of CD40, CD80 and CD86 by flow cytometry.

Taking the lymph node DC population as a whole (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}), Neoral® significantly reduced the number of CD40⁺ cells observed after Zymosan treatment in the mediastinal lymph nodes (Fig. 3.9 A). Intriguingly, Neoral® also significantly reduced the expression intensity of CD40 on DCs in Zymosan-treated mice, as indicated by histograms and MFI values for each mouse (Fig. 3.9 B). Moreover, Neoral® significantly reduced the number of CD80⁺ DCs and their CD80 MFI values in Zymosan-treated mice (Fig. 3.9 A-B). A similar trend was observed CD86⁺ DC numbers and MFI values (Fig. 3.9 A-B).

For CD103⁺ DCs, compared to Zymosan-treated mice, Neoral® significantly reduced the number of CD40⁺ and CD80⁺ cells and also markedly reduced the number of CD86⁺ cells in mediastinal lymph nodes (Fig. 3.10 A). Crucially, histograms and MFI values for each mouse

indicated that Neoral® also significantly lowered the expression of CD40, CD80 and CD86 on CD103⁺ DCs compared to mice treated with Zymosan alone (Fig. 3.10 B). Compared to untreated mice, treatment with Neoral® alone also reduced CD40, CD80 and CD86 MFI values for CD103⁺ DCs (Fig. 3.10 B). It must be noted that CD103⁺ DCs in the mediastinal lymph nodes had greater MFI values for CD40 and CD80 than cells in the lung tissue, suggesting that cells trafficking to the nodes had a more mature phenotype (lung Fig. 3.5 B and node Fig. 3.10 B). For, CD11b⁺ DCs, the number of cells positive for co-stimulatory molecules were detected at much lower levels in the mediastinal lymph nodes, creating difficulty in performing meaningful analysis (Fig. 3.11 A). However, an interesting trend was observed with Neoral® significantly reducing Zymosan-induced influx of CD80⁺ CD11b⁺ DCs (Fig. 3.11 A). Histogram and MFI values indicated that Neoral® marginally lowered CD80 and CD86 expression on CD11b⁺ DCs compared to mice treated with Zymosan alone, while CD40 MFI values were reduced by Neoral® regardless of whether mice received PBS or Zymosan intranasally (Fig. 3.11 B).

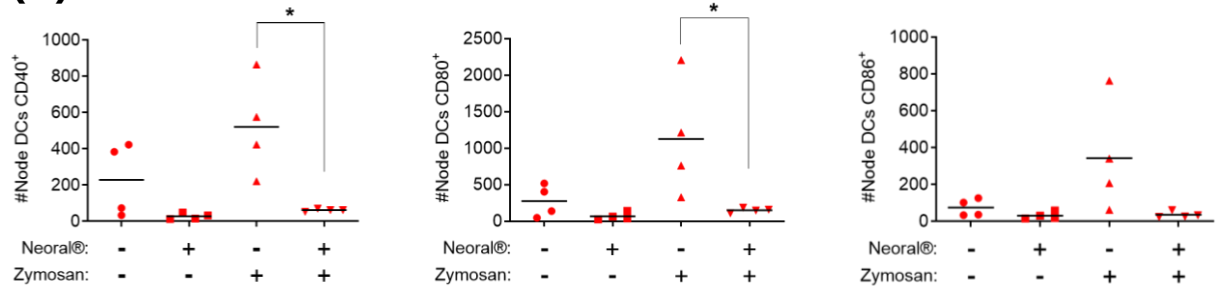
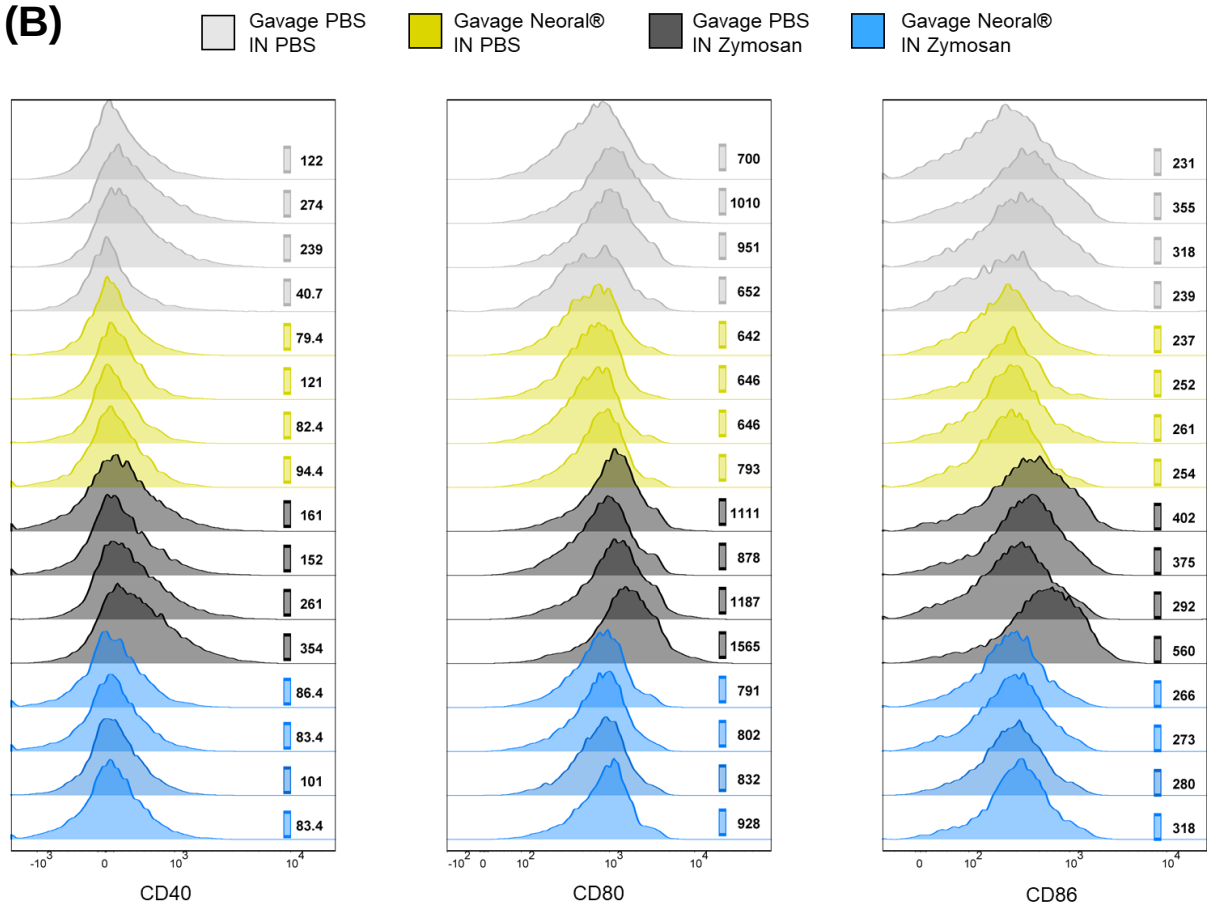
(A)**(B)**

Fig 3.9: Neoral® reduces total DC expression of CD40, CD80 and CD86 in the mediastinal lymph nodes following intranasal Zymosan challenge. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. DCs were gated as live CD3⁺B220⁺Gr1⁺F4/80⁺CD11c⁺MHCII^{hi}. (A) Number of DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for CD40 (left), CD80 (middle) and CD86 (right). *, p < 0.05; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group (n=4).

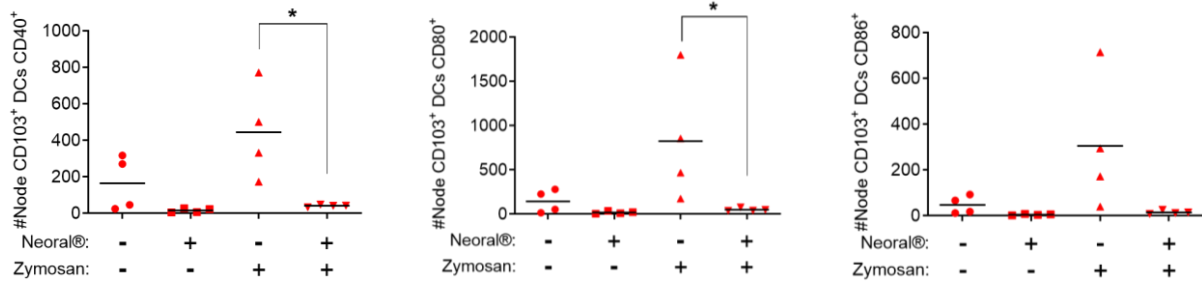
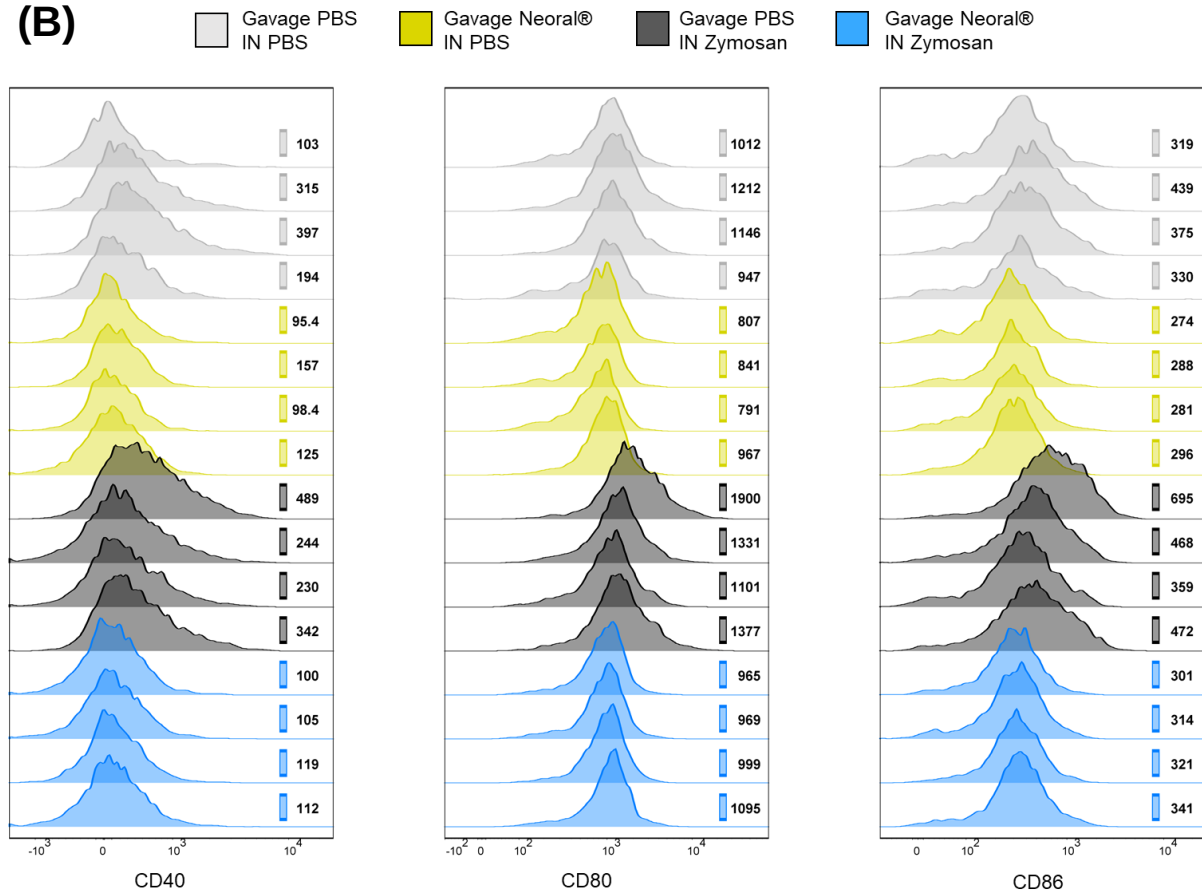
(A)**(B)**

Fig 3.10: Neoral® reduces CD103⁺ DC expression of CD40, CD80 and CD86 in the mediastinal lymph nodes following intranasal Zymosan challenge. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for analysis by flow cytometry. CD103⁺ DCs were gated as live CD3⁺B220⁺Gr1⁺F4/80⁺CD11c⁺MHCII^{hi}CD103⁺. (A) Number of CD103⁺ DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). *, p<0.05; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group (n=4).

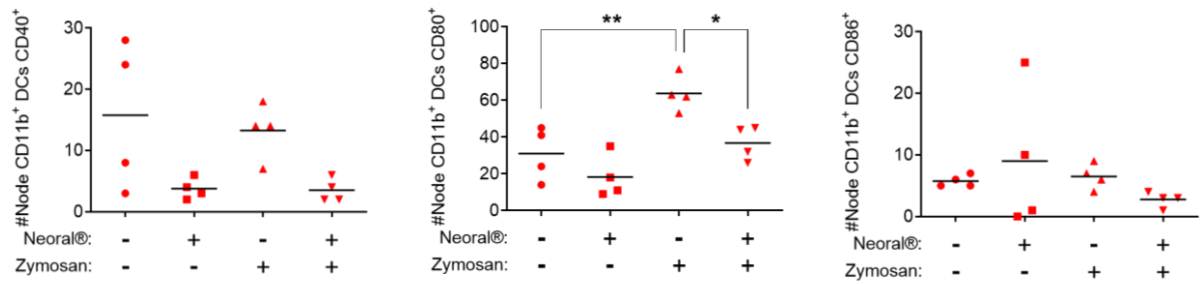
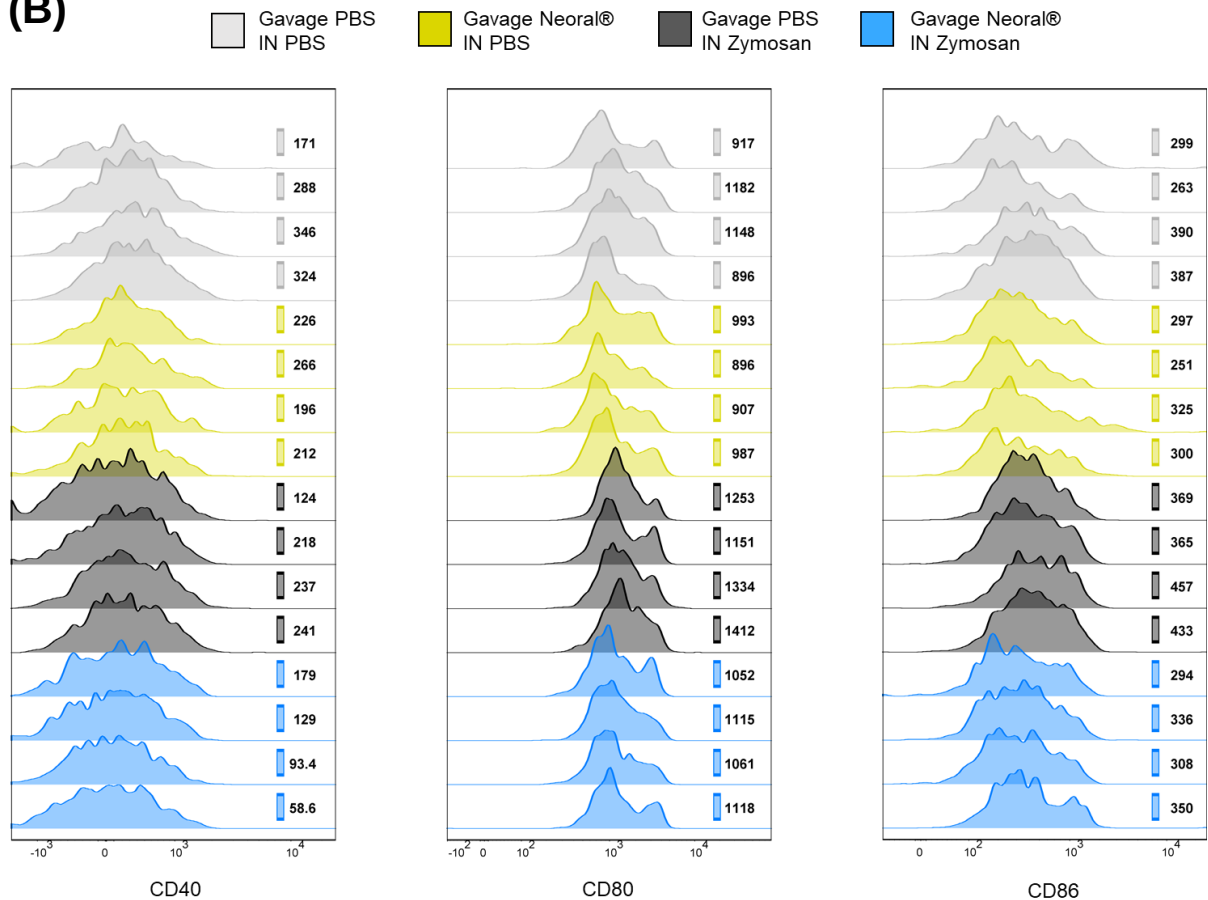
(A)**(B)**

Fig 3.11: Neoral® reduces CD11b⁺ DC expression of CD40, CD80 and CD86 in the mediastinal lymph nodes following intranasal Zymosan challenge. Mice were treated daily with Neoral® or PBS for 4 days and challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. CD11b⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. (A) Number of CD11b⁺ DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). *, p < 0.05; **, p < 0.01; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group (n=4).

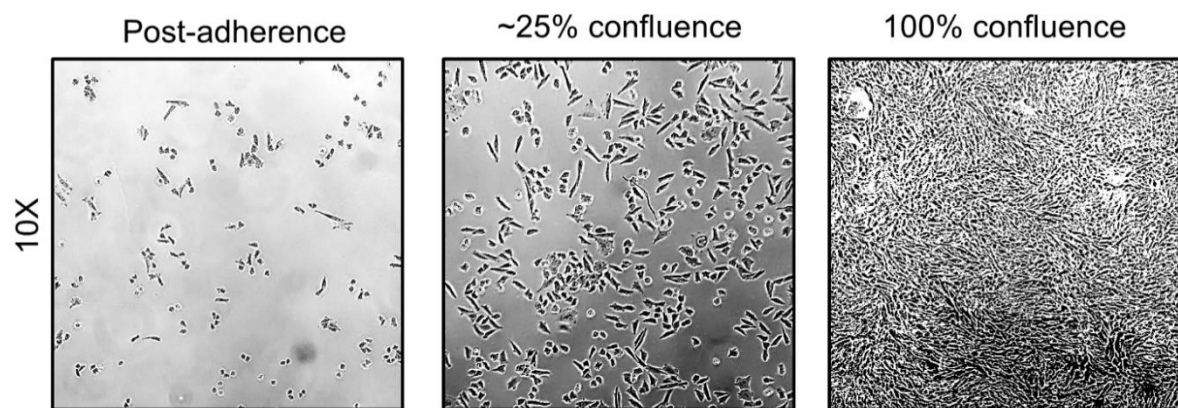
3.5 Generation of CD103⁺ DCs *in vitro*

Mayer *et al* demonstrated that culturing bone marrow cells with GM-CSF and Flt3L generated CD103⁺ DCs, which closely resembled CD103⁺ DCs in mucosal tissues [198]. To establish this protocol in the lab for modulatory studies with CsA, murine Flt3L was generated from a transgenic Chinese hamster ovary (CHO) cell line. Cultured CHO cells were characterised by monitoring their growth rates and morphological changes by light microscopy. These cells rapidly adhered to tissue culture vessels, adopted a filamentous morphology and typically grew to full confluence after 48-96 hours (Fig. 3.12 A). To determine whether CHO cells secreted murine Flt3L, culture supernatants were filter-sterilised and Flt3L measured by ELISA in 3 different aliquots. Since Flt3L concentrations were outside the assay range, the aliquots were added neat to the ELISA plate and a serial 1:2 dilution was performed 20 times. To obtain the most accurate results, 3 sets of diluted samples where the concentration was diluted 1:2 in a linear fashion were used, to calculate an average and final Flt3L concentration. CHO cell supernatants contained substantial concentrations of murine Flt3L, with low variability between each batch produced (Fig. 3.12 B).

To assess whether a combination of GM-CSF and Flt3L induced CD103⁺ DCs with a phenotype distinct to GM-CSF-generated DCs, bone marrow cells were cultured with both growth factors or GM-CSF alone. Cells were then harvested for analysis by flow cytometry. Debris and doublets were excluded using forward and side scatter parameters, while live cells were identified using BV510 viability staining. Live DCs were identified using the markers CD11c and MHC class II. For both types of DC, 95% of cells were negative for surface markers expressed by macrophages (F4/80), pDCs (B220) and splenic cDC1s (CD8 α) (Fig. 3.13 A). In addition, the majority of DCs expressed CD11b (Fig. 3.13 A). In striking contrast to GM-CSF-cultured BMDCs, cells cultured with both GM-CSF and Flt3L expressed

uniformly high levels of CD103 (Fig. 3.13 A-C). Indeed, ~85% of live cells were CD103⁺ DCs, a significantly greater proportion than live GM-CSF-cultured BMDCs (Fig. 3.13 C).

(A)



(B)

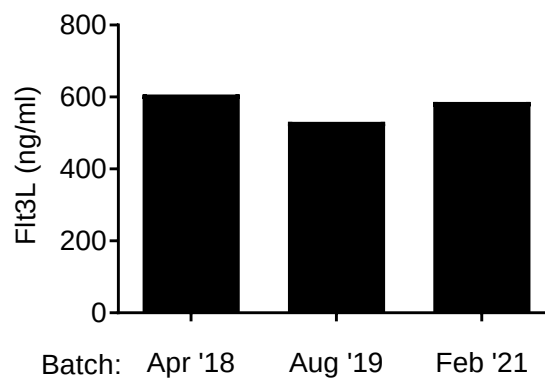
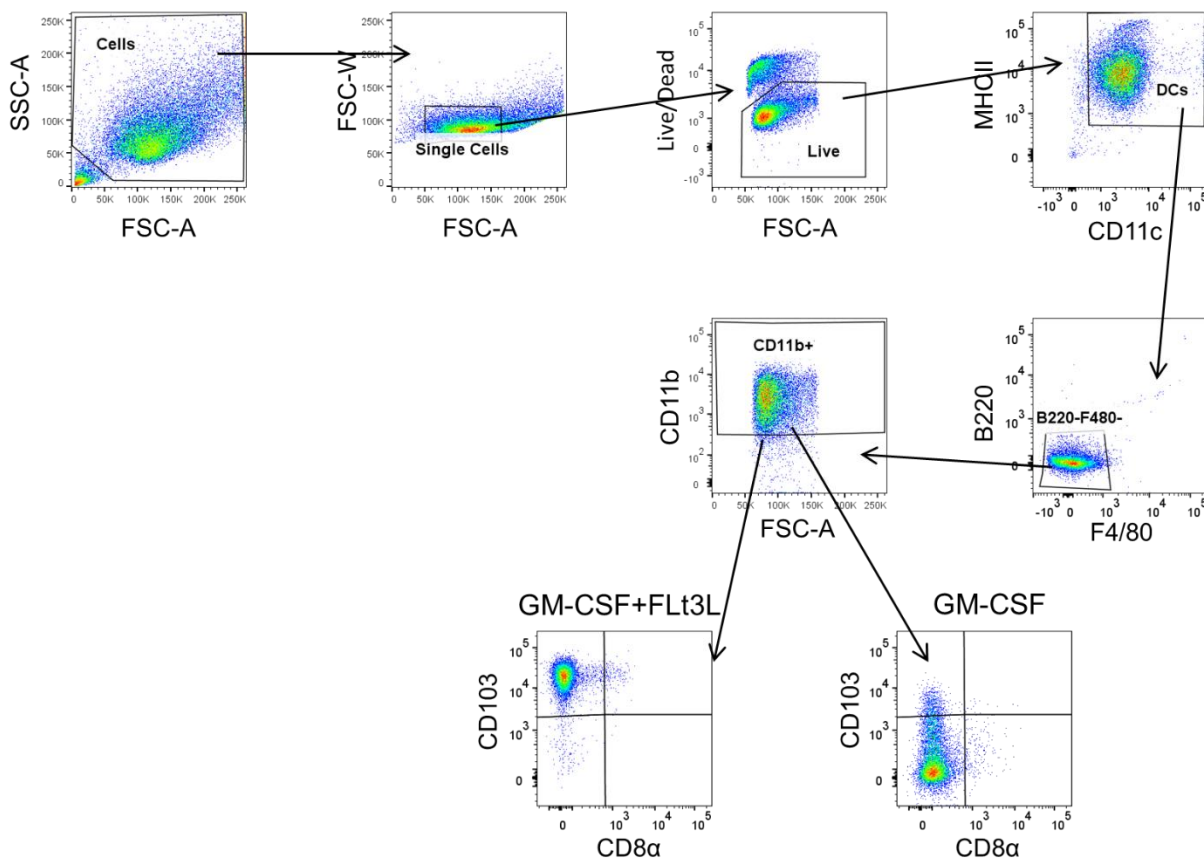
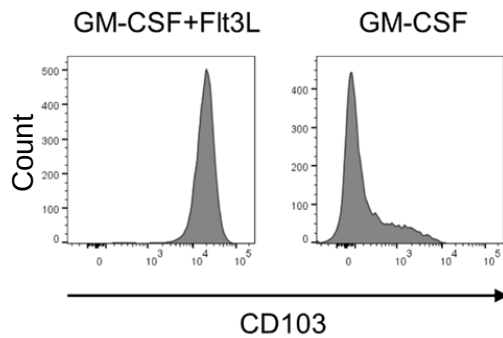


Figure 3.12: Culture of Chinese hamster ovary (CHO) cells expressing murine Flt3L. CHO cells were expanded in culture and supernatants were collected and analysed for murine Flt3L by ELISA. (A) Representative light micrographs of CHO cells at low, moderate and high confluence. (B) Flt3L yield obtained from different batches of expanded CHO cells.

(A)



(B)



(C)

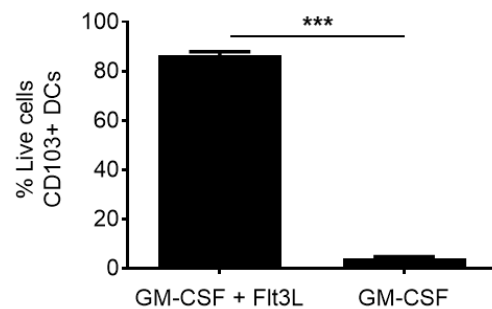


Figure 3.13: Prolonged bone marrow cell culture with both GM-CSF and Flt3L yields an enriched CD103⁺ DC population. iCD103 DCs (GM-CSF + Flt3L) and GM-CSF-generated BMDCs were analysed for surface markers by flow cytometry. (A) Gating strategy. (B) Histograms depicting CD103 expression in iCD103 DCs (GM-CSF+Flt3L) and GM-CSF-generated BMDCs (GM-CSF). (C) Percentage of live cells positive for CD11c, MHC class II, CD11b and CD103 and negative for B220, F4/80 and CD8. Results are pooled from two independent experiments and expressed as mean $\pm$ SEM. ***, $p < 0.001$, unpaired t test.

3.6 Characterisation of cytokine and costimulatory molecule responses in iCD103 DCs and comparison to GM-CSF-generated BMDCs

After successfully generating CD103⁺ DCs, it was important to characterise their responsiveness – in particular to fungal Zymosan – and draw comparisons with the established GM-CSF-generated BMDC model. Thus, cells were incubated with RPMI and a range of PAMPs including Zymosan, LPS, CpG, Poly I:C, Pam3CSK4, R837, R848, Heat-killed (HK) *E. coli* or HK *S. pneumonia* (SP) for 24 hours. Supernatants were collected and analysed for a number of cytokines including IL-6, IL-12p70, and IL-2 by ELISA.

In iCD103 DCs, IL-6 production was highly dependent on the stimulus. For example, high levels of IL-6 were only detected in response to Zymosan, CpG, Poly I:C and HK *E. coli*. By contrast, GM-CSF DCs secreted higher concentrations of IL-6 in response to all stimuli, except poly I:C, which induced higher IL-6 production in iCD103 DCs (Fig. 3.14 A).

In response to Zymosan and CpG, iCD103 DCs secreted significantly higher concentrations of bioactive IL-12p70 than GM-CSF BMDCs. However, secretion was more selective, as BMDCs produced IL-12p70 in response to a wider range of stimuli, including LPS, R837 and R848 (Fig. 3.14 B).

Since IL-2 is an important target of CsA, it was important to establish whether iCD103 DCs secrete this cytokine and to compare levels of production with GM-CSF BMDCs. Consistent with other cytokines, iCD103 DCs only secreted IL-2 in response to Zymosan and CpG, however, intriguingly, iCD103 DCs produced significantly higher IL-2 than GM-CSF BMDCs in response to either stimulus (Fig. 3.14 C).

In addition to measuring cytokine responses, DC expression of co-stimulatory molecules was compared in response to Zymosan, LPS, CpG and Poly I:C. Following overnight stimulation,

cells were harvested for analysis by flow cytometry. After excluding debris and doublets, DCs were identified as live CD11c⁺MHCII⁺ cells (Fig. 3.15). Maturation was compared in GM-CSF BMDCs and iCD103 DCs using median fluorescence intensity (MFI) values obtained for CD40, CD80 and CD86.

When treated with Zymosan, iCD103 DCs expressed greater levels of CD40 and CD86 but significantly less CD80 than GM-CSF DCs (Fig. 3.16 A-C, Fig. 3.17 A-C). In response to LPS, iCD103 DCs express significantly less CD40, CD80 and CD86 than GM-CSF DCs (Fig. 3.16 A-C, Fig. 3.17 A-C). By contrast, CpG stimulation led to greater CD40 expression in iCD103 DCs and comparable levels of CD80 and CD86 (Fig. 3.16 A-C, Fig. 3.17 A-C). Poly I:C stimulation led to greater expression of CD40 and CD86 on iCD103 DCs. Furthermore, although CD80 MFI values were higher in GM-CSF DCs, this was due to higher background levels of CD80 in these cells. As such, Poly I:C induced a greater upregulation of CD80 in iCD103 DC compared to unstimulated cells (Fig. 3.16 A-C, Fig. 3.17 A-C). Indeed, it is important to note that in the absence of stimulation, iCD103 DCs are less mature than GM-CSF BMDCs, as highlighted by lower basal expression of CD40, CD80 and CD86 (Fig. 3.16 A-C, Fig. 3.17 A-C). Therefore, even in cases where the MFI values are lower in iCD103 DCs, PAMP stimulation frequently triggers a higher fold increase in co-stimulatory molecule upregulation compared to GM-CSF DCs.

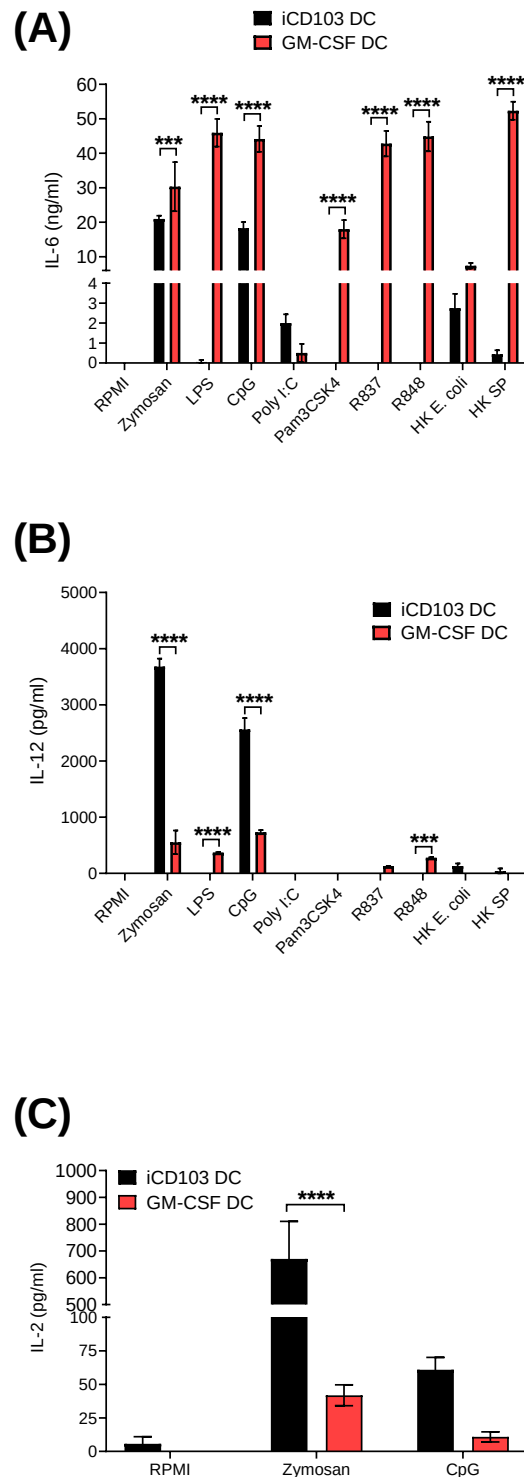


Figure 3.14: iCD103 DCs predominantly secrete cytokines in response to Zymosan and CpG. DCs were stimulated with Zymosan (10 μ g/ml), LPS (10ng/ml), CpG (5 μ g/ml), Poly I:C (5 μ g/ml) Pam3CSK4 (25ng/ml), R837 (4 μ g/ml), R848 (1 μ g/ml), Heat-killed (HK) *E. coli* (1.25x10⁶ bacteria/well) or HK *S. pneumonia* (SP, 15.625x10⁶ bacteria/well) for 24 hours. Supernatants were collected and analysed for IL-6 (A), IL-12p70 (B) and IL-2 (C) by ELISA. Bars are mean $\pm$ SD for triplicate samples. . ***, p<0.001; ****, p<0.0001; Two-Way ANOVA, Sidak's multiple comparison test for GM-CSF DC vs iCD103 DC. Results are representative of two independent experiments.

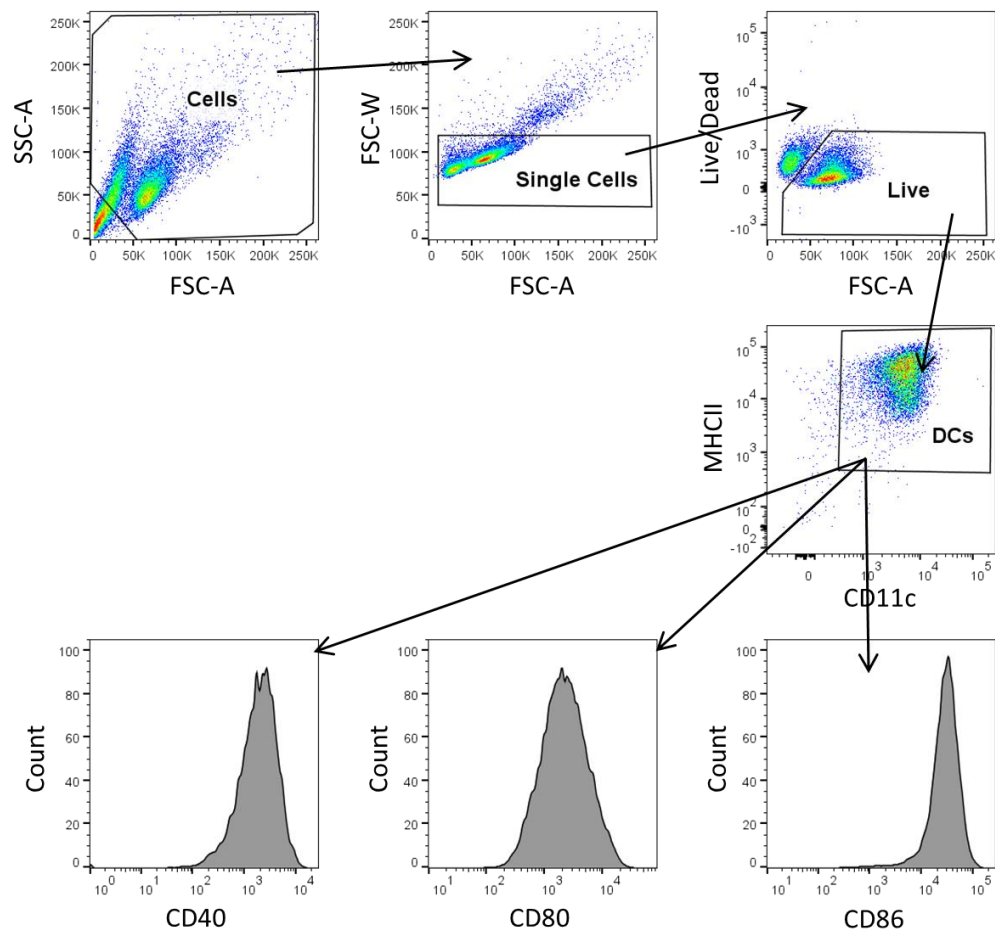


Figure 3.15: Gating strategy to assess maturation marker expression in live iCD103 DCs and GM-CSF-generated BMDCs. DCs were harvested and stained for Live/Dead, CD11c, MHCII, CD40, CD80 and CD86 and acquired on a BD FACSCanto™.

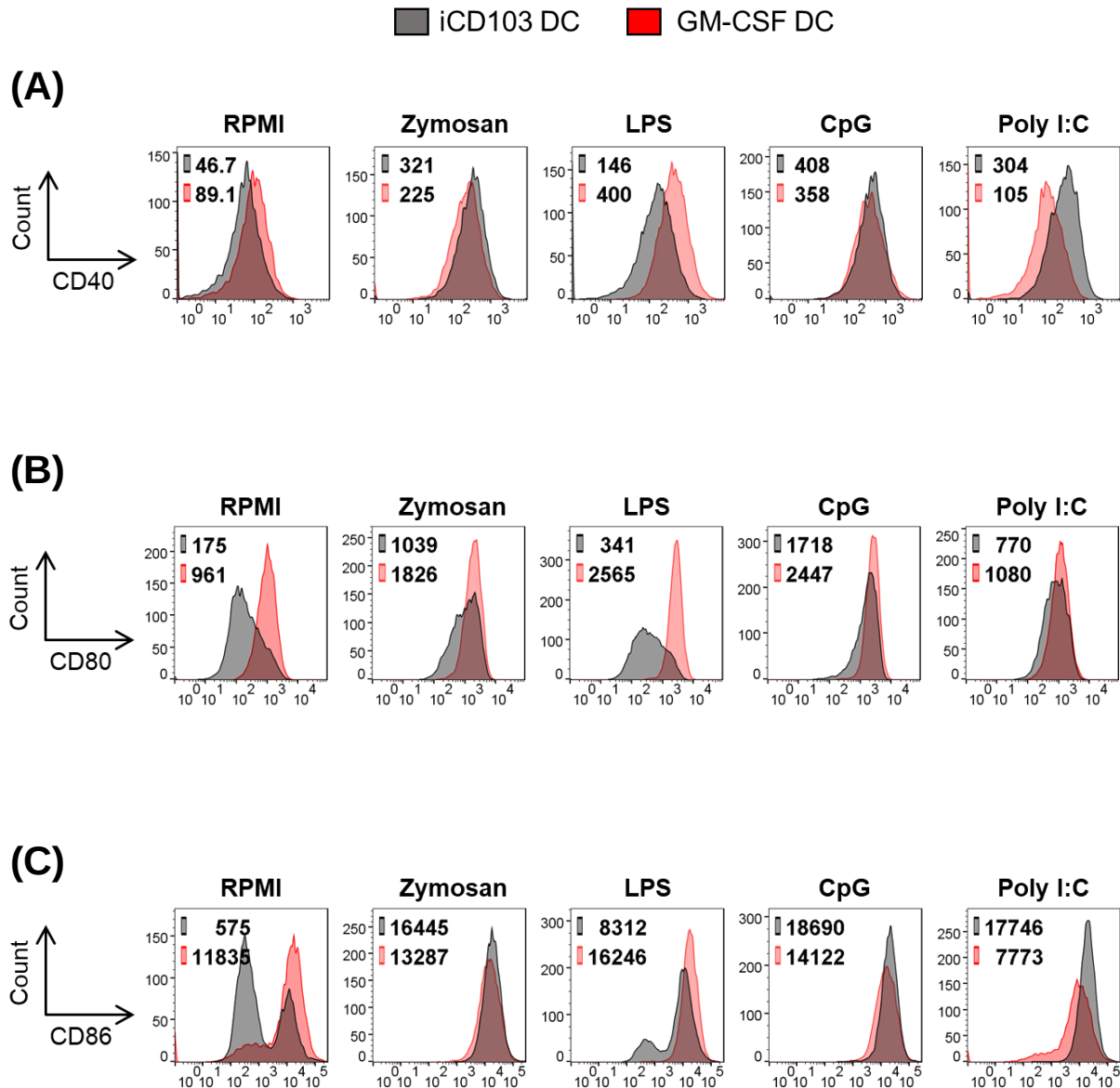


Fig 3.16: Differential maturation of iCD103 DCs and GM-CSF-generated BMDCs in response to PAMPs. iCD103 DCs (grey) and GM-CSF DCs (red) were stimulated with RPMI, Zymosan (5µg/ml), LPS (25ng/ml), CpG (2µg/ml), or Poly I:C (5µg/ml) overnight. Cells were compared for maturation marker expression by flow cytometry. Histograms and corresponding MFI values representing expression of CD40 (A), CD80 (B) and CD86 (C) in response to RPMI or PAMPs. Results are representative of three independent experiments.

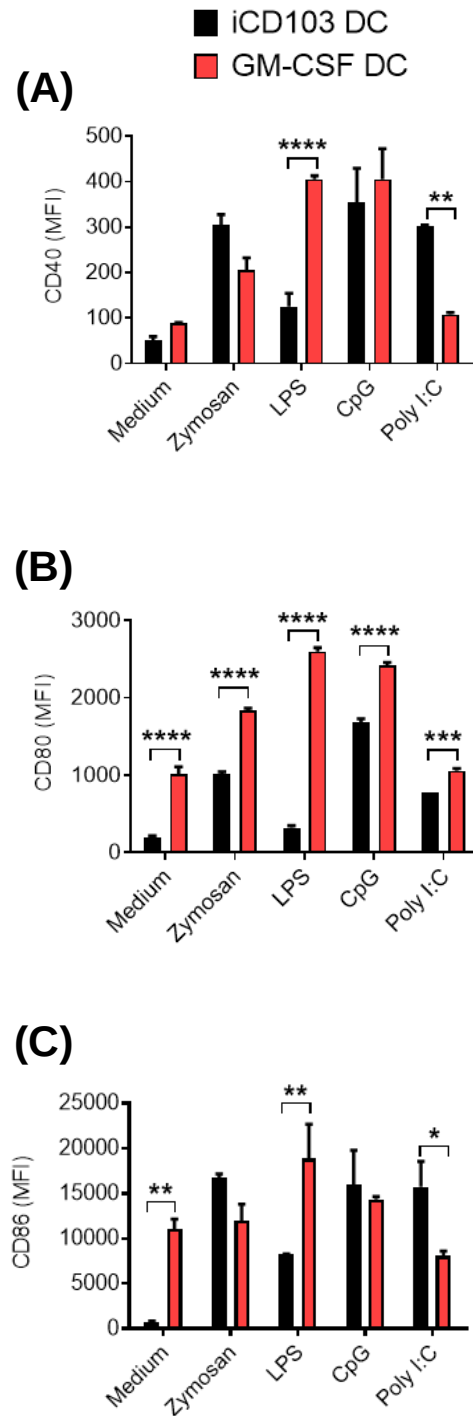


Figure 3.17: Differential maturation of iCD103 DCs and GM-CSF-generated BMDCs in response to PAMPs. DCs were stimulated with RPMI, Zymosan (5µg/ml), LPS (25ng/ml), CpG (2µg/ml) or Poly I:C (5µg/ml) for 24 hours. Cells were compared for maturation marker expression by flow cytometry. Expression of CD40 (A), CD80 (B) and CD86 (C) are represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; Two-Way ANOVA, Sidak's multiple comparison test for iCD103 DC vs GM-CSF DC. Results are representative of three independent experiments.

3.7 CsA significantly inhibits Zymosan-induced IL-2 secretion in iCD103 DCs

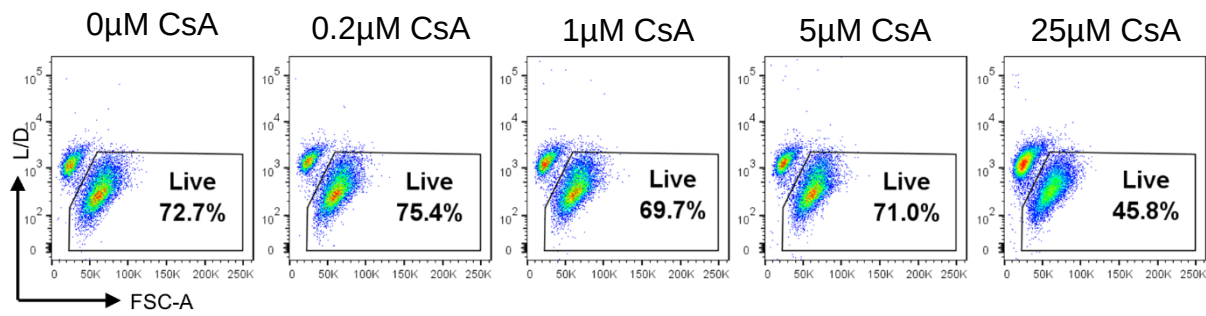
After optimising iCD103 DC cultures and characterising their responses, it was possible to test for immunomodulation by CsA. However, it was first necessary to optimise a CsA concentration range over which iCD103 DCs remained viable, to preclude the possibility of modulation occurring due to reduced viable cell numbers. Thus, cells were treated with CsA overnight and stained with Aqua viability dye. Using the same gating strategy as Fig. 3.15, debris and doublets were excluded and live cells were identified as negative for the live/dead stain. Based on previous CsA studies in the Lavelle lab with GM-CSF DCs, a concentration range of 0-25 μ M was tested, which aligns with low micromolar concentrations typically detected in patient serum [447]. iCD103 DCs were viable at low micromolar concentrations (0-5 μ M) but a significant level of cell death was observed when cells were treated with 25 μ M CsA (Fig. 3.18 A-B).

After determining that low micromolar CsA concentrations were optimal, these findings were validated by LDH assays in which cells were treated with CsA overnight and supernatants were collected and analysed for LDH release, an indicator of cytotoxicity. At low micromolar concentrations (0-5 μ M), no significant cytotoxicity was detected. However, when tested at the high concentration of 25 μ M, a significant 20% decrease in viability was observed (Fig. 3.18 C).

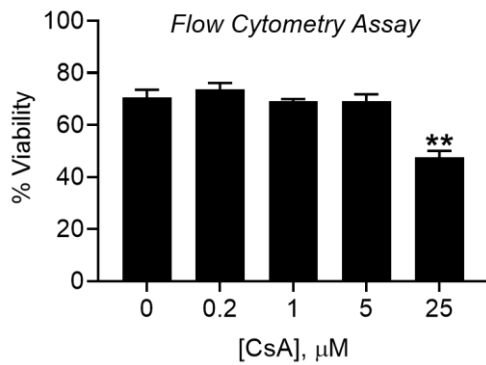
After characterising the CsA concentration at which iCD103 DCs remained viable, it was important to determine whether CsA could modulate cytokine responses elicited by fungal PAMPs. Previous studies have highlighted that CsA can inhibit Zymosan-induced IL-2 secretion in other BMDC culture models, including GM-CSF DCs [3]. As highlighted in section 3.2, iCD103 DCs secreted higher IL-2 than GM-CSF-generated BMDCs, which was

consistent with superior IL-2 secretion observed for CD103⁺ DC in the gut and lungs [336]. A pivotal study for this research found that IL-2 – produced specifically by CD103⁺ DCs – was essential for optimal priming of Th17 cells that protected against *Aspergillus fumigatus* infection [3, 232]. Thus, it was important to confirm whether CsA inhibition of IL-2 was reproducible in the iCD103 DC model. To this end, iCD103 DCs were pre-treated with 5µM CsA for 1h and stimulated with medium or zymosan overnight. Supernatants were collected and analysed for IL-2 by ELISA. CsA had no effect on the steady-state production of IL-2 in iCD103 DCs. However, CsA significantly reduced iCD103 DC secretion of IL-2 in response to Zymosan (Fig. 3.19).

(A)



(B)



(C)

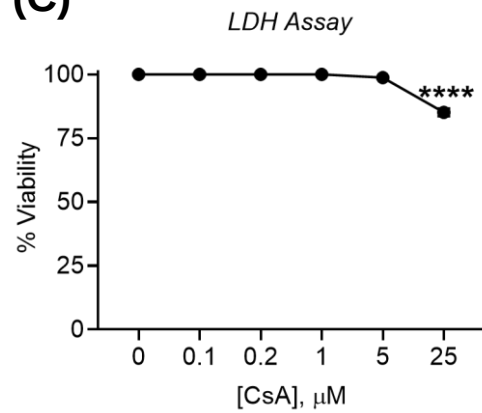


Figure 3.18: CsA is not cytotoxic to iCD103 DCs at low micromolar concentrations. iCD103 DCs were treated with CsA for 18 hours. (A) Cells were stained with viability dye (L/D) and viability was analysed in single cells by flow cytometry. (B) Flow cytometry viability data with bars depicting mean values for duplicate samples. (C) Supernatants were collected and cytotoxicity was assessed by LDH assay, with symbols depicting the mean values for triplicate samples. **, $p < 0.01$; ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test for treated versus untreated samples.

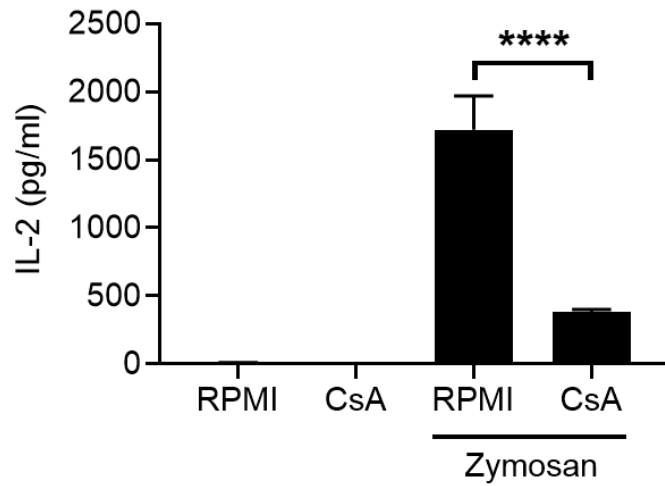


Figure 3.19: CsA inhibits Zymosan-induced IL-2 secretion by iCD103 DCs. iCD103 DCs were pre-treated with CsA (5 μ M) for 1 hour and stimulated with medium or zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for IL-2 by ELISA. Bars are mean $\pm$ SD for triplicate samples. ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of two independent experiments.

3.8 CsA significantly reduces Zymosan-induced IL-12p70 but not TNF α secretion in iCD103 DCs

As discussed in section 1.10, previous studies have highlighted the importance of TNF α secretion by myeloid cells during fungal infection and CD103⁺ DCs are likely to contribute to these responses in peripheral tissues [257, 260-262]. Of high relevance to this research, CsA was found to inhibit TNF α production in human monocyte-derived DCs and GM-CSF DCs in response to LPS stimulation [333, 343, 351, 352]. To assess whether this effect also occurred in iCD103 DCs stimulated with Zymosan, cells were pre-treated with CsA for 1h and stimulated overnight with medium or Zymosan. Supernatants were collected and analysed for the pro-inflammatory cytokine TNF α by ELISA. In contrast to IL-2, CsA only moderately reduced Zymosan-induced TNF α secretion by iCD103 DCs (Fig. 3.20 A).

CD103⁺ DCs are a dominant source of IL-12 and were shown to produce this cytokine during *Histoplasma capsulatum* infection, enhancing type I immunity and promoting fungal clearance, suggesting that IL-12 inhibition by CsA would potentially impact antifungal immunity [152]. In previous studies, CsA inhibited IL-12 secretion by DCs derived from PBMCs and GM-CSF DCs in response to LPS [353, 355]. More relevant to this research, CsA also inhibited Zymosan-induced IL-12 secretion in GM-CSF DCs [130]. It was important to determine whether this was reproducible in iCD103 DCs, particularly as Zymosan was found to elicit significantly greater levels of IL-12 in these cells than GM-CSF DCs (see Fig. 3.14 B). To address this question, iCD103 DCs were pre-treated with CsA for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for IL-12p70 by ELISA. CsA failed to alter steady-state production of IL-12p70. However, CsA significantly inhibited Zymosan-induced IL-12p70 secretion in iCD103 DCs (Fig. 3.20 B).

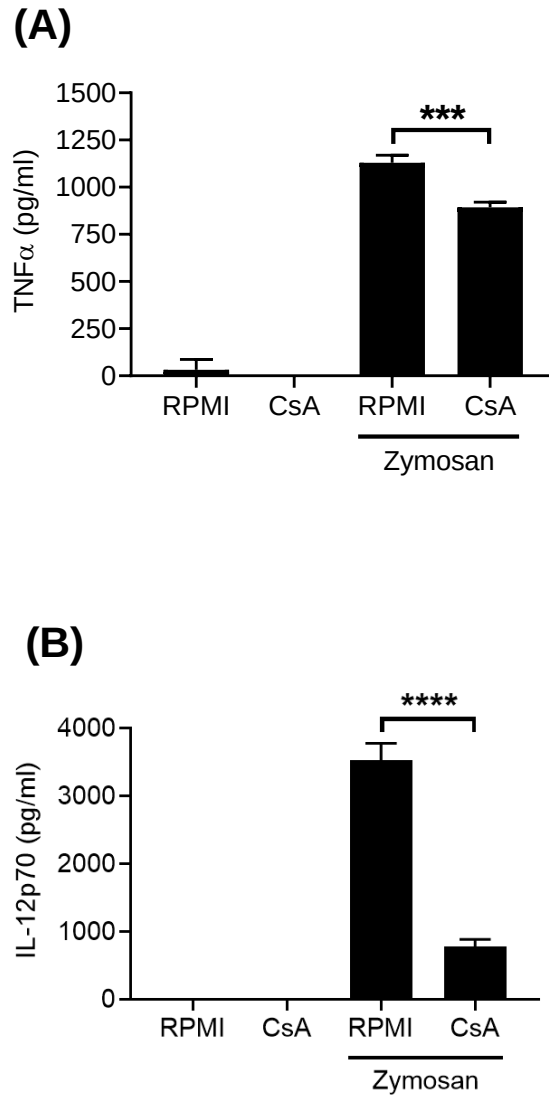


Figure 3.20: CsA treatment has a marginal impact on Zymosan-induced TNF α but potently inhibits IL-12p70 secretion in iCD103 DCs. iCD103 DCs were pre-treated with CsA (5 μ M) for 1 hour and stimulated with medium or Zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for TNF α (A) and IL-12p70 (B) by ELISA. Bars are mean $\pm$ SD for triplicate samples. ***, $p < 0.001$; ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

3.9 CsA modulates DC production of pro-Th17 cell cytokines

Th17 cell responses are vital for clearing fungal infections, with production of IL-17 being particularly important for the mobilisation of phagocytes [291]. The induction of Th17 cells requires secretion of innate cytokines such as IL-6, IL-23 and IL-1 β , which are produced by DCs during fungal infection [4, 232]. After confirming that CsA can modulate zymosan-induced production of the pro-Th1 cell cytokine IL-12 in iCD103 DCs, it was important to assess pro-Th17 cell cytokines, as inhibition would have significant implications for susceptibility to fungal infections. Thus, iCD103 DCs were pre-treated with CsA and stimulated with medium or Zymosan. Supernatants were collected and analysed for IL-6 and IL-23 by ELISA. Intriguingly, CsA caused a marked inhibition of iCD103 DC production of IL-6 in response to Zymosan (Fig 3.21 A). Regarding IL-23, CsA also significantly inhibited Zymosan-induced secretion (Fig. 3.21 B).

Secretion of IL-1 β – following processing by the NLRP3 inflammasome and caspase-1 – is also important for the induction of Th17 cell responses [448-450]. Previous Lavelle lab members found that GM-CSF DCs can secrete IL-1 β in response to a single PAMP, such as LPS, in the absence of an inflammasome activator, although responses were significantly enhanced by inflammasome activators such as Alum or Chitosan [39]. In recent studies, GM-CSF DC cultures were found to yield a heterogenous population of DCs and macrophages, with macrophages being the dominant producer of IL-1 β [451, 452]. Considering this, it was important to extensively characterise whether iCD103 DCs were capable of secreting IL-1 β , before conducting studies with CsA. Thus, cells were primed with Zymosan, LPS, CpG or Poly I:C for 3 hours and stimulated with the inflammasome activators Alum, Chitosan or the K⁺ ionophore nigericin overnight. Supernatants were harvested and analysed for IL-1 β by

ELISA. In the absence of inflammasome activators, iCD103 DCs stimulated with PAMPs alone failed to secrete IL-1 β , with levels being comparable to unstimulated cells (Fig 3.22 A-C). When inflammasome activators were included, Alum and Chitosan responses were strongest when cells were pre-primed with LPS (Fig 3.22 A-B). By contrast, nigericin induced IL-1 β secretion regardless of the initial PAMP, hence providing a useful model for studying IL-1 β responses primed by Zymosan (Fig 3.22 C). After characterising IL-1 β secretion, cells were pre-treated with CsA for 1 hour and stimulated as before. Importantly, during nigericin stimulation, CsA significantly inhibited IL-1 β secretion in cells primed with Zymosan but failed to inhibit this response in cells primed with LPS, CpG or Poly I:C (Fig 3.23).

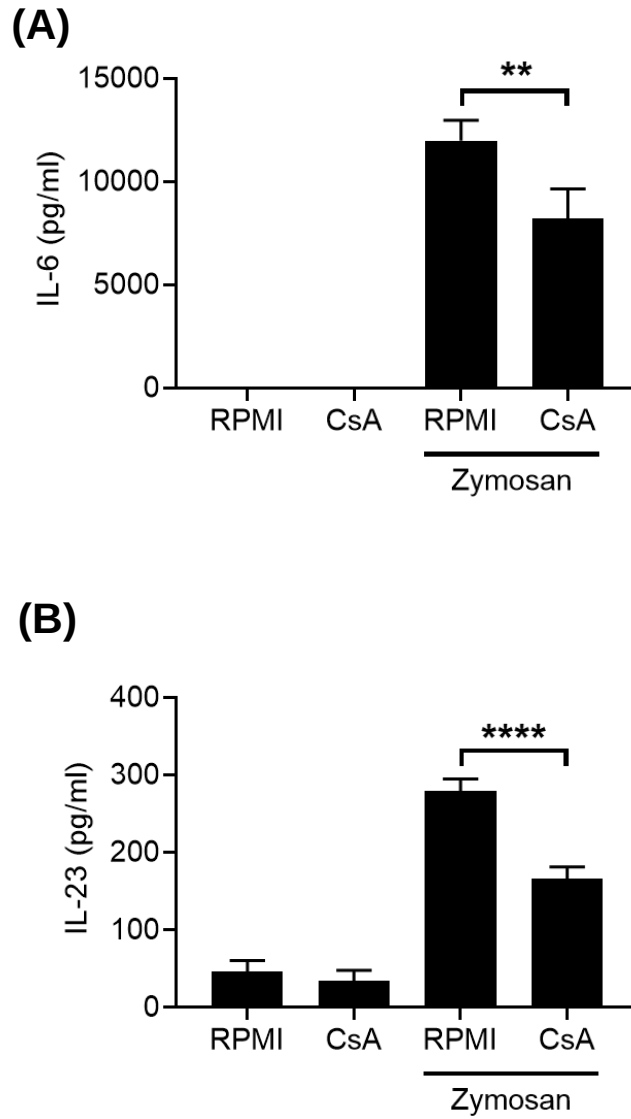


Figure 3.21: CsA inhibits Zymosan-induced secretion of pro-Th17 cell cytokines IL-6 and IL-23 by iCD103 DCs. iCD103 DCs were pre-treated with CsA (5 μ M) for 1h and stimulated with medium or Zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for IL-6 (A) and IL-23 (B) by ELISA. Bars are mean $\pm$ SD for triplicate samples. **, $p < 0.01$; ****, $p < 0.0001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

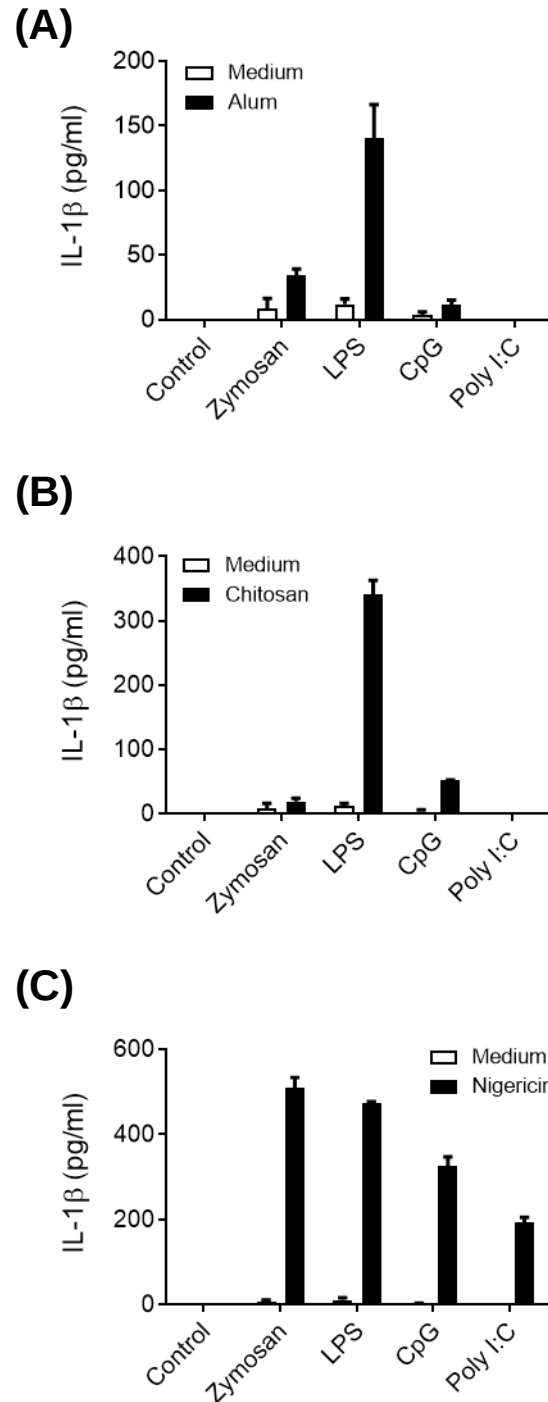


Figure 3.22: Inflammasome activation by nigericin enables optimal Zymosan-induced IL-1 β secretion by iCD103 DCs. iCD103 DCs were stimulated with Zymosan (5 μ g/ml), LPS (500ng/ml), CpG (4 μ g/ml) or Poly I:C (5 μ g/ml) for 3 hours and subsequently incubated with the inflammasome activators Alum (A; 100 μ g/ml), Chitosan (B; 5 μ g/ml) or Nigericin (C; 20 μ M) overnight. Supernatants were collected and analysed for IL-1 β by ELISA. Bars are mean $\pm$ SD for triplicate samples. Results are representative of two independent experiments.

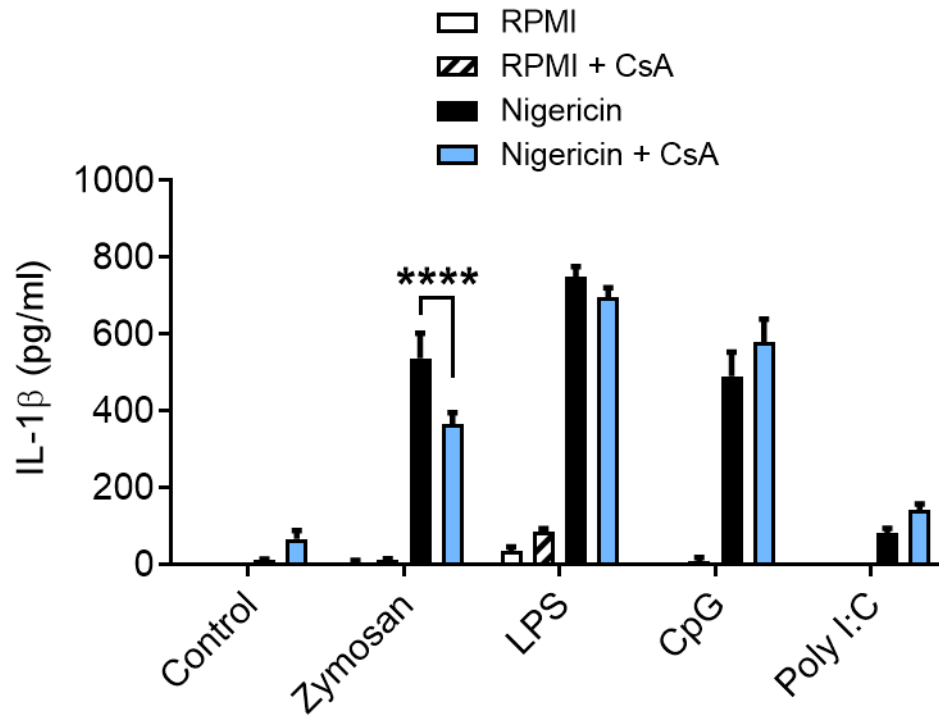


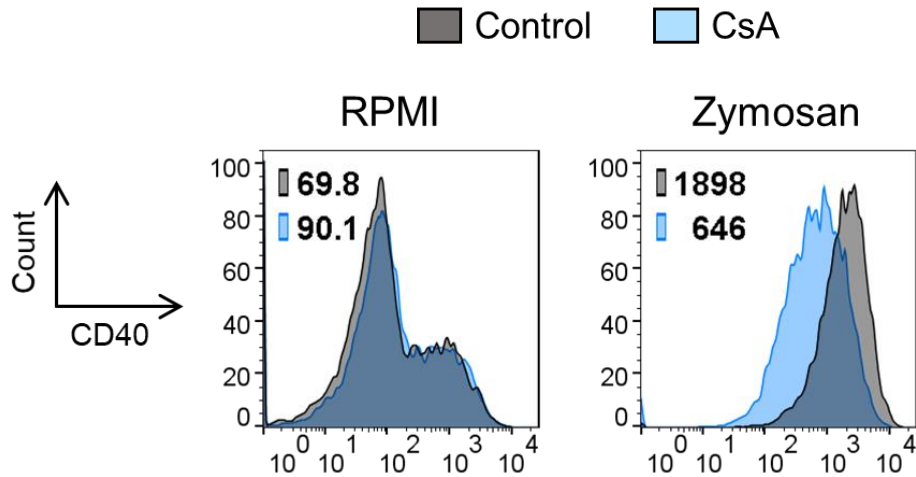
Figure 3.23: CsA reduces Zymosan-induced IL-1 β secretion by iCD103 DCs. iCD103 DCs were pre-treated with CsA (5 μ M) for 1h, stimulated with Zymosan (5 μ g/ml) or CpG (4 μ g/ml) for 3 hours and subsequently incubated with the inflammasome activator Nigericin (15 μ M) overnight. Supernatants were collected and analysed for IL-1 β by ELISA. Bars are mean $\pm$ SD for triplicate samples. ****, $p < 0.0001$; Two-Way ANOVA, Tukey's multiple comparison test. Results are representative of two independent experiments.

3.10 CsA inhibits iCD103 DC upregulation of CD40, CD80 and CD86 in response to Zymosan

DC depletion studies highlight that these cells are essential for priming protective T cell responses during fungal infection [16]. Upon recognition of fungal PAMPs, DCs upregulate the co-stimulatory molecules CD40, CD80 and CD86, which provide a vital activation signal to T cell co-stimulatory receptors, such as CD40L and CD28 [4, 290]. Therefore, modulation of Zymosan-induced maturation in iCD103 DCs by CsA would help to explain how this drug increases susceptibility to infection. Since Neoral® modulated CD103⁺ DC maturation *in vivo* (see section 3.4), it was important to determine whether this was reproducible in iCD103 DCs. Therefore, cells were pre-treated with CsA and stimulated overnight with Zymosan. Following stimulation, cells were harvested, stained for viability, CD11c, MHC II, CD40, CD80 and CD86 and analysed by flow cytometry. Live DCs were identified using the same gating strategy as in figure 3.15. From this population, MFI values were obtained for CD40, CD80 and CD86.

CsA significantly inhibited iCD103 DC upregulation of CD40 in response to Zymosan (Fig. 3.24 A-B). The impact of CsA on CD80 expression by iCD103 DCs stimulated with Zymosan was also significant, with a near 3-fold reduction observed (Fig. 3.25 A-B). In addition, CsA had a significant impact on Zymosan-induced expression of CD86 (Fig. 3.26 A-B).

(A)



(B)

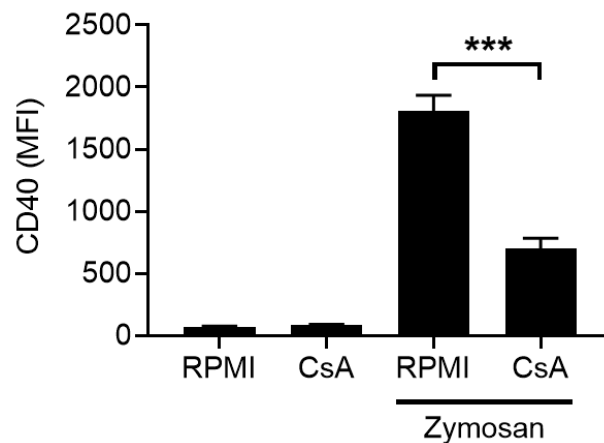
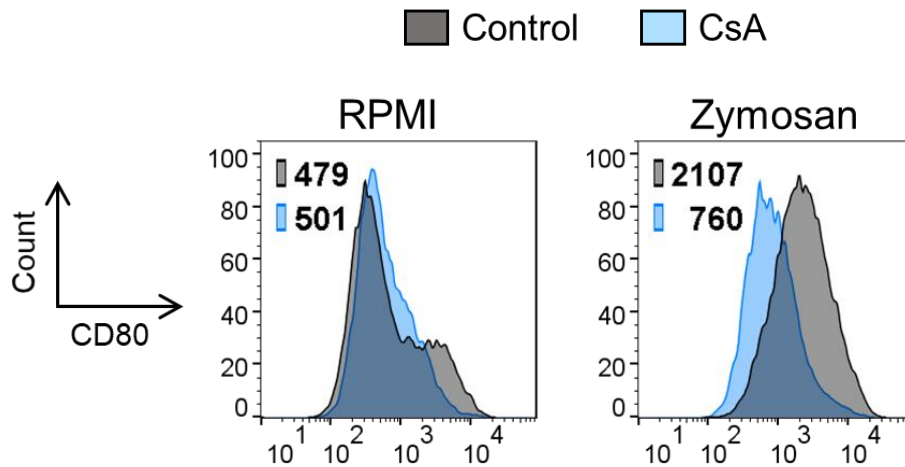


Fig 3.24: CsA inhibits iCD103 DC upregulation of CD40 in response to Zymosan. iCD103 DCs were pre-treated with 5 μ M CsA for 1h and stimulated overnight with zymosan (1 μ g/ml) or RPMI. Maturation marker expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CD40 expression represented as histograms with corresponding MFI values. (B) CD40 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ***, $p < 0.001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

(A)



(B)

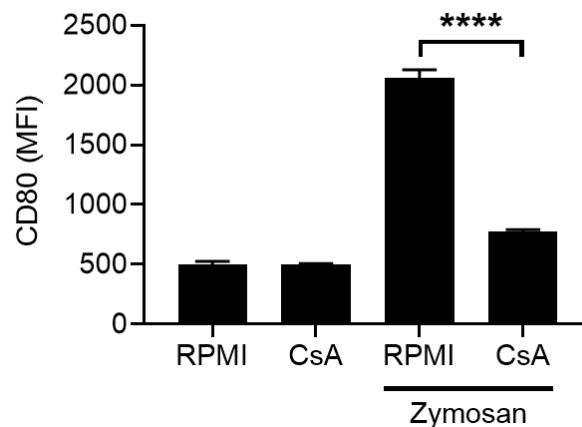
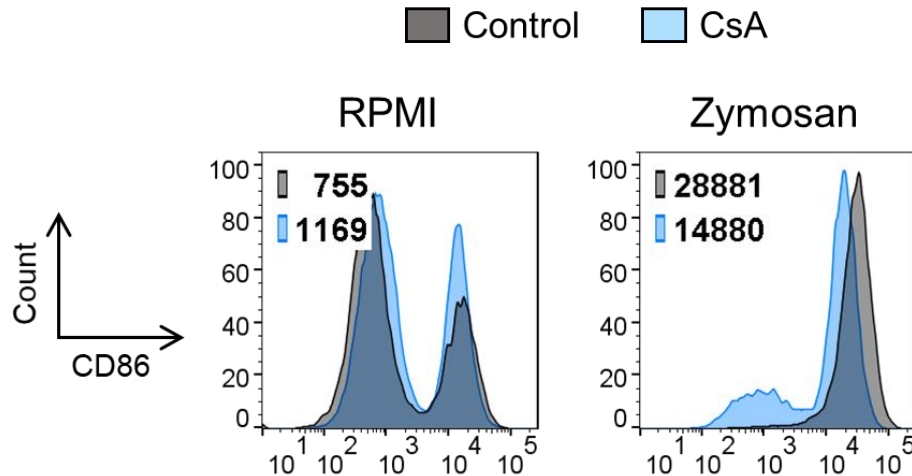


Fig 3.25: CsA significantly reduces iCD103 DC upregulation of CD80 in response to Zymosan.

iCD103 DCs were pre-treated with 5 μ M CsA for 1h and stimulated overnight with zymosan (1 μ g/ml) or RPMI. Maturation marker expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry.

(A) CD80 expression represented as histograms with corresponding MFI values. (B) CD80 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

(A)



(B)

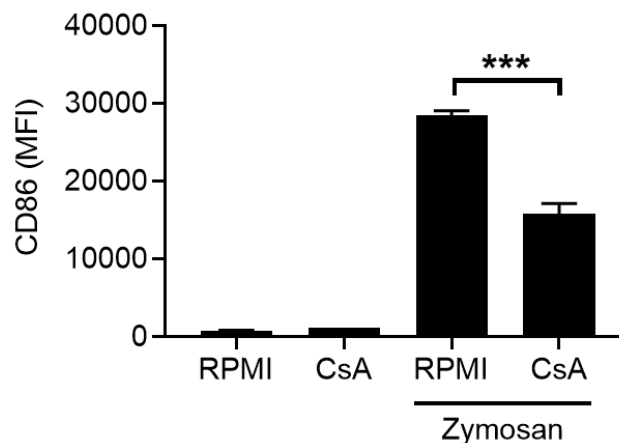


Fig 3.26: CsA inhibits iCD103 DC upregulation of CD86 induced by Zymosan. iCD103 DCs were pre-treated with 5 μ M CsA for 1h and stimulated overnight with zymosan (1 μ g/ml) or RPMI. Maturation marker expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CD86 expression represented as histograms with corresponding MFI values. (B) CD86 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ***, $p < 0.001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

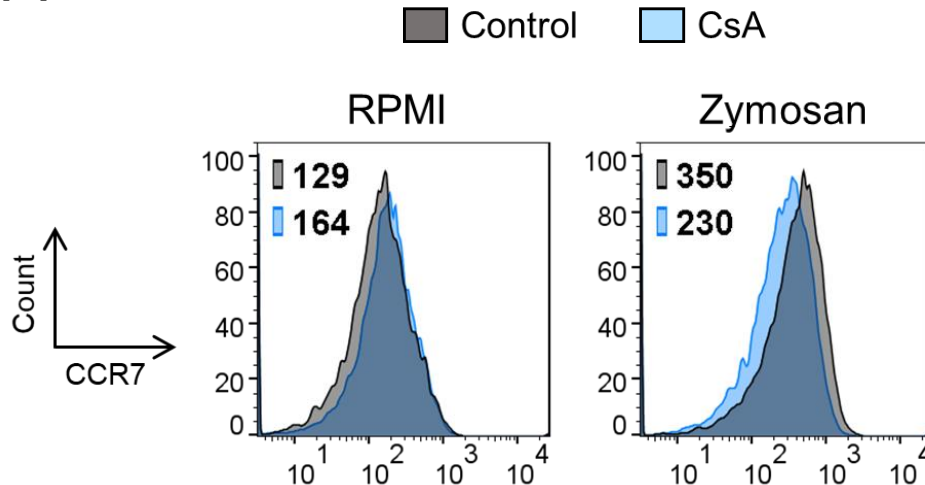
3.11 CsA reduces Zymosan-induced expression of CCR7 in iCD103 DCs and impairs migration to CCL19 *in vitro*

Microbial uptake by DCs and subsequent migration to the draining lymph nodes is essential for inducing adaptive T cell responses, including during cases of fungal infection [288]. As such, it was significant that initial *in vivo* studies found that Neoral® reduced CD103⁺ DC localisation to mediastinal lymph nodes upon intranasal Zymosan challenge (Fig. 3.8 A), as this was suggestive of impaired antifungal immunity. As highlighted, CCR7 is an essential mediator of DC migration to lymph nodes [161]. Therefore, it was decided to test whether Zymosan stimulation led to an upregulation of CCR7 in the iCD103 DC model and whether this was inhibited by CsA. Therefore, iCD103 DCs were pre-treated with CsA for 1h and stimulated with Zymosan overnight. Cells were harvested, stained for viability, CD11c, MHCII and CCR7 and analysed by flow cytometry. Live DCs were identified using the same gating strategy as in figure 3.15 and analysed for CCR7 expression. Interestingly, Zymosan increased CCR7 expression compared to unstimulated cells (Fig. 3.27 A-B). Moreover, this upregulation was markedly inhibited by CsA (Fig. 3.27 A-B).

After observing reduced CCR7 expression on cells treated with CsA, it was decided to assess whether this was associated with a defect in iCD103 DC migration. As discussed in section 1.5, CCR7 drives DC migration by directly binding to the ligand CCL19 [164, 165]. This chemokine is expressed in a soluble form, or on the surface of lymphatic endothelial cells [164, 165]. CCL19 forms a gradient of increasing concentrations that enables CCR7 to drive DC migration from peripheral tissues, through lymphatic vessels, into lymph nodes and towards the T cell zone [164, 165, 171-175]. A chemotaxis assay can be used to measure DC migration *in vitro*, using a transwell with recombinant CCL19 in the lower chamber [453]. Using this approach, iCD103 DCs were pre-treated with CsA for 1h and stimulated with Zymosan overnight. Recombinant CCL19 was added to the lower chamber of each transwell

and a spontaneous control was included, whereby medium was added to the lower chamber. Stimulated cells were harvested and added to the upper chamber of the transwell and the plates were incubated for 3h. As expected, light microscopy indicated minimal migration in the spontaneous control well compared to CCL19-treated wells (Fig. 3.28 A). Compared to unstimulated cells, Zymosan triggered an increase in DC confluence that was reduced by CsA pre-treatment (Fig. 3.28 A). After obtaining light micrographs, cells were harvested and counted by trypan blue staining and the spontaneous migration count was subtracted from each sample. From the final data, it was observed that Zymosan caused a significant increase in iCD103 DC migration and this was significantly reduced by CsA treatment (Fig. 3.28 B).

(A)



(B)

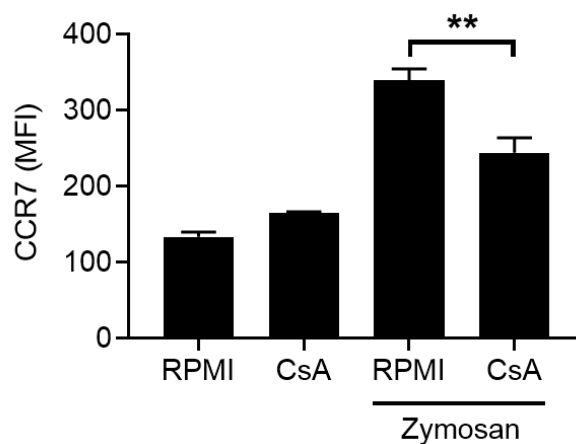
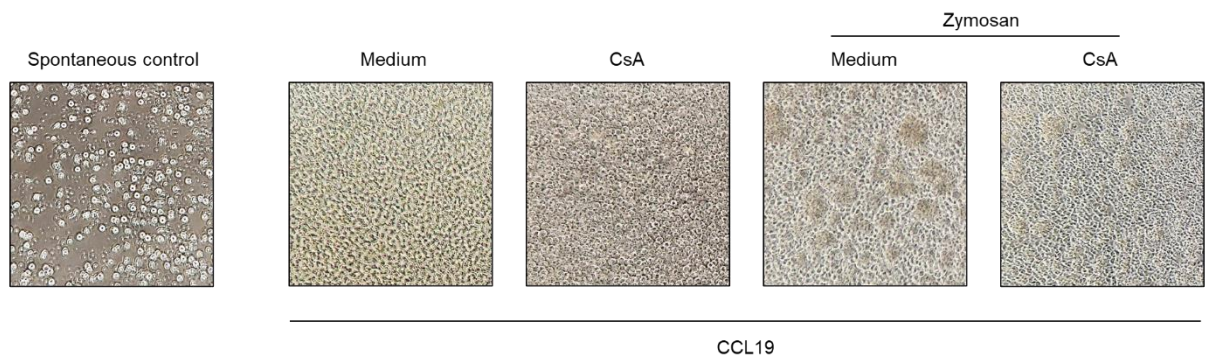


Fig 3.27: CsA inhibits iCD103 DC upregulation of CCR7 in response to Zymosan. iCD103 DCs were pre-treated with 5 μ M CsA for 1h and stimulated overnight with zymosan (1 μ g/ml). Surface CCR7 expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CCR7 expression in iCD103 DCs represented as histograms with corresponding MFI values. (B) CCR7 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. **, $p < 0.01$; One-way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

(A)



(B)

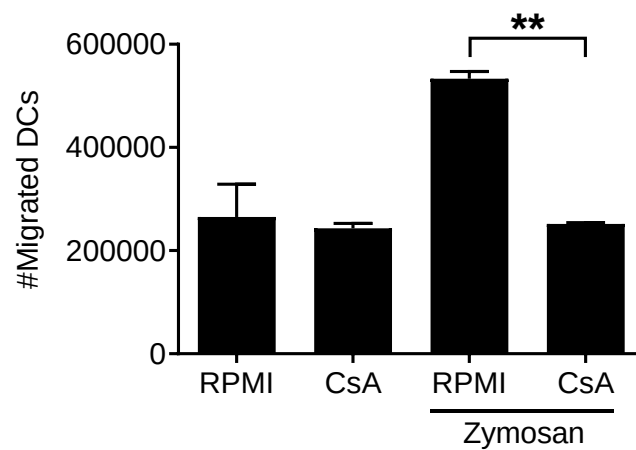


Fig 3.28: CsA reduces iCD103 DC migration towards CCL19 after Zymosan stimulation. iCD103 DCs were pre-treated with 5 μ M CsA for 1h and stimulated with zymosan (300ng/ml) overnight. Cells were harvested and added to the upper chamber of a transwell containing CCL19 in the lower chamber. Cells were incubated for 3h and migration was analysed using light microscopy (A) and trypan blue staining (B). Bars are mean $\pm$ SD for duplicate samples. **, $p < 0.01$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

3.12 Discussion

This work renders CD103⁺ DCs as potentially important targets of CsA. This may have implications for why the drug is highly effective against inflammatory diseases. However, more importantly for this research, the inhibition of Zymosan-elicited responses in these cells may help to explain why the drug increases susceptibility to fungal infections in patients. Indeed, CD103⁺ DCs were recently found to be essential for protection against a number of common fungal infections, such as *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Candida albicans* [152, 232, 248].

The *in vivo* studies found that intranasal Zymosan treatment increased the number of DCs in lung-draining mediastinal lymph nodes. As opposed to CD11b⁺ DCs, a higher proportion of lymph node DCs were CD103⁺, which is consistent with their strong migratory capacity compared to other DC subsets [216, 217]. Crucially, Neoral® treatment significantly reduced the number of CD103⁺ DCs in the lymph nodes after intranasal Zymosan challenge. This highlights that CsA can impact DC trafficking *in vivo*, which would significantly impair antifungal immunity. Indeed, while DCs provide a source of antigen, cytokines and co-stimulation, a physical interaction with naïve T cells in draining lymph nodes is also required for priming adaptive immunity [161]. One study demonstrated this in a fungal setting, by showing that during a live infection lung DCs uptake *Aspergillus fumigatus*, upregulate co-stimulatory molecules and migrate to lung-draining lymph nodes to prime adaptive T cell responses [288]. As highlighted, a key function of DCs is to upregulate co-stimulatory molecules upon recognition of fungal PAMPs, to provide an essential priming signal for antifungal naïve T cells [4, 290]. Neoral® reduced the number of CD103⁺ DCs expressing CD40, CD80 and CD86 in the lung-draining lymph nodes upon intranasal Zymosan challenge. Of note, CD103⁺ DCs in the lymph nodes appeared to have a more mature phenotype than cells in the lung tissue. Importantly, Neoral® lowered the expression intensity of co-

stimulatory molecules on CD103⁺ DCs. Together, these findings implicate reduced DC maturation and migration in response to fungal PAMPs as an additional mechanism of susceptibility to infection caused by CsA.

The iCD103 DC model was optimised in the lab to generate a large, pure population of CD103⁺ DCs *in vitro*. This enabled a more rigorously controlled investigation into the mechanistic impact of CsA on these cells. Based on previous studies on CD103⁺ DCs in the gut and lungs, it was expected that iCD103 DCs would produce significantly greater levels of IL-2 than the GM-CSF DC model also used in the lab [232, 336]. In addition, with the reported role of NFAT in DC IL-2 secretion, it was consistent that CsA significantly reduced Zymosan-induced IL-2 production in iCD103 DCs [127, 334]. This may be highly relevant for fungal susceptibility. For instance, Zelante et al. found that mice deficient for IL-2 production in CD103⁺ DCs were significantly more susceptible to *Aspergillus fumigatus* infection, with uncontrolled fungal growth in the lungs [232]. This was caused by dysregulated production of IL-23, excessive priming of IL-17⁺ Th17 cell responses and harmful tissue damage, due to uncontrolled neutrophil recruitment [232]. In contrast to IL-2, CsA inhibition of Zymosan-induced TNF α secretion in iCD103 DCs was only marginal. However, any reduction may impact antifungal immunity, as previous work demonstrates that TNF α secretion by myeloid cells promotes killing of *Aspergillus fumigatus* in the lungs [262]. In addition, gene polymorphisms linked with reduced expression of TNF α or its receptor were associated with an increased risk of pulmonary aspergillosis [454, 455]. Regarding IL-12, NFAT was found to promote upregulation in other DC culture models, in response to LPS or Zymosan stimulation [353, 355, 356]. As such, it was expected that CsA would also significantly reduce IL-12 secretion in Zymosan-stimulated iCD103 DCs. This finding is significant as IL-12 is well-established to prime Th1 cells, which are a key source of IFN γ , a cytokine that was necessary to prevent systemic dissemination of *Candida albicans* and *Cryptococcus*

neoformans [297, 298]. Of high relevance to the inhibition of IL-12, one study found that during *Histoplasma capsulatum* infection CD103⁺ DCs were the dominant source of IL-12 and this enhanced protective IFN γ ⁺ T cell responses [152]. Therefore, demonstrating that CsA can inhibit Zymosan-induced IL-12 secretion in a BATF3-dependent CD103⁺ DC culture model is a significant finding.

Th17 cell responses are key for antifungal immunity. Indeed, studies using mice deficient for IL-17, the IL-17R or treated with anti-IL-17 have reported reduced survival during *Candida albicans* and *Pneumocystis carinii* infections [291-293]. A key role of this cytokine is to recruit and activate neutrophils that promote fungal killing [291]. Consistently, deficiencies in Th17 cell-polarising cytokines, such as IL-6, IL-23 and IL-1 β , which are produced by DCs, has also been found to impair antifungal immunity [293, 456, 457]. For these reasons, it was significant that CsA could inhibit Zymosan-induced secretion of IL-6, IL-23 and IL-1 β in iCD103 DCs, as this may contribute to increased susceptibility during a fungal infection.

Another important question was whether the effects of Neoral® on *in vivo* DC maturation would translate to the iCD103 DC model. When assessing this, CsA did indeed reduce iCD103 DC upregulation of the co-stimulatory molecules CD40, CD80 and CD86 in response to Zymosan. This further supports the idea that CsA treatment may predispose to fungal infections by impairing DC maturation.

After observing impaired trafficking of CD103⁺ DCs into the draining lymph nodes of mice receiving Neoral®, it was predicted that this was caused by Neoral® impacting the expression of CCR7 on DCs. Another possibility was that Neoral® impaired DC migration indirectly, for example, by reducing the production of CCR7 ligands in the lungs, such as the chemokines CCL19 and CCL21 [164, 165]. Previous studies using Human monocyte-derived DCs detected an upregulation of CCR7 when treated with *Aspergillus fumigatus* [458]. Consistently, iCD103 DCs upregulated CCR7 in response to Zymosan. Subsequently, it was

interesting to observe that CsA reduced both CCR7 expression and *in vitro* migration to the ligand CCL19. Together, the data generated suggest that CsA can directly reduce the capacity of DCs to migrate to the draining lymph nodes, which, as discussed, is likely to reduce the magnitude of antifungal T cell responses.

The work in this chapter enhances our knowledge of the mechanisms by which CsA can increase susceptibility to fungal infection. The research specifically focuses on CD103⁺ DCs, a cell type that has recently emerged as an important mediator of antifungal immunity. Overall, CsA reduces a range of antifungal responses elicited by Zymosan in CD103⁺ DCs. These include the secretion of cytokines, upregulation of co-stimulatory molecules, chemotaxis and trafficking into lung-draining lymph nodes.

Chapter 4: Results (ii)

**Investigating the Mechanisms by which CsA
Modulates CD103⁺ Dendritic Cells**

4.1 Introduction, hypothesis and aims

CsA has a strong track record of ameliorating inflammatory diseases and protecting against graft rejection [321, 322, 324, 325, 441]. Alongside these benefits are a number of potential side effects, most notably for this research, the increased risk of opportunistic fungal infections [366, 368, 391, 393-401]. Initially, this was attributed to CsA reducing T cell proliferation, by inhibiting a calcineurin-NFAT-IL-2 pathway [323]. However, it is now clear that the immunosuppressive effects of CsA are not restricted to T cells and the drug can also modulate innate immune cells, including DCs, macrophages and neutrophils [3]. This is highly relevant to fungal susceptibility, as antifungal Th1 and Th17 cell responses require priming by DCs. In addition, Th1 and Th17 cells do not directly kill fungi but instead facilitate this process by engaging effector phagocytes, which include macrophages and neutrophils [301].

A number of studies provide insight into the mechanisms by which CsA targets innate immune cells. For example, in macrophages, activation of NFAT led to an increased expression of iNOS, a key antimicrobial mediator, by directly binding to its promoter [364]. Accordingly, CsA inhibited macrophage expression of iNOS and subsequent nitrite production [364]. In other studies, neutrophils treated with CsA were less capable of killing *Candida albicans* or *Aspergillus fumigatus* *ex vivo* [366, 368]. This is likely explained by CsA inhibition of neutrophil ROS, as well as NETs, both key antimicrobial mediators in these cells [367, 369]. Despite this, the mechanisms of ROS or NET inhibition by CsA in neutrophils were never addressed. In DCs and macrophages, CsA has been shown to reduce secretion of the cytokines IL-2, IL-12, TNF α and IL-1 β in response to a range of PAMPs [333, 343, 351-354, 365]. Despite this progress, the specific impact of CsA on CD103⁺ DCs, which play an important role in antifungal immunity, had yet to be addressed prior to the current work [152, 232, 248]. This chapter will explore the mechanisms by which CsA inhibits antifungal responses in CD103⁺ DCs.

The two best-described mechanisms of CsA action are inhibition of the calcineurin-NFAT pathway and prevention of DAMP release through the MPT pore [3]. One pivotal study used a D1 DC line stably expressing an NFAT transgene and, through chromatin immunoprecipitation sequencing, found that NFAT binds to a number of antifungal genes, such as *Il2*, *Il2b*, *Il23a* and *Tnf* [356]. In a microarray analysis, the calcineurin inhibitor tacrolimus was found to reduce the expression of these genes in curdlan-stimulated D1 DCs [356]. In the same microarray analysis, it was clear that calcineurin significantly influenced the DC transcriptional landscape. For instance, curdlan upregulated 515 genes in D1 DCs and 171 of these were reduced by co-treating with tacrolimus [356]. Moreover, curdlan downregulated 323 genes and tacrolimus impaired this reduction for 82 genes [356]. In contrast to calcineurin inhibition, CsA prevention of mitochondrial DAMP release through the MPT pore has yet to be linked to a broad change in DC gene expression. However, CsA inhibition of mitochondrial ROS release was found to inhibit IL-1 β secretion by BMDMs treated with LPS and ATP, as well as by J774A.1 monocytes stimulated with LPS and silica or nigericin [73, 374, 385].

Despite these advances, it remains to be determined whether CsA reduces antifungal responses in CD103⁺ DCs by inhibiting the calcineurin-NFAT pathway, mitochondrial DAMP release or another unknown pathway. Furthermore, the effects of CsA are broad and novel mechanisms of suppression are likely to be discovered. In the context of antifungal immunity, an interesting set of studies with relevance for this project demonstrated that CD103⁺ DCs and type I IFNs are essential for protection against fungal infections [150-152, 232, 248, 435, 436, 439]. Crucially, one study measured the source of type I IFNs during a lung fungal infection and found that CD103⁺ DCs were dominant producers [152]. Therefore, an intriguing route of investigation will be to assess whether CsA impacts on type I IFN production by CD103⁺ DCs. Moreover, as discussed in section 1.5, type I IFN signalling leads to a broad change in gene

expression, significantly enhancing DC activation. This would suggest that any impact of CsA on type I IFN responses in CD103⁺ DCs would also lead to a secondary impact on other antifungal responses.

The hypothesis underlying this chapter is that CD103⁺ DC reactivity to fugal PAMPs is dependent on NFAT and mitochondrial DAMPs, and that CsA impacts on type I IFN secretion and IFNAR signalling by CD103⁺ DCs. As such, this chapter will address the following aims:

1. Determine the importance of NFAT and mitochondrial ROS for Zymosan-induced responses in iCD103 DCs.
2. Assess whether CsA impacts Zymosan-induced type I IFN secretion and IFNAR signalling by iCD103 DCs.
3. Determine whether CsA inhibition of type I IFN secretion leads to a secondary reduction of other Zymosan-induced responses in iCD103 DCs.
4. Investigate whether IFNAR signalling is required for Zymosan-induced CD103⁺ DC activation *in vivo*.

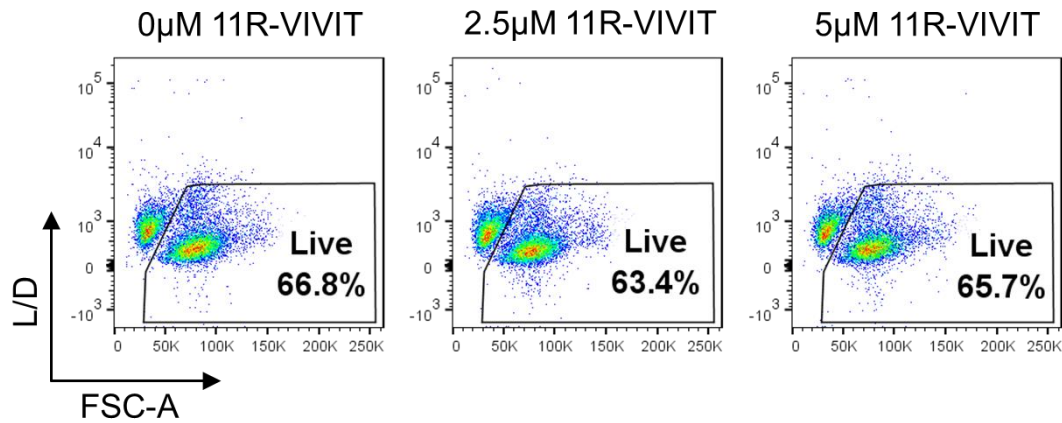
4.2 iCD103 DC secretion of IL-2 in response to Zymosan requires NFAT

In a number of cell types, CsA has been shown to inhibit the phosphatase calcineurin and thereby prevent the activation of NFAT. To date, this mechanism has been linked to the inhibition of IL-2, IL-12 and TNF α in DCs [130, 330, 334, 336, 337, 356]. Despite this, a number of Zymosan responses measured in chapter 3 remain to be assessed for their dependency on NFAT. Thus, it was decided to determine whether specific inhibition of NFAT reduced iCD103 DC production of other antifungal cytokines and the expression of co-stimulatory molecules. This was tested using the cell permeable peptide 11R-VIVIT, which directly interacts with the calcineurin-binding site for NFAT, inhibiting its nuclear translocation without affecting upstream calcineurin activity [459, 460]. This enables assessment of whether antifungal responses in iCD103 DCs are dependent on NFAT, which would be suggestive of the mechanism of CsA inhibition. Before testing for immunomodulation, it was first important to determine whether 11R-VIVIT affected iCD103 DC viability. Thus, cells were treated with 11R-VIVIT overnight. Cells were stained with BV510 Aqua viability dye and assessed for viability by flow cytometry. At the concentrations tested, 11R-VIVIT caused no significant decrease in viability compared to untreated cells (Fig. 4.1 A-B). In separate studies, supernatants were collected and assessed for cytotoxicity by LDH assay. Consistent with the flow cytometry data, 11R-VIVIT had no significant impact on iCD103 DC viability compared to untreated cells in LDH assays (Fig. 4.1 C).

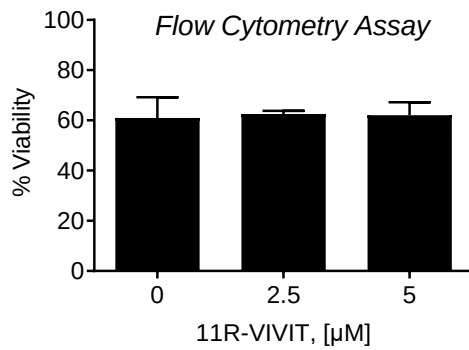
Upon confirming that 11R-VIVIT was not cytotoxic to iCD103 DCs, it was important to assess for inhibition of IL-2. Indeed, this cytokine has an established link with NFAT and was found to be produced by CD103⁺ DCs to protect against fungal infection in the lungs [232]. Thus, iCD103 DCs were pre-treated with 11R-VIVIT for 1h and stimulated with medium or

zymosan overnight. Supernatants were collected and analysed for IL-2 by ELISA. Consistent with CsA (Fig. 3.19), 11R-VIVIT significantly reduced Zymosan-induced secretion of IL-2 by iCD103 DCs (Fig. 4.2).

(A)



(B)



(C)

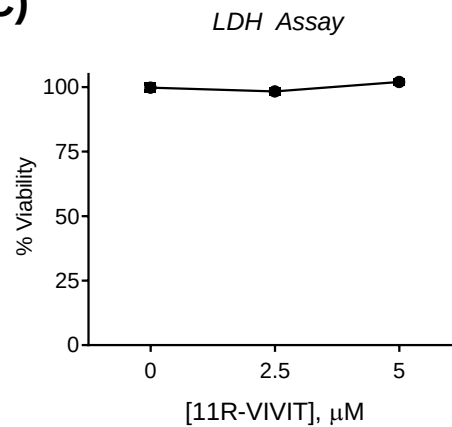


Fig. 4.1: iCD103 DCs are viable when cultured with the NFAT inhibitor 11R-VIVIT. iCD103 DCs were treated with 11R-VIVIT overnight. (A) Cells were stained with viability dye (L/D) and viability was analysed in single cells by flow cytometry. (B) Flow cytometry viability data with bars depicting mean values for duplicate samples. (C) Supernatants were collected and cytotoxicity was assessed by LDH assay, with symbols depicting the mean values for triplicate samples. Results are representative of two independent experiments.

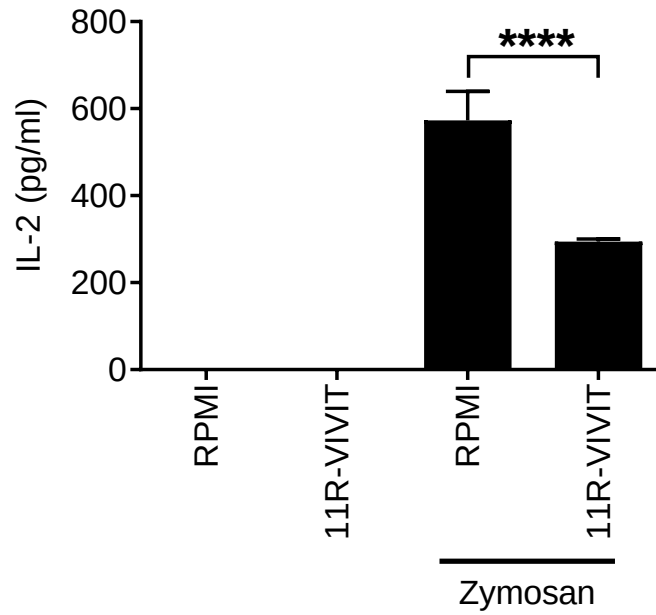
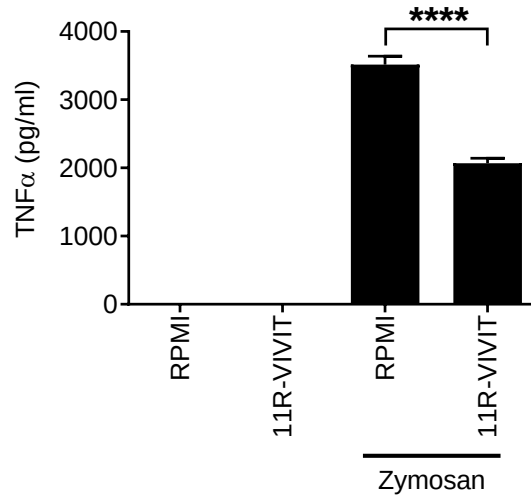


Fig. 4.2: 11R-VIVIT significantly inhibits Zymosan-induced IL-2 secretion by iCD103 DCs. iCD103 DCs were pre-treated with 11R-VIVIT (5 μ M) for 1 hour and stimulated with medium or zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for IL-2 by ELISA. Bars are mean $\pm$ SD for triplicate samples. ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test.

4.3 NFAT is required for optimal iCD103 DC secretion of TNF α and IL-12 in response to Zymosan

As discussed in chapter 1, TNF α and IL-12 are important mediators of antifungal immunity, highlighting the significance of CsA impairing secretion of these cytokines in iCD103 DCs (Fig. 3.20). Regarding the mechanism by which CsA reduces expression of TNF α and IL-12, one key study used the alternative calcineurin inhibitor tacrolimus and found that NFAT binds to the promoters of TNF α and IL-12 to enhance their expression [356]. Moreover, another group found that the NFAT inhibitor 11R-VIVIT impaired Zymosan-induced secretion of IL-12p70 in GM-CSF DCs [130]. To confirm whether NFAT was important for these responses in iCD103 DCs, cells were pre-treated with 11R-VIVIT for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for TNF α and IL-12p70 by ELISA. 11R-VIVIT significantly inhibited both Zymosan-induced secretion of TNF α and IL-12p70 by iCD103 DCs (Fig. 4.3 A-B).

(A)



(B)

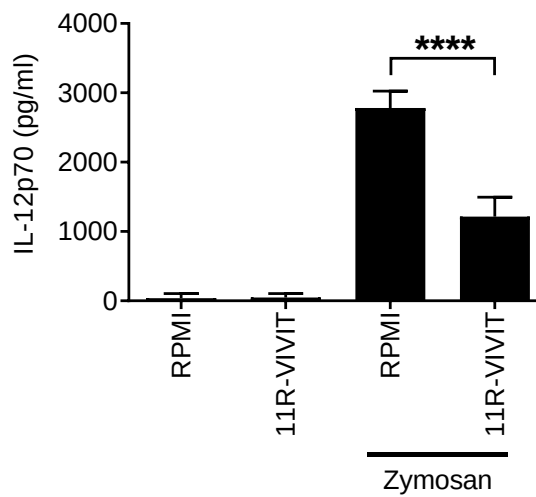
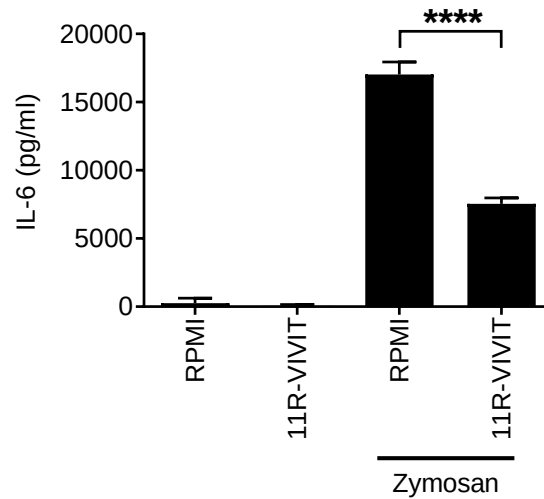


Fig. 4.3: Zymosan-induced TNF α and IL-12p70 secretion by iCD103 DCs is dependent on NFAT. iCD103 DCs were pre-treated with 11R-VIVIT (5 μ M) for 1 hour and stimulated with medium or zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for TNF α (A) and IL-12p70 (B) by ELISA. Bars are mean $\pm$ SD for triplicate samples. ****, $p < 0.0001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

4.4 Zymosan induction of IL-6 and IL-23 in iCD103 DCs is dependent on NFAT

The induction of Th17 cells is vital for preventing pathogenic fungal infections, particularly as these cells secrete IL-17, which is known to enable the recruitment of antifungal phagocytes to the site of infection [291]. Th17 cell differentiation is driven by a range of DC-produced cytokines, including IL-6 and IL-23 [4, 232, 291]. The ability of CsA to reduce IL-6 and IL-23 production by iCD103 DCs (Fig. 3.21) was therefore suggestive of impaired antifungal immunity. Importantly, the relevance of NFAT to these responses remained to be characterised. Thus, iCD103 DCs were pre-treated with 11R-VIVIT for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for IL-6 and IL-23 by ELISA. Interestingly, 11R-VIVIT significantly decreased IL-6 production by almost 3-fold (Fig. 4.4 A). In addition, 11R-VIVIT significantly inhibited Zymosan-induced secretion of IL-23 by iCD103 DCs (Fig. 4.4 B).

(A)



(B)

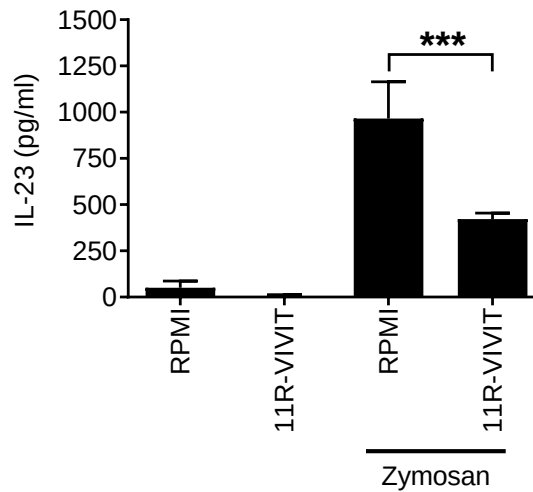


Fig. 4.4: NFAT inhibition leads to reduced IL-6 and IL-23 secretion by iCD103 DCs stimulated with Zymosan. iCD103 DCs were pre-treated with 11R-VIVIT (5 μ M) for 1 hour and stimulated with medium or zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for IL-6 (A) and IL-23 (B) by ELISA. Bars are mean $\pm$ SD for triplicate samples. ***, $p < 0.001$; ****, $p < 0.0001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

4.5 CsA increases the levels of mitochondrial reactive oxygen species induced by Zymosan

Previous work highlights that mitochondrial ROS can enter the cytosol – through the MPT pore – and act as intracellular signalling molecules that aid innate immune cell activation. For example, in BMDMs, ROS can contribute to the activation of the NLRP3 inflammasome, which is responsible for processing of IL-1 β [74, 385]. Across other studies, mitochondrial ROS have been found to promote the activation of MAVS and the cGAS-STING pathway, leading to secretion of type I IFNs [39, 113, 377]. Intriguingly, ROS were also found to increase human blood-derived DC maturation, in a mechanism that was dependent on NF κ B [461, 462]. As discussed, CsA inhibits opening of the MPT pore, preventing mitochondrial ROS – as well as other DAMPs, such as oxidised DNA – from being released into the cytosol to trigger these aforementioned pathways [3]. Therefore, CsA treatment was expected to cause an increase in the levels of ROS inside the mitochondria of iCD103 DCs stimulated with Zymosan.

To confirm this, cells were pre-treated with CsA, stimulated with Zymosan and stained for flow cytometry analysis with the mitochondrial ROS indicator MitoSOX. This detects superoxide generated specifically in the mitochondria [463]. MitoSOX fluorescence on live cells was analysed through histograms and MFI values. CsA had no significant impact on basal mitochondrial ROS production (Fig. 4.5 A-B). Zymosan marginally increased the level of MitoSOX fluorescence (Fig. 4.5 A-B). Importantly, CsA increased mitochondrial ROS levels in Zymosan-stimulated cells (Fig. 4.5 A-B).

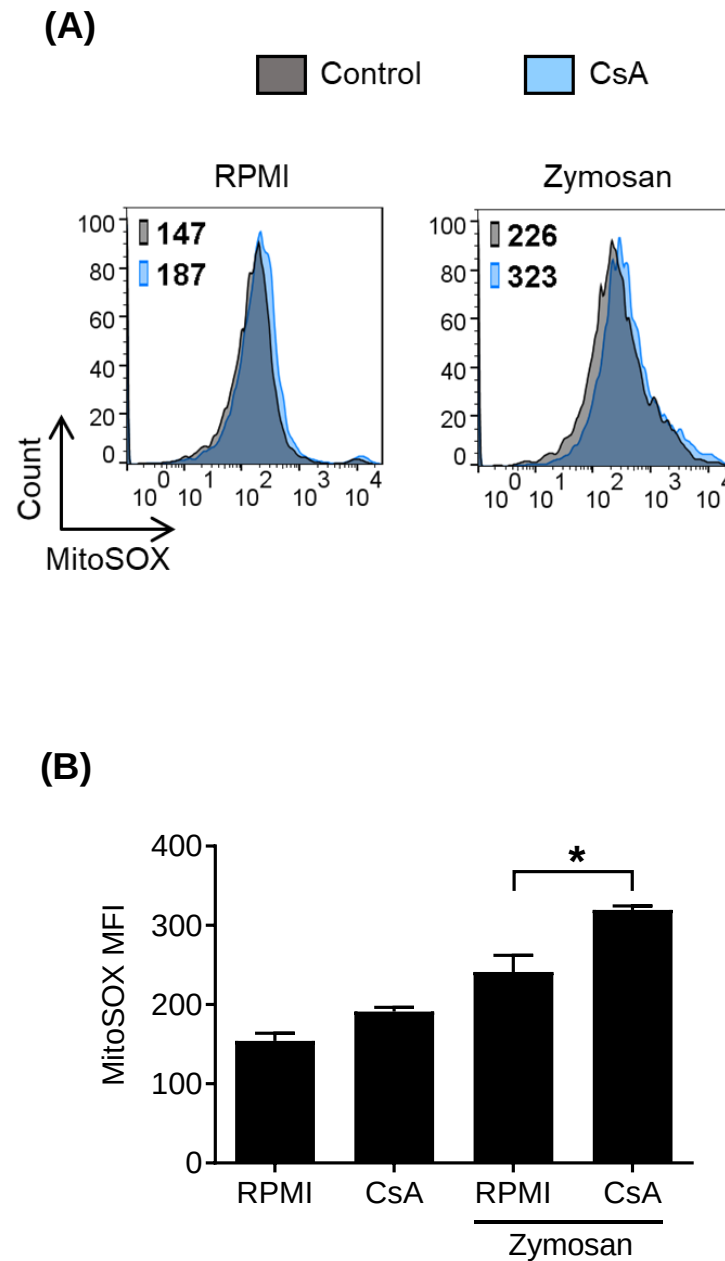


Fig. 4.5: CsA increases Zymosan-induced levels of mitochondrial ROS in iCD103 DCs. iCD103 DCs were pre-treated with 5 μ M CsA for 1h and stimulated with medium or zymosan (5 μ g/ml) for 1.5h. Cells were harvested and analysed for mitochondrial ROS production by MitoSOX flow cytometry assay. (A) Representative MitoSOX histograms with corresponding MFI values. (B) MitoSOX MFI values. Bars are mean $\pm$ SEM for two independent experiments. *, $p < 0.05$; One-way ANOVA, Tukey's multiple comparison test.

4.6 Mitochondrial ROS are important for Zymosan-induced secretion of IL-23, but not TNF α , IL-12p70 or IL-6 by iCD103 DCs

TNF α and IL-12 play key antifungal roles, enabling recruitment of neutrophils and priming of IFN γ ⁺ Th1 cells during fungal infection [152, 262]. Figures 3.20 and 4.3 highlight that CsA inhibits secretion of TNF α and IL-12 elicited by Zymosan in iCD103 DCs and this may occur through inhibition of NFAT, due to its important role in these responses. To better understand the mechanism of inhibition, the role of mitochondrial ROS species was assessed by using MitoTEMPO. This compound combines the antioxidant properties of piperidine nitroxide with a membrane-permeant cation that selectively accumulates within the mitochondria and quenches ROS [464]. Cells were pre-treated with MitoTEMPO for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for TNF α and IL-12p70 by ELISA. MitoTEMPO failed to inhibit secretion of TNF α induced by Zymosan, while IL-12p70 production was also unaffected (Fig. 4.6 A-B).

Previous studies found that mice genetically deficient for IL-6 and IL-23 were significantly more susceptible to infection by including *Candida albicans* and *Pneumocystis carinii* [292, 293, 456]. Indeed, key roles of these cytokines include polarisation of Th17 cells, which secrete IL-17, an essential cytokine for antifungal immunity due to its ability to mobilise protective neutrophils [4, 232, 291]. Regarding IL-6 and IL-23, CsA was found to inhibit Zymosan-induced secretion in iCD103 DCs, which required NFAT activity for optimal responses (Figures 3.21 and 4.4). The mechanism of inhibition was further explored by assessing whether mitochondrial ROS are required for these responses. Thus, iCD103 DCs were pre-treated with MitoTEMPO for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for IL-6 and IL-23 by ELISA. It was found that

MitoTEMPO treatment had no significant impact on IL-6 secretion (Fig. 4.6 C). However, MitoTEMPO caused a significant decrease in iCD103 DC secretion of IL-23 in response to Zymosan (Fig. 4.6 D).

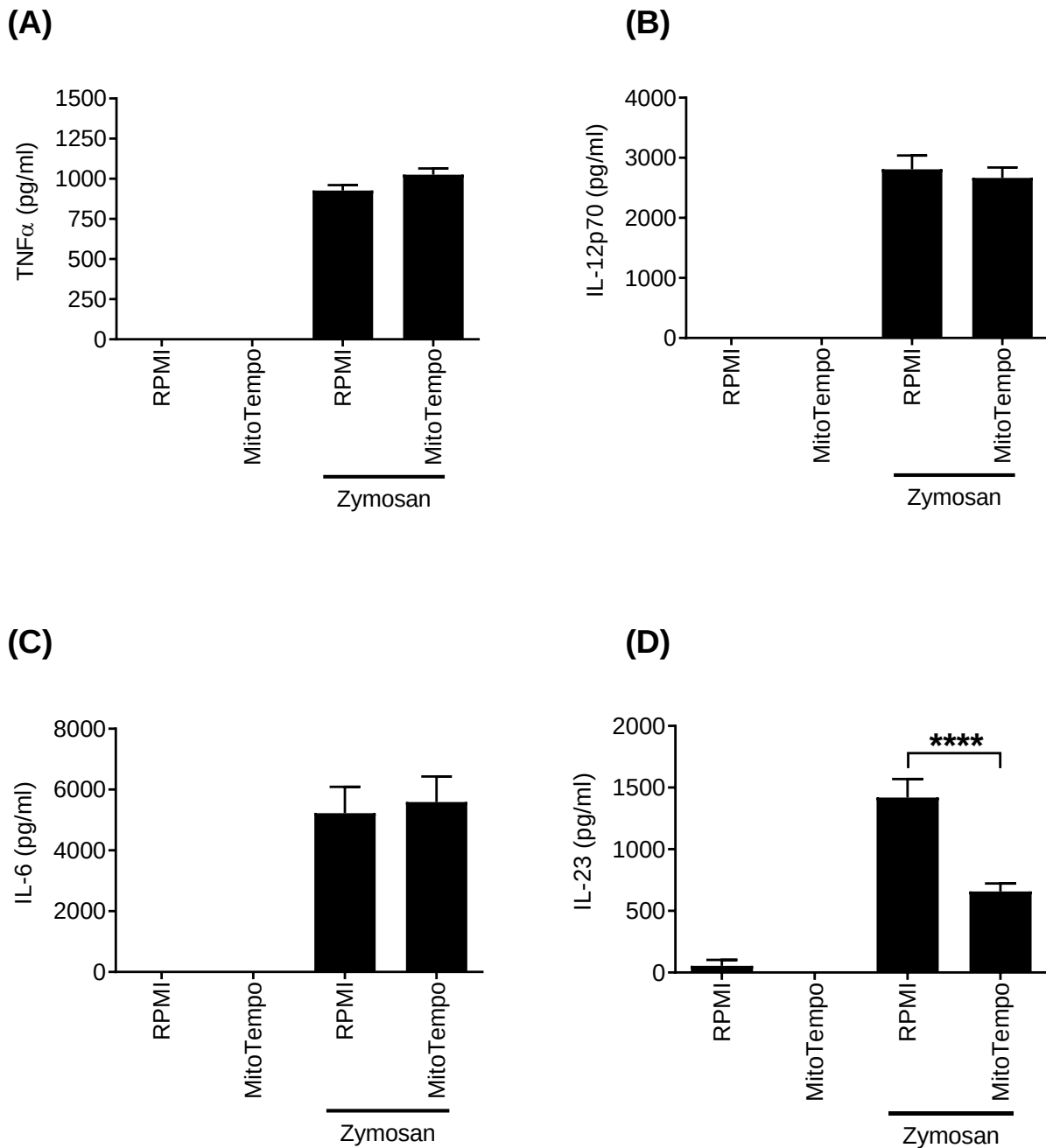


Fig. 4.6: Mitochondrial ROS facilitate Zymosan-induced secretion of IL-23 but not TNF α , IL-12p70 or IL-6 by iCD103 DCs. iCD103 DCs were pre-treated with MitoTEMPO (500 μ M) for 1 hour and stimulated with medium or zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for TNF α (A), IL-12p70 (B), IL-6 (C) and IL-23 (D) by ELISA. Bars are mean $\pm$ SD for triplicate samples. ****, $p < 0.0001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

4.7 NFAT is required for optimal upregulation of co-stimulatory molecules by iCD103 DCs stimulated with Zymosan

In both *in vitro* and *in vivo* models, detection of fungal PAMPs by DCs leads to upregulation of CD40, CD80 and CD86 [4, 290]. This equips these cells to prime protective T cell responses within lymph nodes proximal to the site of infection [4]. CsA is likely to impair the ability of CD103⁺ DCs to prime T cells, as highlighted by Neoral® reducing co-stimulatory molecule expression on these cells in lung-draining lymph nodes (Fig. 3.10), as well as CsA-mediated inhibition of co-stimulatory molecules on iCD103 DCs (Fig. 3.24-3.26). Regarding the mechanism, the role of NFAT itself in DC maturation remains to be demonstrated. Thus, to assess this, iCD103 DCs were pre-treated with the NFAT inhibitor 11R-VIVIT for 1h and stimulated with Zymosan overnight. Cells were harvested and stained for viability, CD11c, MHC II, CD40, CD80 and CD86 and analysed by flow cytometry. Live DCs were identified using the same gating strategy as in figure 3.15. From this population, histograms and MFI values were obtained for CD40, CD80 and CD86. NFAT inhibition led to significantly reduced expression of CD40 in iCD103 DCs treated with Zymosan (Fig. 4.7 A-B). Moreover, 11R-VIVIT had a limited effect on Zymosan-induced CD80 (Fig. 4.8 A-B) but significantly reduced the upregulation of CD86 (Fig. 4.9 A-B).

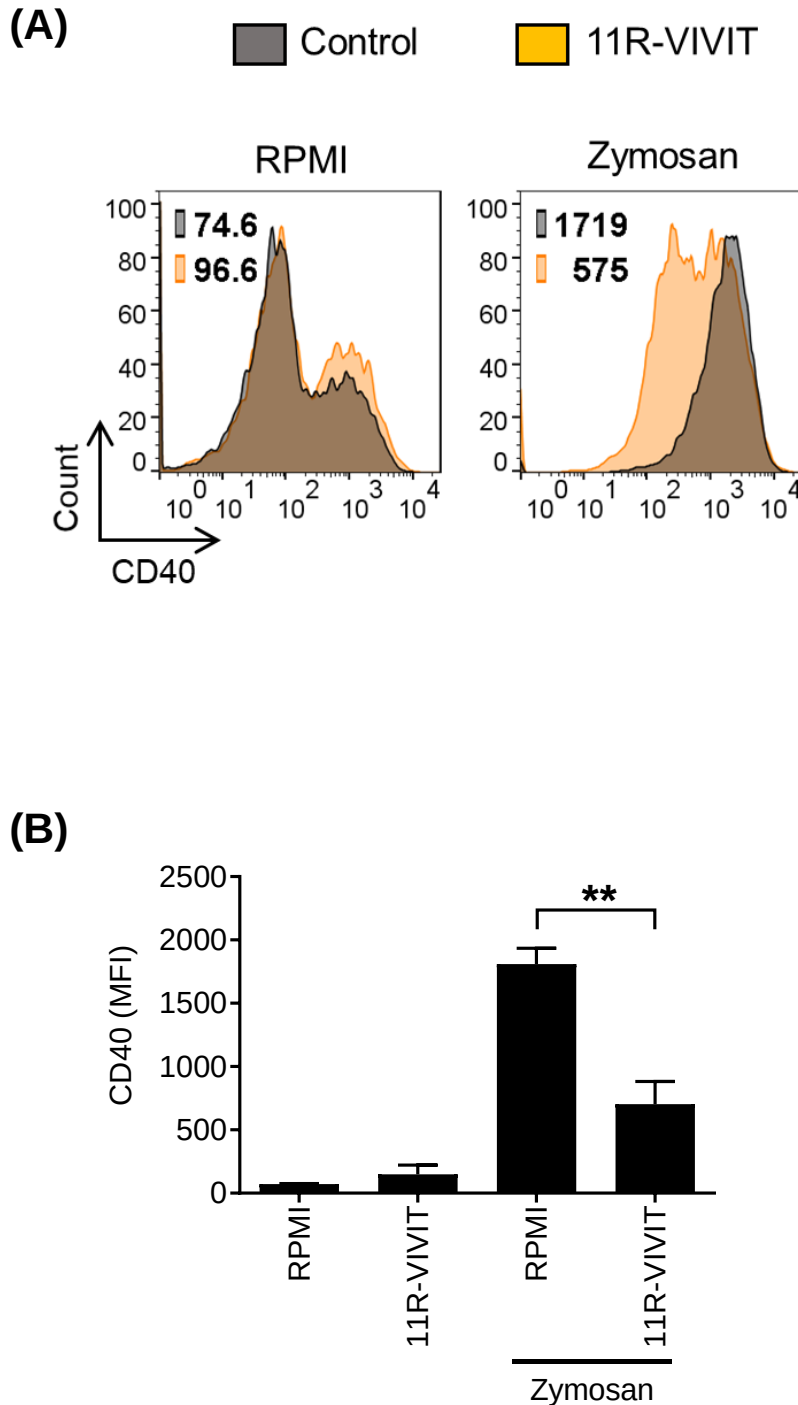


Fig. 4.7: Direct NFAT inhibition significantly impairs CD40 upregulation in Zymosan-stimulated iCD103 DCs. iCD103 DCs were pre-treated with 5 μ M 11R-VIVIT for 1h and stimulated overnight with zymosan (1 μ g/ml). Expression of CD40 was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CD40 expression in iCD103 DCs represented as histograms with corresponding MFI values. (B) CD40 expression represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. **, $p < 0.01$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

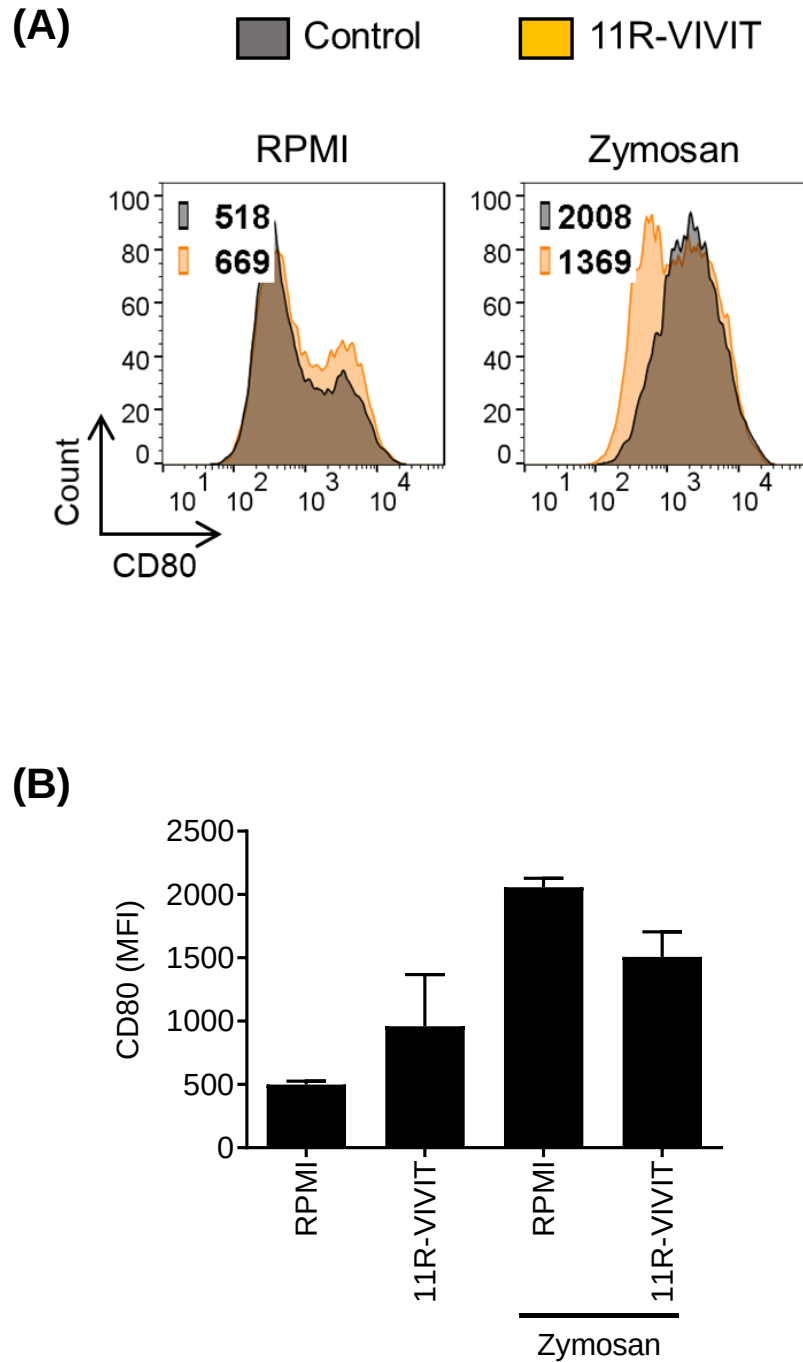


Fig. 4.8: Direct NFAT inhibition leads to impaired upregulation of CD80 in iCD103 DCs stimulated with Zymosan. iCD103 DCs were pre-treated with 5 μ M 11R-VIVIT for 1h and stimulated overnight with zymosan (1 μ g/ml). Expression of CD80 was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CD80 expression in iCD103 DCs represented as histograms with corresponding MFI values. (B) CD80 expression represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. Results are representative of three independent experiments.

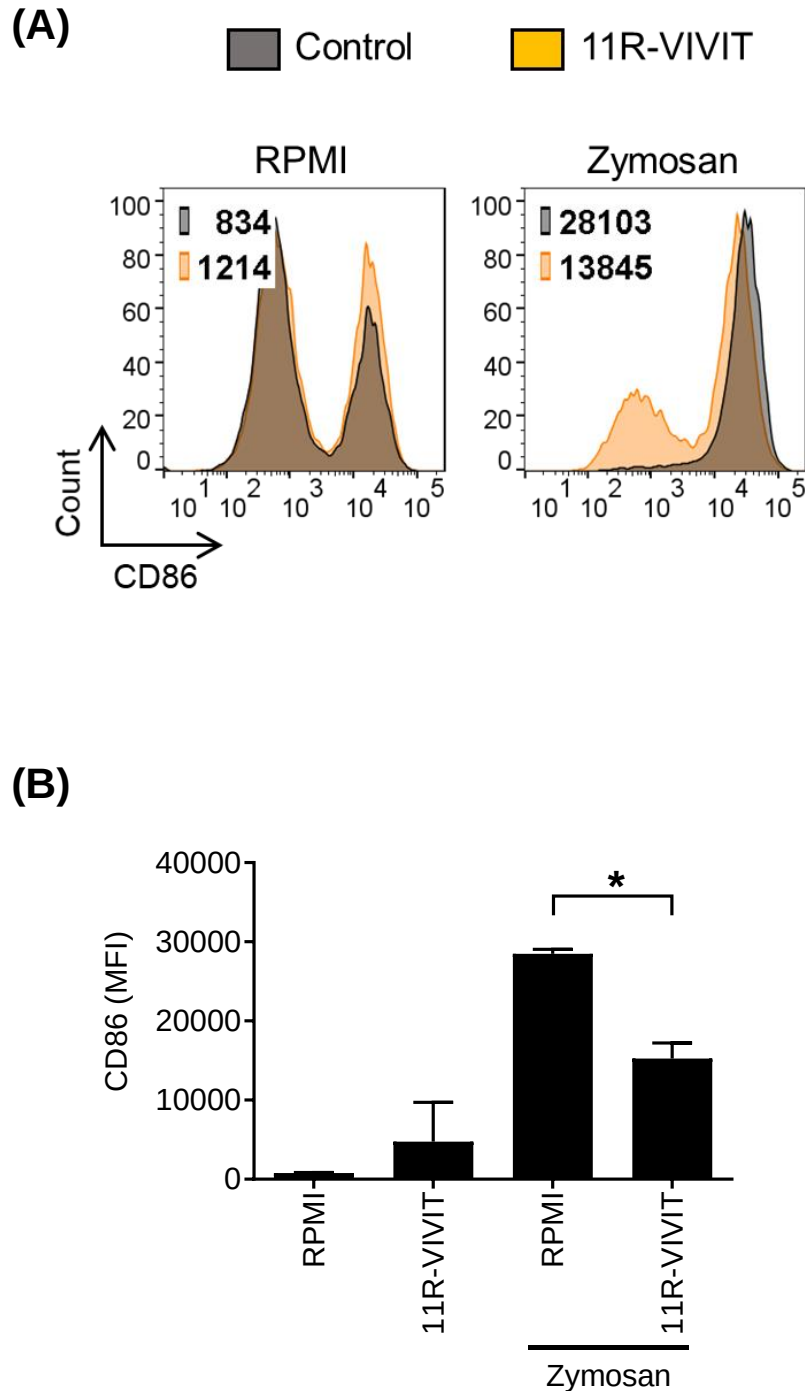


Fig. 4.9: Direct NFAT inhibition significantly reduces the upregulation of CD86 in iCD103 DCs stimulated with Zymosan. iCD103 DCs were pre-treated with 5 μ M 11R-VIVIT for 1h and stimulated overnight with zymosan (1 μ g/ml). Expression of CD86 was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CD86 expression in iCD103 DCs represented as histograms with corresponding MFI values. (B) CD86 expression represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. *, $p < 0.05$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of three independent experiments.

4.8 NFAT inhibition moderately reduces Zymosan-induced upregulation of CCR7 by iCD103 DCs

DC uptake of fungi and migration to the draining lymph nodes is essential for inducing adaptive T cell responses, which ultimately enables the clearance of pathogenic fungal infections [288]. A key mediator of DC migration is CCR7 [161]. Therefore, the reduction of CCR7 expression in iCD103 DCs by CsA (Fig. 3.27) would be consistent with the impaired antifungal immunity observed with this drug. With an interest in the mechanism of inhibition, it was decided to assess whether NFAT specifically was playing a role in the Zymosan-induced CCR7 response. Hence, iCD103 DCs were pre-treated with the NFAT inhibitor 11R-VIVIT for 1h and stimulated with Zymosan overnight. Cells were harvested and stained for viability, CD11c, MHC II and CCR7 and analysed by flow cytometry. Live DCs were identified using the same gating strategy as in figure 3.15. From this population, histograms and MFI values were obtained for CCR7. It was observed that NFAT inhibition by 11R-VIVIT reduced the upregulation of CCR7 induced by Zymosan stimulation (Fig. 4.10 A-B).

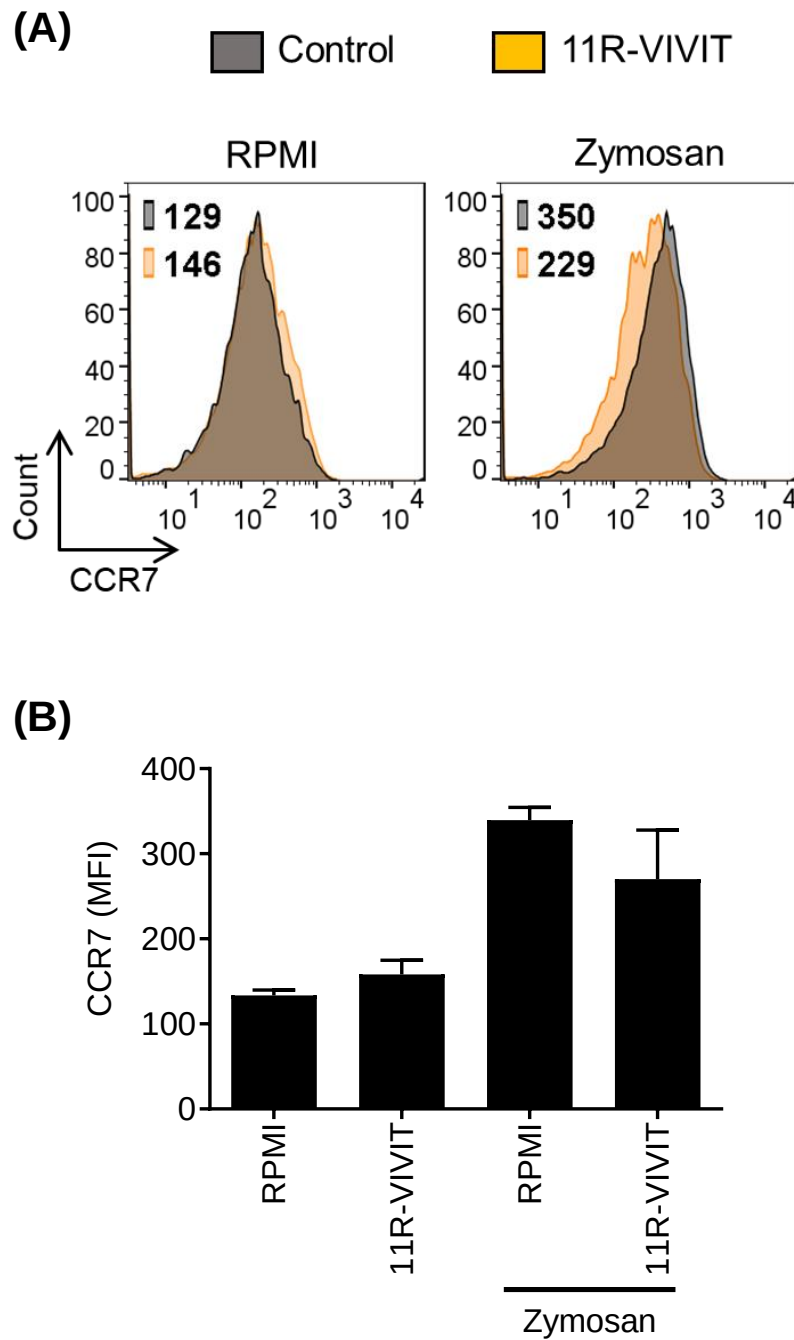


Fig 4.10: NFAT is required for optimal upregulation of CCR7 on iCD103 DCs stimulated with Zymosan. iCD103 DCs were pre-treated with 5 μ M 11R-VIVIT for 1h and stimulated overnight with zymosan (1 μ g/ml). Expression of CCR7 was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) CCR7 expression in iCD103 DCs represented as histograms with corresponding MFI values. (B) CCR7 expression represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. Results are representative of three independent experiments.

4.9 MitoTEMPO treatment marginally reduces Zymosan-induced iCD103 DC upregulation of CD40 and CD80 but not CD86

DC maturation in response to fungal PAMPs is a pre-requisite for the priming of antifungal T cell responses, including Th17 and Th1 cells that enable the clearance of pathogenic infections [290]. With CsA inhibiting Zymosan-induced co-stimulatory molecules expression in iCD103 DCs (Fig. 3.24-3.26), and the importance of NFAT for these responses being confirmed (Fig. 4.7-4.9), it was important to address whether mitochondrial ROS also played a role. Indeed, mitochondrial ROS can contribute to DC activation in some settings and CsA is known to prevent the release of these mediators into the cytosol [3, 39, 74, 113, 377, 385].

To test this, iCD103 DCs were pre-treated with MitoTEMPO for 1h and stimulated with Zymosan overnight. Cells were harvested and stained for viability, CD11c, MHC II, CD40, CD80 and CD86 and analysed by flow cytometry. Live DCs were identified using the same gating strategy as in figure 3.15. From this population, histograms and MFI values were obtained for each co-stimulatory molecule. MitoTEMPO treatment marginally reduced Zymosan-induced CD40 responses (Fig. 4.11 A-B). Similarly, MitoTEMPO led to a marginal reduction in Zymosan-induced upregulation of CD80 (Fig. 4.12 A-B). However, CD86 expression in response to Zymosan was not impacted by MitoTEMPO (Fig. 4.13 A-B).

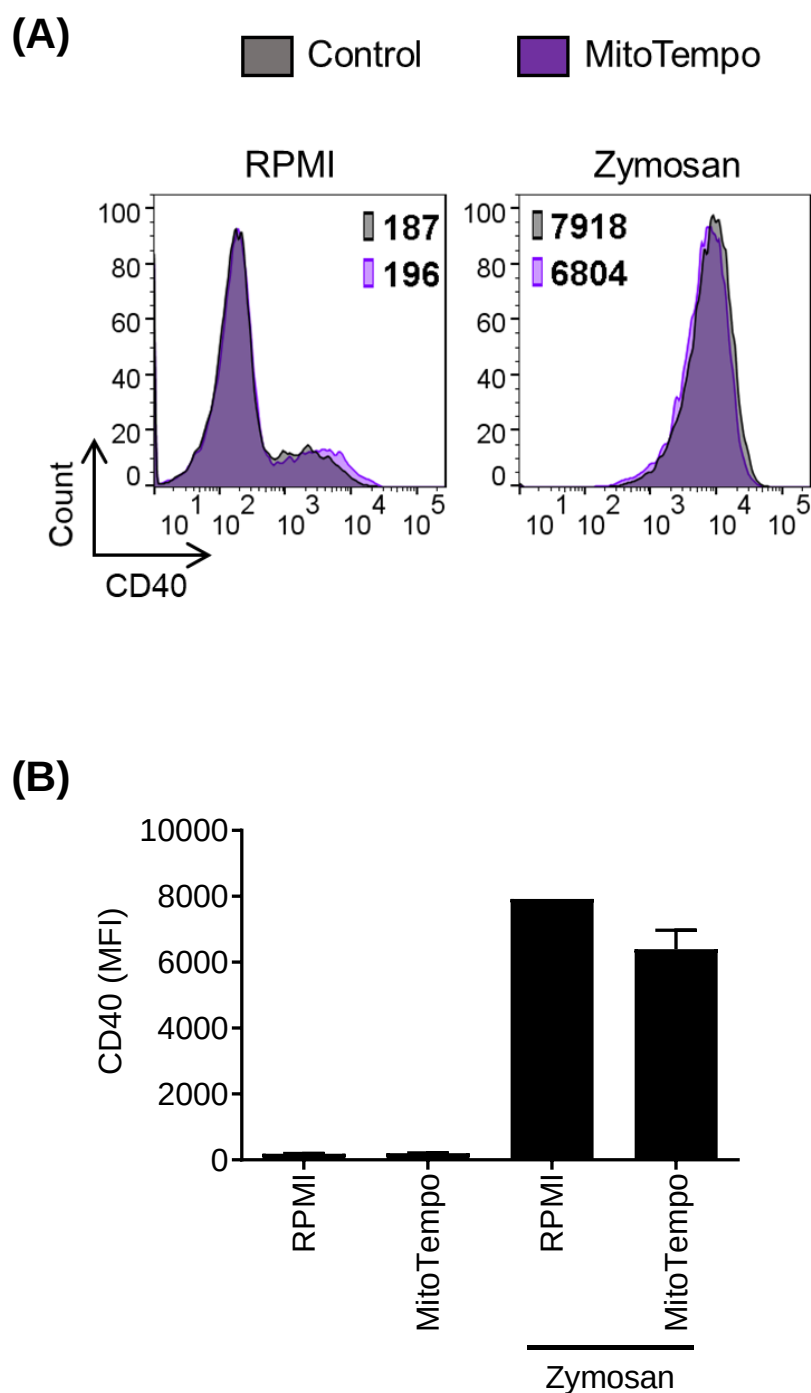


Fig. 4.11: MitoTempo treatment marginally impairs the upregulation of CD40 in iCD103 DCs stimulated with Zymosan. iCD103 DCs were pre-treated with 500 μ M MitoTEMPO for 1h and stimulated overnight with zymosan (1 μ g/ml). (A) Representative histograms and corresponding MFI values for CD40 expression in iCD103 DCs. (B) CD40 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. Results are representative of two independent experiments.

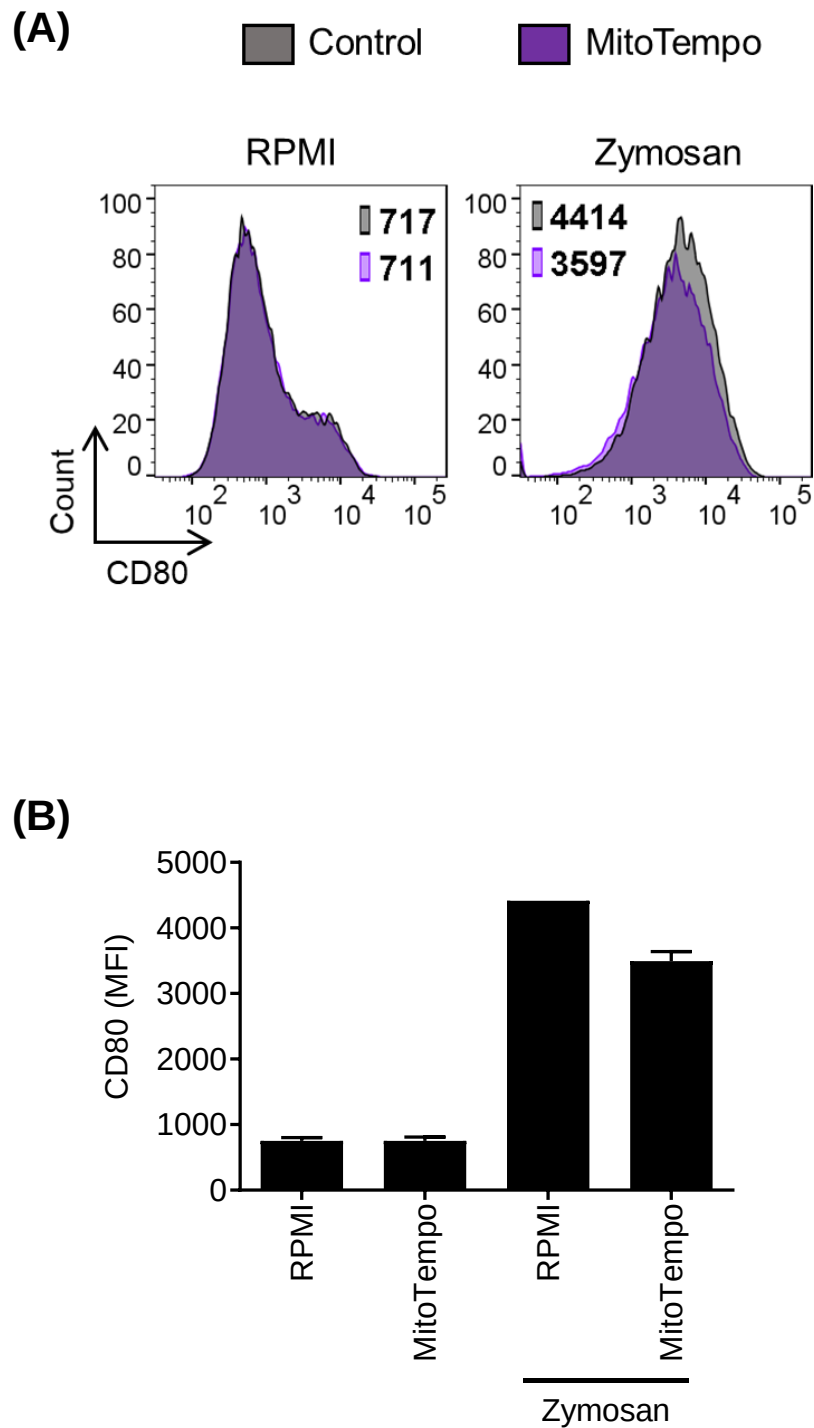


Fig. 4.12: MitoTempo moderately reduces Zymosan-induced CD80 expression in iCD103 DCs. iCD103 DCs were pre-treated with 500 μ M MitoTEMPO for 1h and stimulated overnight with zymosan (1 μ g/ml). (A) Representative histograms and corresponding MFI values for CD80 expression in iCD103 DCs. (B) CD80 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. Results are representative of two independent experiments.

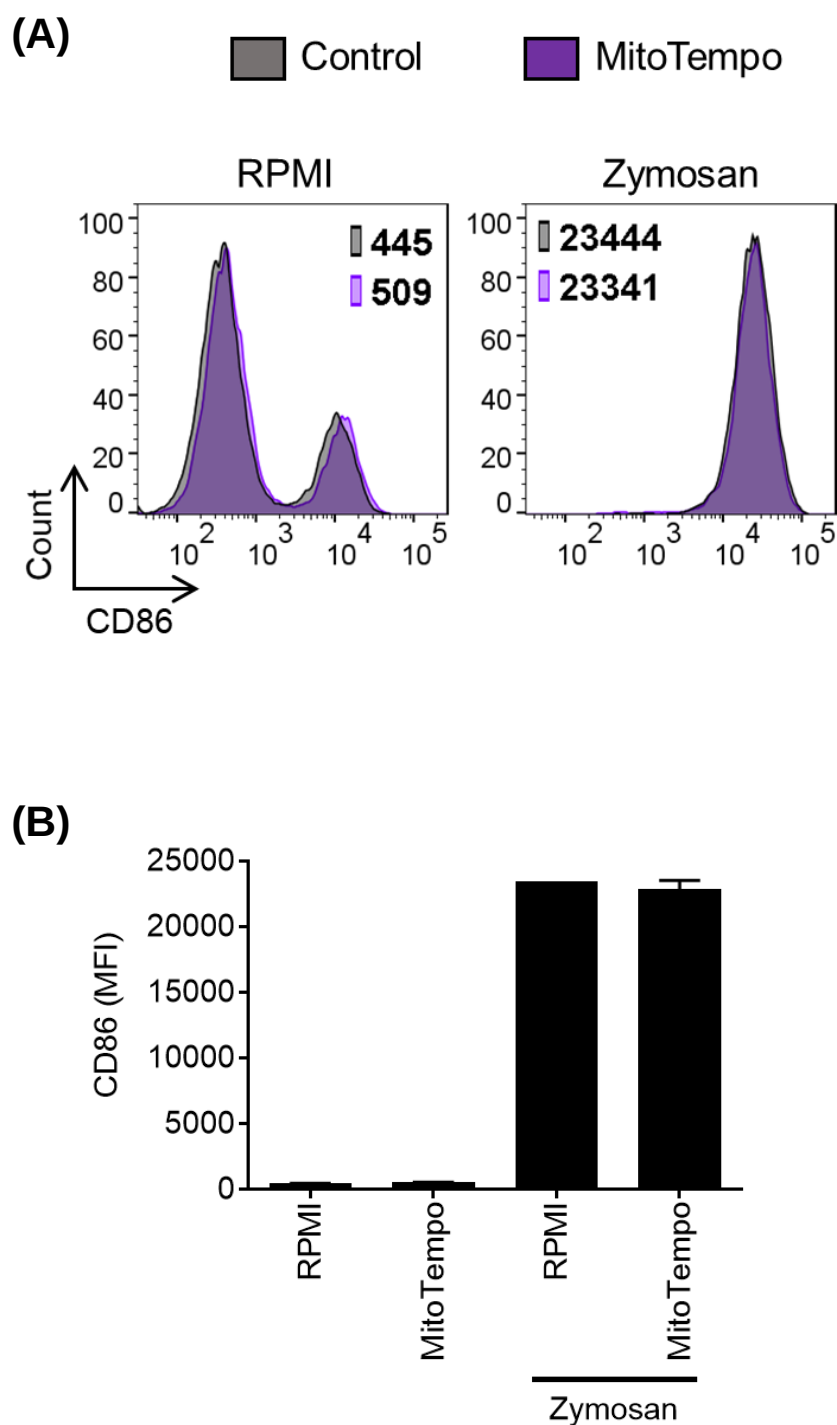


Fig. 4.13: MitoTempo treatment does not significantly impact Zymosan-induced CD86 expression in iCD103 DCs. iCD103 DCs were pre-treated with 500 μ M MitoTEMPO for 1h and stimulated overnight with zymosan (1 μ g/ml). (A) Representative histograms and corresponding MFI values for CD86 expression in iCD103 DCs. (B) CD86 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. Results are representative of two independent experiments.

4.10 CsA inhibits iCD103 DC production of type I IFNs, which requires NFAT activity but not mitochondrial ROS

A number of studies have reported that type I IFNs protect against fungal infections [150-152, 435, 436, 439]. Key roles of type I IFNs include enhancing IL-12-dependent induction of IFN γ ⁺ T cells, which are known to activate antifungal macrophages [152, 436]. A strong type I immune response may also provide a counterbalance that prevents excessive Th17 cell responses, where dysregulated neutrophil recruitment is avoided [152, 436]. Of great interest to this research, one group found that CD103⁺ DCs were the dominant producer of type I IFNs in the lungs during infection with *Histoplasma capsulatum* [152]. Thus, it was highly important to assess whether iCD103 DCs secreted type I IFNs in response to Zymosan and whether this was inhibited by CsA. To test this, iCD103 DCs were pre-treated with CsA for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for IFN β secretion by ELISA. Intriguingly, CsA significantly inhibited Zymosan-induced IFN β production in iCD103 DCs stimulated with Zymosan (Fig. 4.14 A).

The specific role of NFAT in IFN β secretion has not been extensively studied and it was therefore important to analyse the impact of 11R-VIVIT treatment in the iCD103 DC model. Thus, cells were pre-treated with 11R-VIVIT for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for IFN β by ELISA. Intriguingly, 11R-VIVIT abrogated Zymosan-induced IFN β production in iCD103 DCs (Fig. 4.14 B).

Since fungal type I IFN responses have previously been linked to mitochondrial signalling [465], it was decided to assess whether there was a similar dependency on mitochondrial ROS in this model. Therefore, iCD103 DCs were pre-treated with MitoTEMPO for 1h and stimulated with Zymosan overnight. Supernatants were collected and analysed for IFN β by

ELISA. MitoTEMPO failed to inhibit Zymosan-induced IFN β secretion by iCD103 DCs (Fig. 4.14 C).

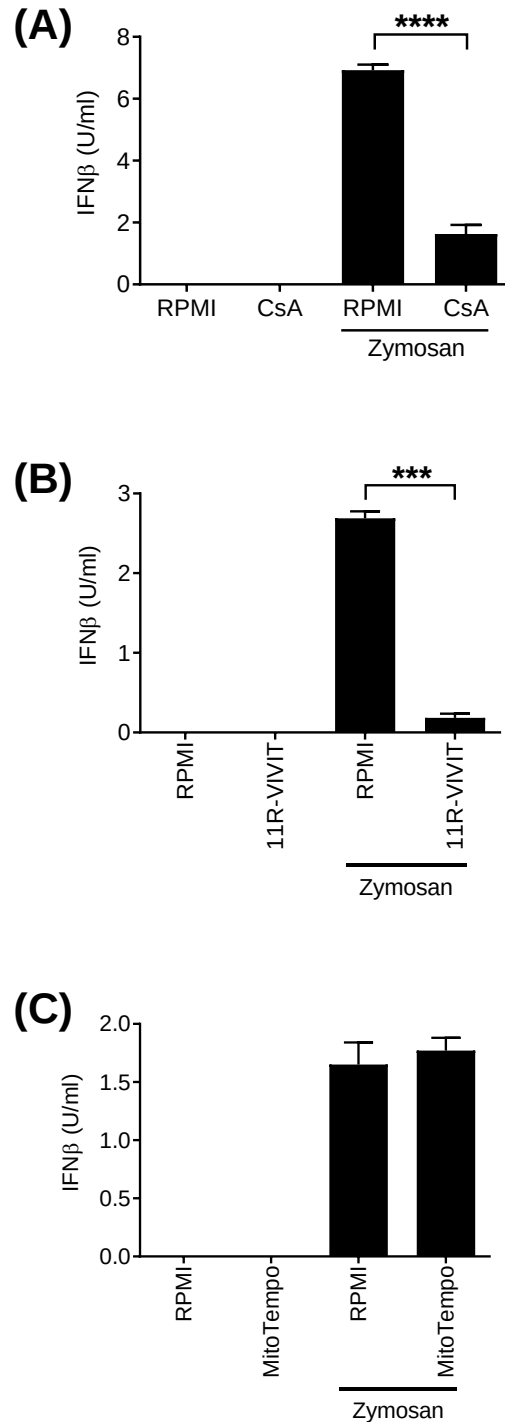


Fig 4.14: CsA significantly inhibits Zymosan-induced type I IFN secretion in iCD103 DCs which requires NFAT but not Mitochondrial ROS. iCD103 DCs were pre-treated with 5 μ M CsA (A), 5 μ M 11R-VIVIT (B) or 500 μ M MitoTempo (C) for 1h and stimulated with Zymosan (5 μ g/ml) overnight. Supernatants were collected and analysed for IFN β by ELISA. Bars are mean $\pm$ SD for triplicate samples. ***, $p < 0.001$; ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

4.11 CsA inhibits Zymosan-induced IFNAR signalling in iCD103 DCs

While type I IFNs protect against fungi, the mechanism by which this occurs is signalling through their receptor, IFNAR [150-152, 435, 436, 439]. After demonstrating that CsA significantly reduced antifungal type I IFN secretion by iCD103 DCs, a more important question was whether CsA reduced IFNAR signalling in these cells. As highlighted in section 1.4, the key effector of IFNAR signalling is the transcription factor STAT1. IFNAR signalling activates the kinases JAK1 and TYK2, which subsequently activate STAT1 by phosphorylation. Therefore, it was decided to assess the impact of CsA on IFNAR signalling by western blotting for phosphorylated STAT1 (pSTAT1). iCD103 DCs were pre-treated with CsA for 1h and stimulated with Zymosan for 2h or recombinant IFN β for 15min. Cells were lysed and analysed for STAT1 phosphorylated at residue Y701, as well as total STAT1 and β -actin for loading controls. As expected, cells that were unstimulated or treated with CsA alone had no detectable pSTAT1 (Fig. 4.15). Interestingly, Zymosan caused a strong increase in pSTAT1 and this was significantly reduced by CsA treatment (Fig. 4.15). Direct treatment with IFN β also engaged IFNAR signalling, inducing a strong pSTAT1 response but this was not inhibited by prior CsA treatment (Fig. 4.15).

After confirming that Zymosan induces iCD103 DCs to secrete type I IFNs and activate IFNAR signalling, it was important to confirm whether pSTAT1 activation was dependent on IFNAR or through another mechanism triggered by Zymosan. Indeed, this would confirm whether CsA inhibition of pSTAT1 activation was due to impaired type I IFN secretion or through a different mechanism specific to Zymosan. To test this, both WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with Zymosan for 2h or IFN β for 15min. As expected, unstimulated cells exhibited no pSTAT1 activation and IFN β -induced pSTAT1 failed to occur in IFNAR-

deficient cells (Fig. 4.16). Interestingly, pSTAT1 activation induced by Zymosan was also entirely dependent on IFNAR (Fig. 4.16).

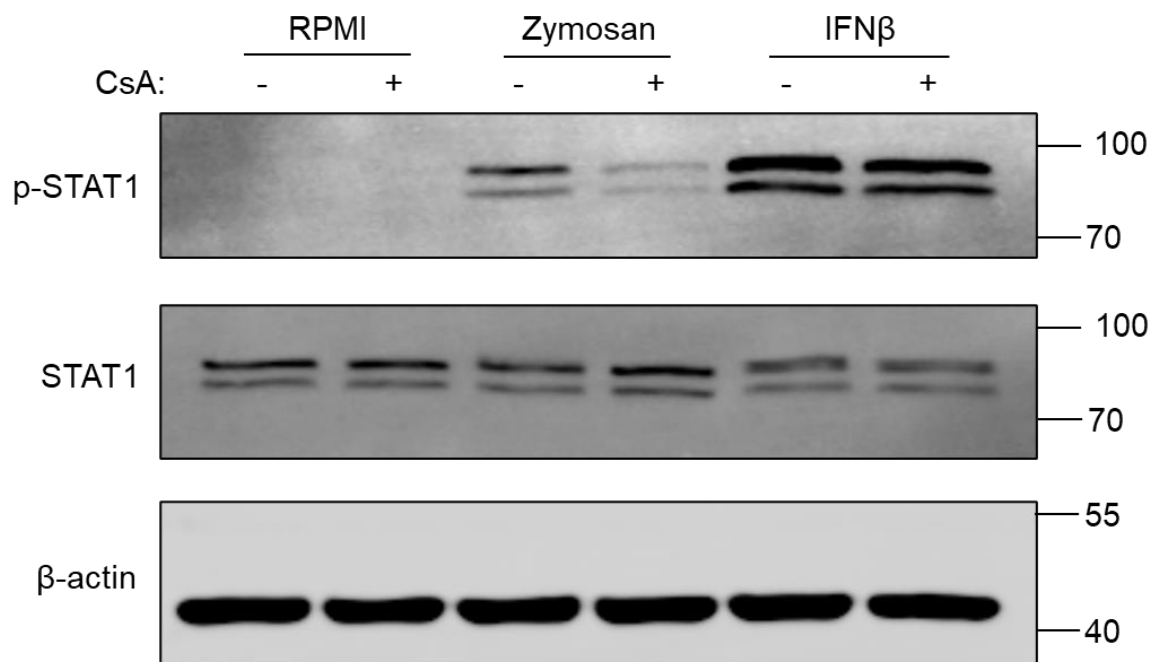


Fig 4.15: CsA significantly inhibits Zymosan-induced STAT1 activation in iCD103 DCs. iCD103 DCs were pre-treated with 5μM CsA for 1h and stimulated with Zymosan (10μg/ml) for 2h or IFNβ (40U/ml) for 15min. Cells were lysed and analysed for pSTAT1 protein by western blot, with total STAT1 and β-actin as loading controls. Results are representative of three independent experiments.

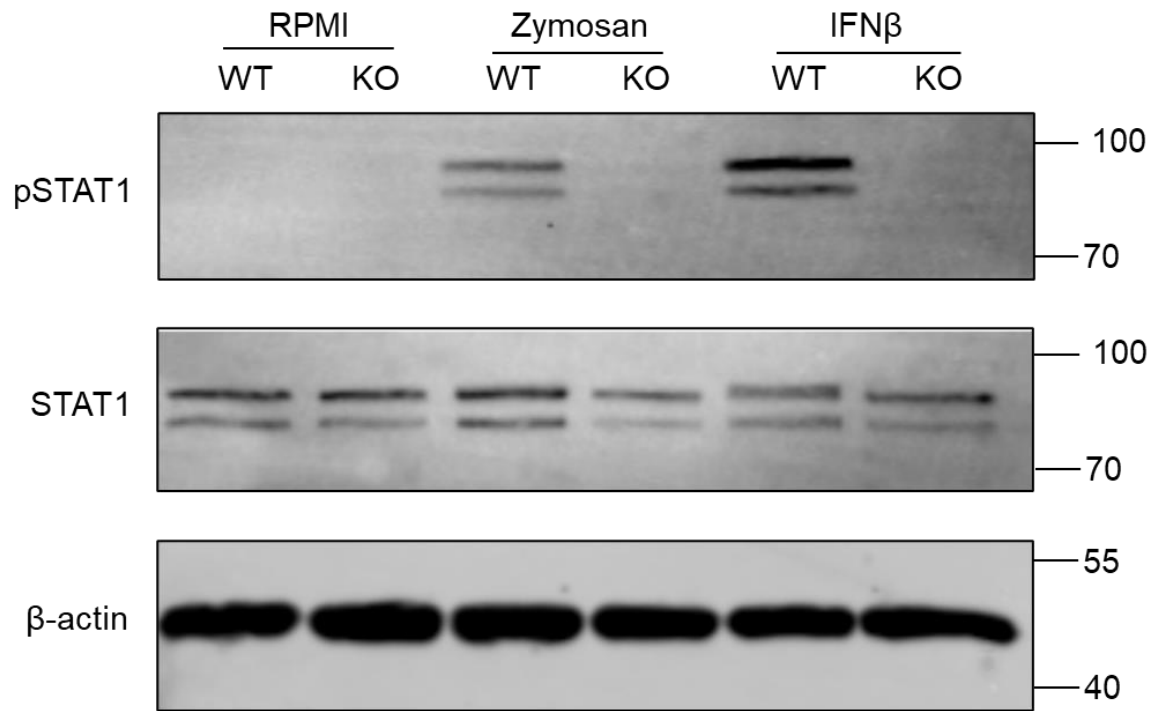


Fig 4.16: Zymosan-induced STAT1 activation in iCD103 DCs is entirely IFNAR-dependent. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with Zymosan (10 μ g/ml) for 2h or IFN β (40U/ml) for 15min. Cells were lysed and analysed for pSTAT1 protein by western blot, with total STAT1 and β -actin as loading controls. Results are representative of two independent experiments.

4.12 iCD103 DC maturation in response to Zymosan requires IFNAR signalling

These studies have characterised CsA inhibition of antifungal responses by CD103⁺ DCs and implicated NFAT as a potential mechanistic target. A particularly important finding was that CsA significantly inhibited Zymosan-induced type I IFN secretion and subsequent IFNAR signalling (section 4.10-4.11). As discussed, type I IFN secretion and IFNAR signalling are now established as essential mechanisms of protection against fungal infections [150-152, 435, 436, 439]. In DCs, IFNAR signalling has been found to significantly alter the DC transcriptional landscape, yielding an activated phenotype that is more equipped to prime adaptive T cell responses (see section 1.5 for detailed discussion) [136, 160]. As such, it was hypothesised that the inhibition of iCD103 DC secretion of type I IFNs may contribute to the suppression of other Zymosan responses, such as the upregulation of maturation markers and CCR7 and cytokine production. Therefore, the role of IFNAR signalling in Zymosan responses in the iCD103 DC model was addressed. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan overnight. Cells were harvested and stained for viability, CD11c, MHC II, CD40, CD80, CD86 and CCR7 and analysed by flow cytometry. Live DCs were identified using the same gating strategy as in figure 3.15. From this population, histograms and MFI values were obtained for CD40, CD80, CD86 and CCR7.

Regarding CD40, *Ifnar*^{-/-} iCD103 DCs expressed significantly lower levels than WT cells after Zymosan stimulation, as indicated by histograms (Fig. 4.17 A) and graphed MFI values (Fig. 4.17 B). Similarly, Zymosan-induced CD80 upregulation in iCD103 DCs was significantly impaired by IFNAR deficiency (Fig. 4.18 A-B). Moreover, when assessing CD86, IFNAR-deficient iCD103 DCs expressed significantly lower levels than WT cells in response to Zymosan (Fig. 4.19 A-B). In addition to analysing co-stimulatory molecules, it was important to assess expression of CCR7, as CsA was found to inhibit the upregulation of this protein

(Fig. 3.27). Intriguingly, IFNAR deficiency significantly reduced Zymosan-induced CCR7 expression (Fig. 4.20 A-B).

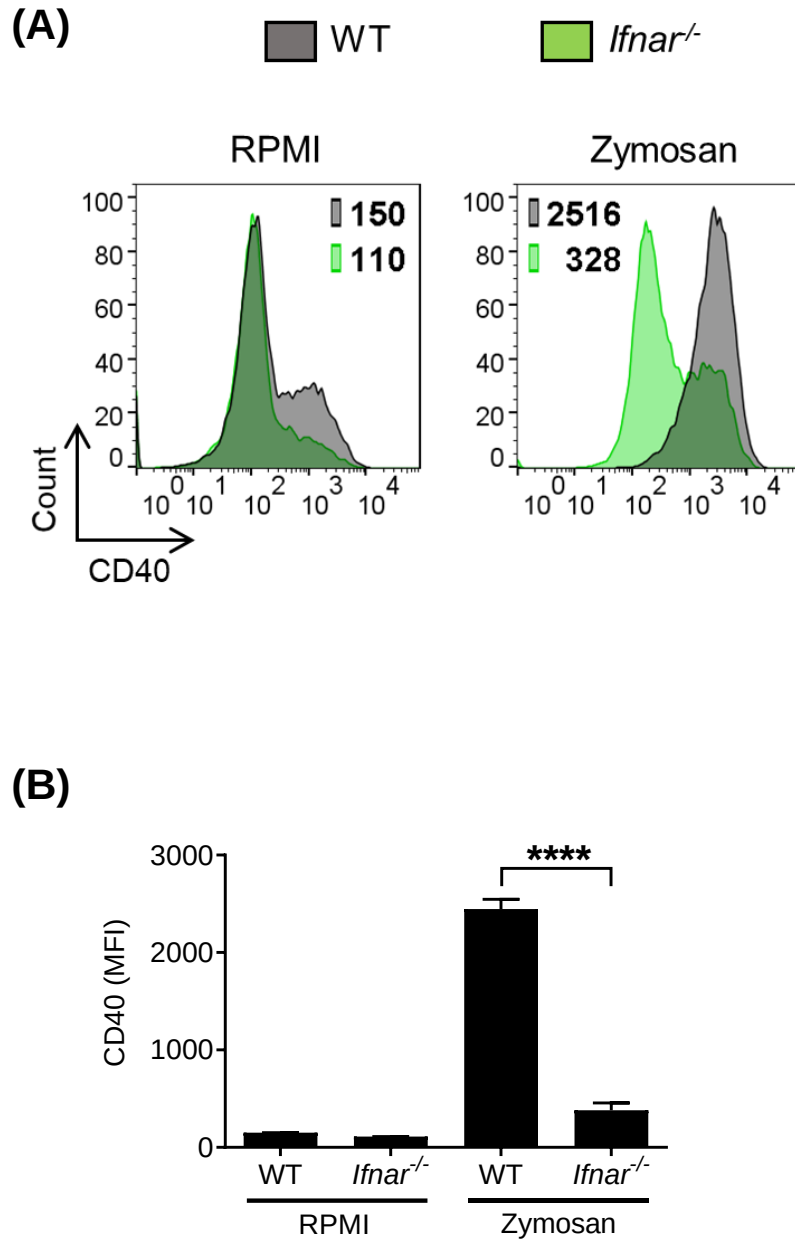


Fig 4.17: IFNAR signalling is essential for CD40 upregulation in iCD103 DCs stimulated with Zymosan. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan (125ng/ml) overnight. CD40 expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) Representative histograms and corresponding MFI values for CD40 expression in iCD103 DCs. (B) CD40 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ****, $p < 0.0001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of two independent experiments.

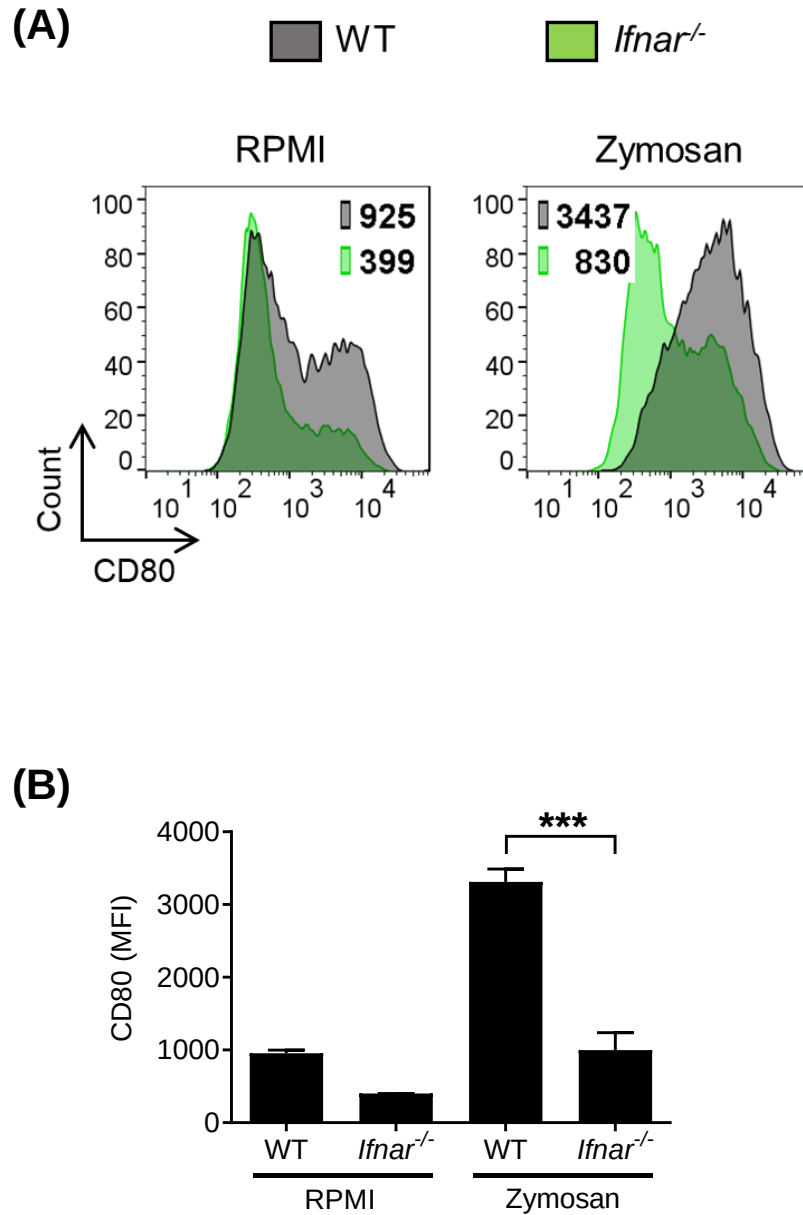


Fig 4.18: IFNAR deficiency significantly impairs Zymosan-induced upregulation of CD80 in iCD103 DCs. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan (125ng/ml) overnight. CD80 expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) Representative histograms and corresponding MFI values for CD80 expression in iCD103 DCs. (B) CD80 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ***, $p < 0.001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of two independent experiments.

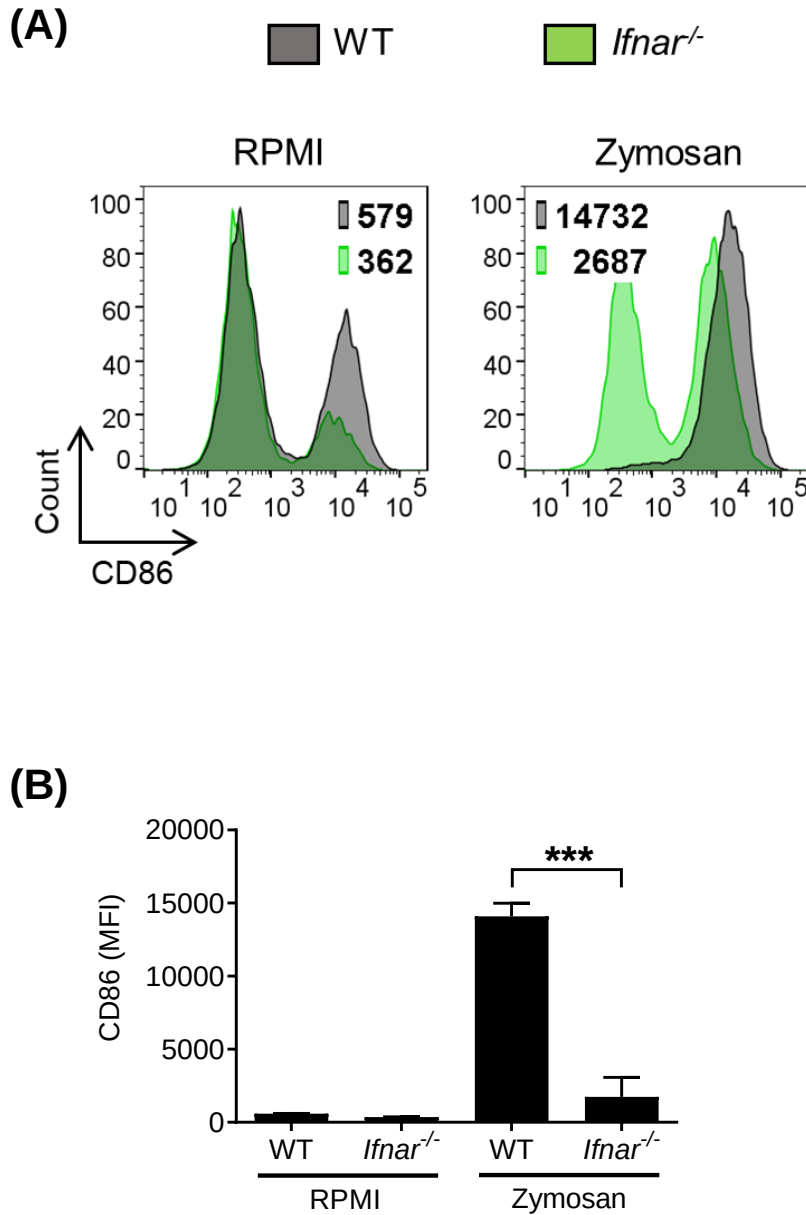


Fig 4.19: IFNAR is essential for zymosan-induced upregulation of CD86 in iCD103 DCs. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan (125ng/ml) overnight. CD86 expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) Representative histograms and corresponding MFI values for CD86 expression in iCD103 DCs. (B) CD86 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ***, p<0.001; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of two independent experiments.

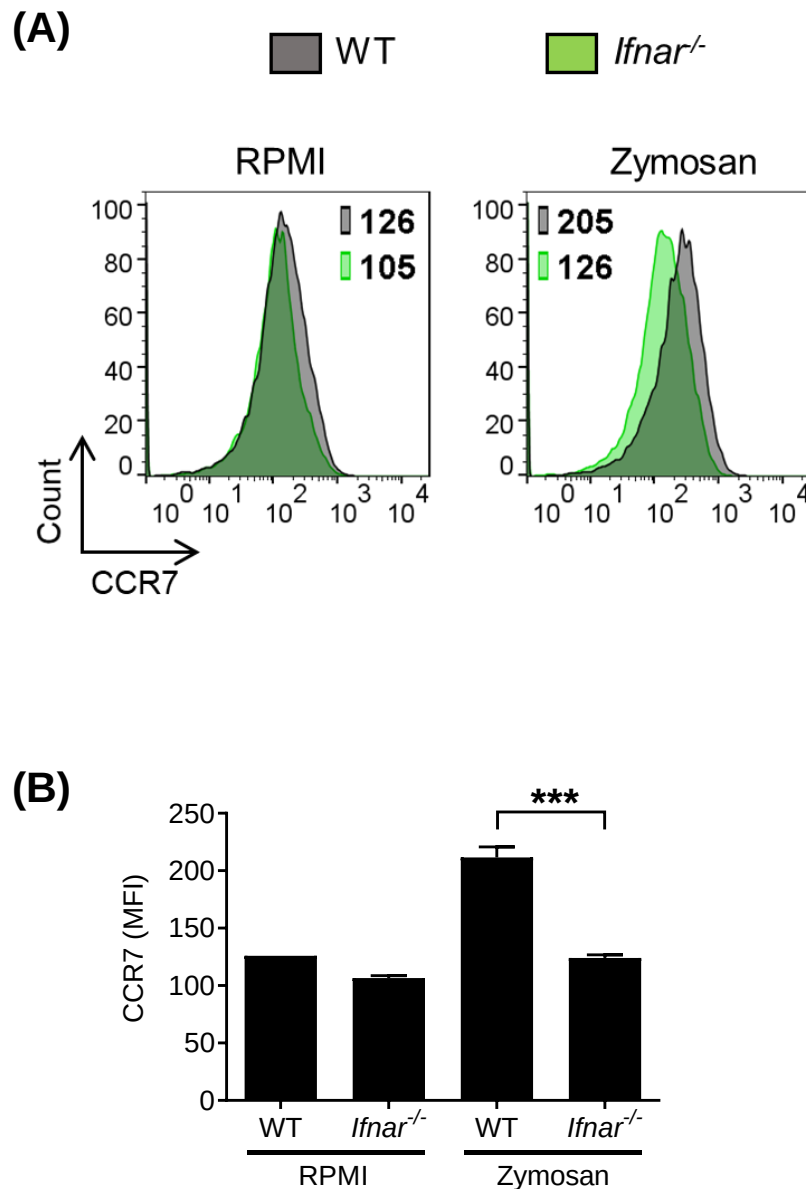


Fig 4.20: IFNAR signalling is required for optimal upregulation of CCR7 in iCD103 DCs stimulated with Zymosan. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan (125ng/ml) overnight. CCR7 expression was analysed in live CD11c⁺MHCII⁺ cells by flow cytometry. (A) Representative histograms and corresponding MFI values for CCR7 expression in iCD103 DCs. (B) CCR7 expression in DCs represented as MFI values. Bars are mean $\pm$ SD for duplicate samples. ***, $p < 0.001$; One-Way ANOVA, Tukey's multiple comparison test. Results are representative of two independent experiments.

4.13 IFNAR signalling is required for optimal cytokine secretion by iCD103 DCs stimulated with Zymosan

In addition to DC surface maturation markers, IFNAR signalling has been linked to a range of other genes that include pro-inflammatory cytokines [160, 466]. Therefore, it was hypothesised that CsA inhibition of type I IFNs and IFNAR signalling in iCD103 DCs (section 4.10-4.11) may contribute to the inhibition of Zymosan cytokine responses. In a viral context, a well-established role of type I IFNs is the formation of a positive feedback loop, whereby IFNAR signalling promotes further production of type I IFNs [467]. However, this concept is less established in the context of antifungal immunity. To test this, WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan overnight. Supernatants were collected and analysed for IFN β by ELISA. Interestingly, *Ifnar*^{-/-} iCD103 DCs produced significantly less IFN β than WT cells in response to Zymosan (Fig 4.21).

After confirming that Zymosan triggered a type I IFN feedback loop in iCD103 DCs, it was important to assess the importance of IFNAR signalling for other cytokine responses in this model. Therefore, WT and *Ifnar*^{-/-} cells were stimulated overnight as before and supernatants were collected and analysed for TNF α , IL-12p70, IL-6 and IL-23 by ELISA. Regarding TNF α , IFNAR deficiency led to significantly reduced secretion by iCD103 DCs stimulated with Zymosan (Fig. 4.22 A). For IL-12p70, *Ifnar*^{-/-} iCD103 DCs stimulated with Zymosan also produced significantly lower levels compared to WT cells (Fig. 4.22 B). Similarly, IFNAR-deficient cells produced significantly less IL-6 in response to Zymosan (Fig. 4.22 C). Finally, IL-23 secretion was markedly reduced in *Ifnar*^{-/-} iCD103 DCs stimulated with Zymosan (Fig. 4.22 D).

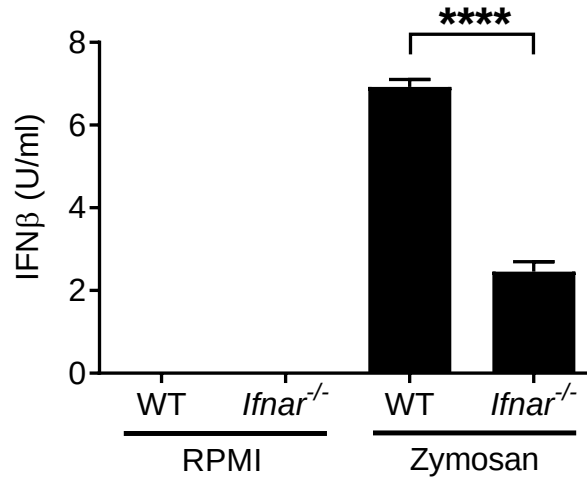


Fig 4.21: IFNAR deficiency significantly impairs Zymosan-induced secretion of type I IFNs in iCD103 DCs. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan (5μg/ml) overnight. Supernatants were collected and analysed for IFNβ by ELISA. Bars are mean ± SD for triplicate samples. ****, $p < 0.0001$; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

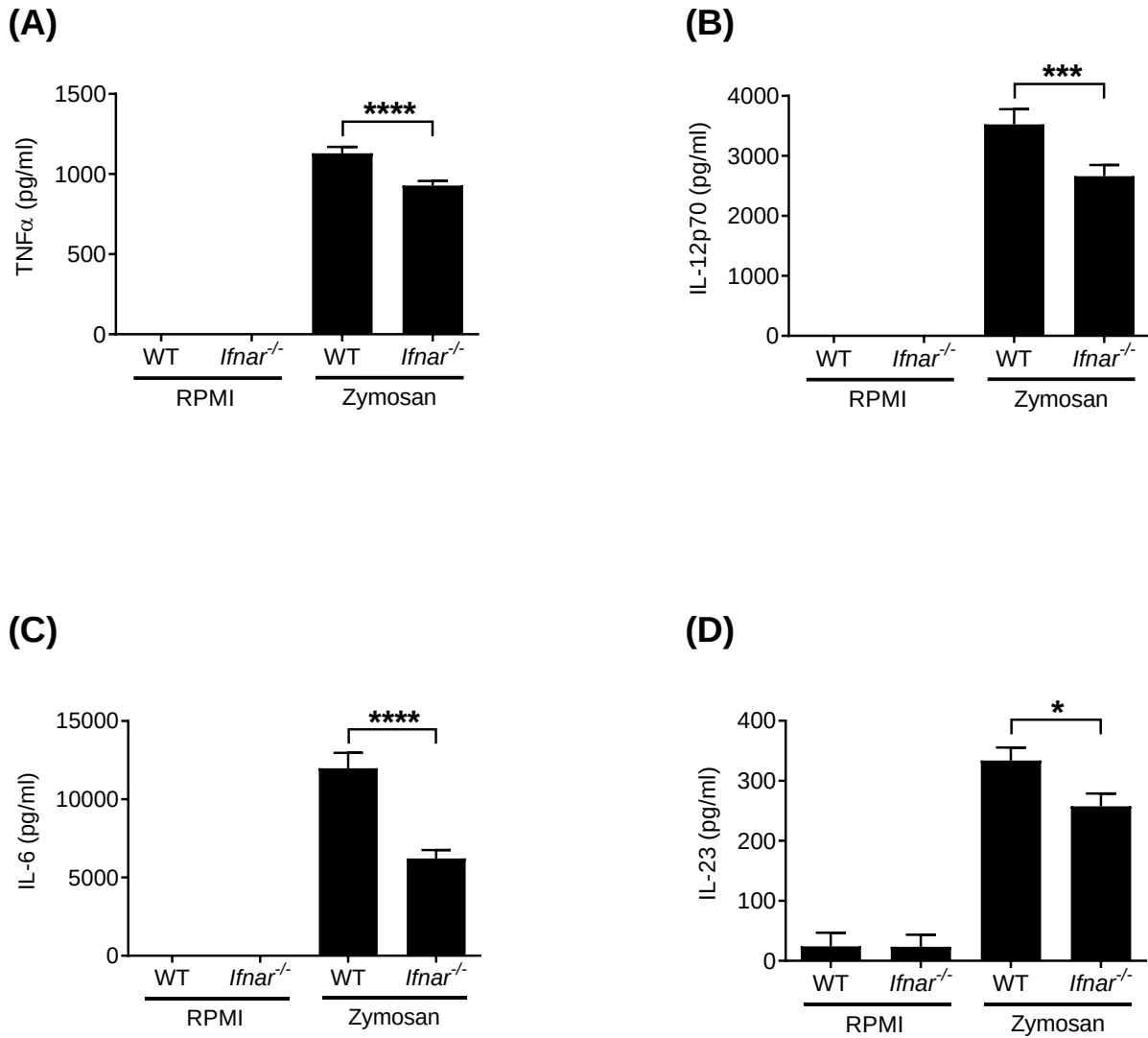


Fig 4.22: IFNAR deficiency impairs Zymosan-induced production of TNFα, IL-12p70, IL-6 and IL-23 in iCD103 DCs. WT and *Ifnar*^{-/-} iCD103 DCs were stimulated with medium or Zymosan (5μg/ml) overnight. Supernatants were collected and analysed for TNFα (A), IL-12p70 (B), IL-6 (C) and IL-23 (D) by ELISA. Bars are mean ± SD for triplicate samples. *, p<0.05; ***, p<0.001; ****, p<0.0001; One-Way ANOVA Tukey's multiple comparison test. Results are representative of three independent experiments.

4.14 IFNAR deficiency does not significantly impair neutrophil recruitment or DC activation in the lungs 12hr after intranasal Zymosan challenge

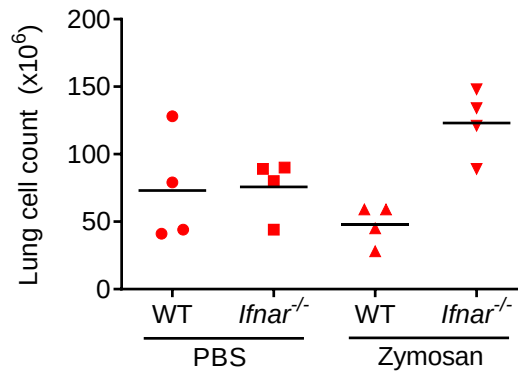
Oral administration of Neoral® was found to impact CD103⁺ DCs *in vivo* and this was corroborated with the iCD103 DC model, in which the inhibition of IFNAR signalling was particularly significant. To determine whether IFNAR signalling was also important for CD103⁺ DC activation *in vivo*, it was decided to re-examine responses in the lungs of WT and *Ifnar*^{-/-} mice upon intranasal treatment with Zymosan. Lung-draining mediastinal lymph nodes – a key site of T cell priming during respiratory infection – were also assessed. Similar to the Neoral® studies, key parameters included the total number of CD103⁺ DCs in lung tissue and draining lymph nodes, which provides an indication for DC trafficking. In addition, cells were assessed for expression of the key co-stimulatory molecules CD40, CD80 and CD86.

WT and *Ifnar*^{-/-} mice were anaesthetised and treated intranasally with 400µg Zymosan in a total volume of 40µL (20µL per nostril). This high volume reportedly enables the dose to reach the lower respiratory tract [443]. After 12h, lung and mediastinal lymph nodes were isolated and analysed by flow cytometry. Cells populations were analysed using the same gating strategy as Fig. 3.1.

In the lung tissue, IFNAR-deficient mice had an increased number of cells compared to WT mice (Fig 4.23 A). This was reflected by an increased number of neutrophils (Fig. 4.23 B), total DCs (Fig. 4.24 A), CD103⁺ DCs (Fig. 4.24 B) and CD11b⁺ DCs (Fig. 4.24 C). By extension, IFNAR-deficient mice had a higher number of DCs positive for the co-stimulatory molecules CD40, CD80 and CD86 (Fig. 4.25 A). This trend was also observed for CD103⁺ DCs (Fig. 4.26 A) and CD11b⁺ DCs (Fig. 4.27 A). The expression of co-stimulatory molecules on the DC surface was assessed using histograms and MFI values. For total DCs, there was

no significant defect in the expression of CD40, CD80 or CD86 caused by IFNAR deficiency (Fig. 4.25 B). This same trend was observed for CD103⁺ DCs (Fig. 4.26 B) and CD11b⁺ DCs (Fig. 4.27 B).

(A)



(B)

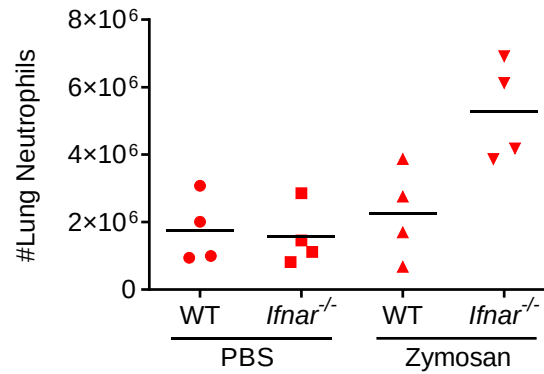


Fig 4.23: IFNAR-deficiency does not reduce total lung cell and neutrophil counts. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. (A) Trypan blue staining and light microscopy were used to obtain total cell counts. Following this, 5x10⁶ cells were stained for flow cytometry. (B) Neutrophils were gated as live CD3⁺B220⁻Gr1⁺F4/80⁻. For each sample, the number of neutrophils were divided by the number of single cells and subsequently multiplied by the corresponding total lung cell count. Horizontal bars are mean for each group (n=4).

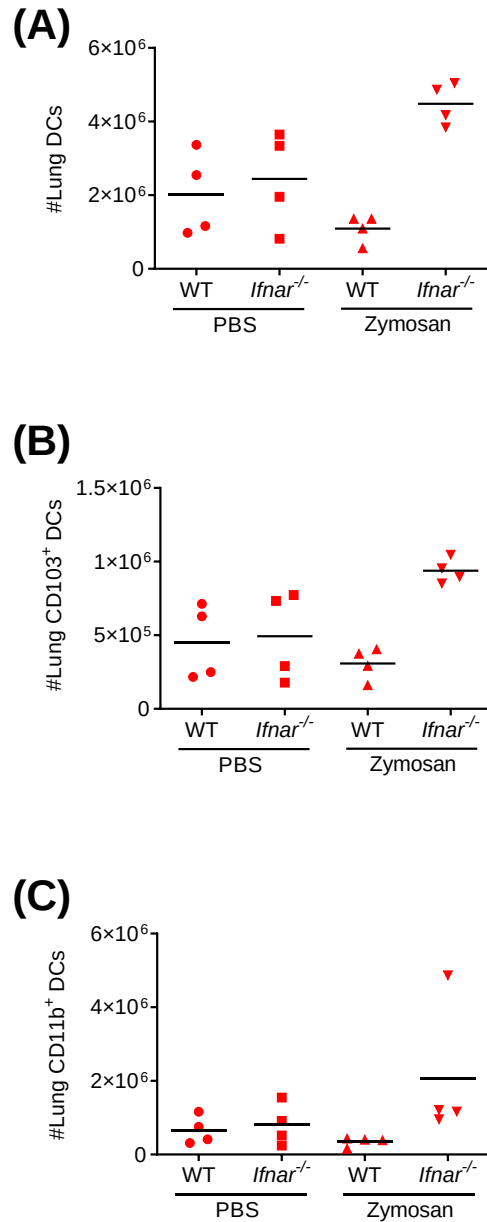


Fig 4.24: *Ifnar*^{-/-} mice have increased numbers of lung DCs after intranasal Zymosan treatment.

WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5 × 10⁶ cells were stained for flow cytometry. (A) Total DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}. (B) CD103⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺. (C) CD11b⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. For each sample, the number of total DCs (A), CD103⁺ DCs (B) or CD11b⁺ DCs (C) were divided by the number of single cells and subsequently multiplied by the corresponding total lung cell count (Fig. 4.23 A). Horizontal bars are mean for each group (n=4).

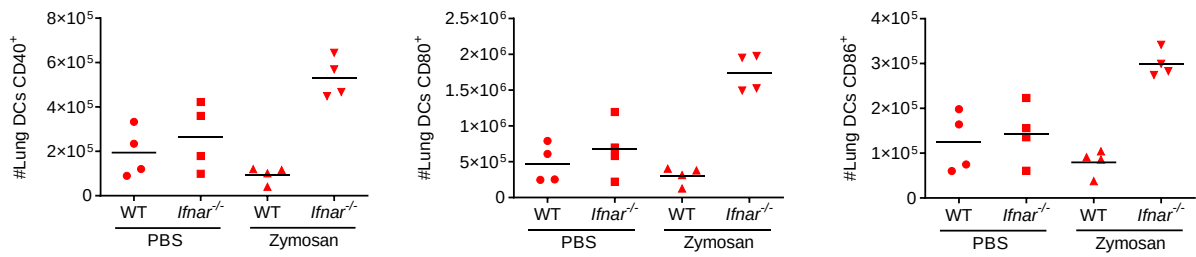
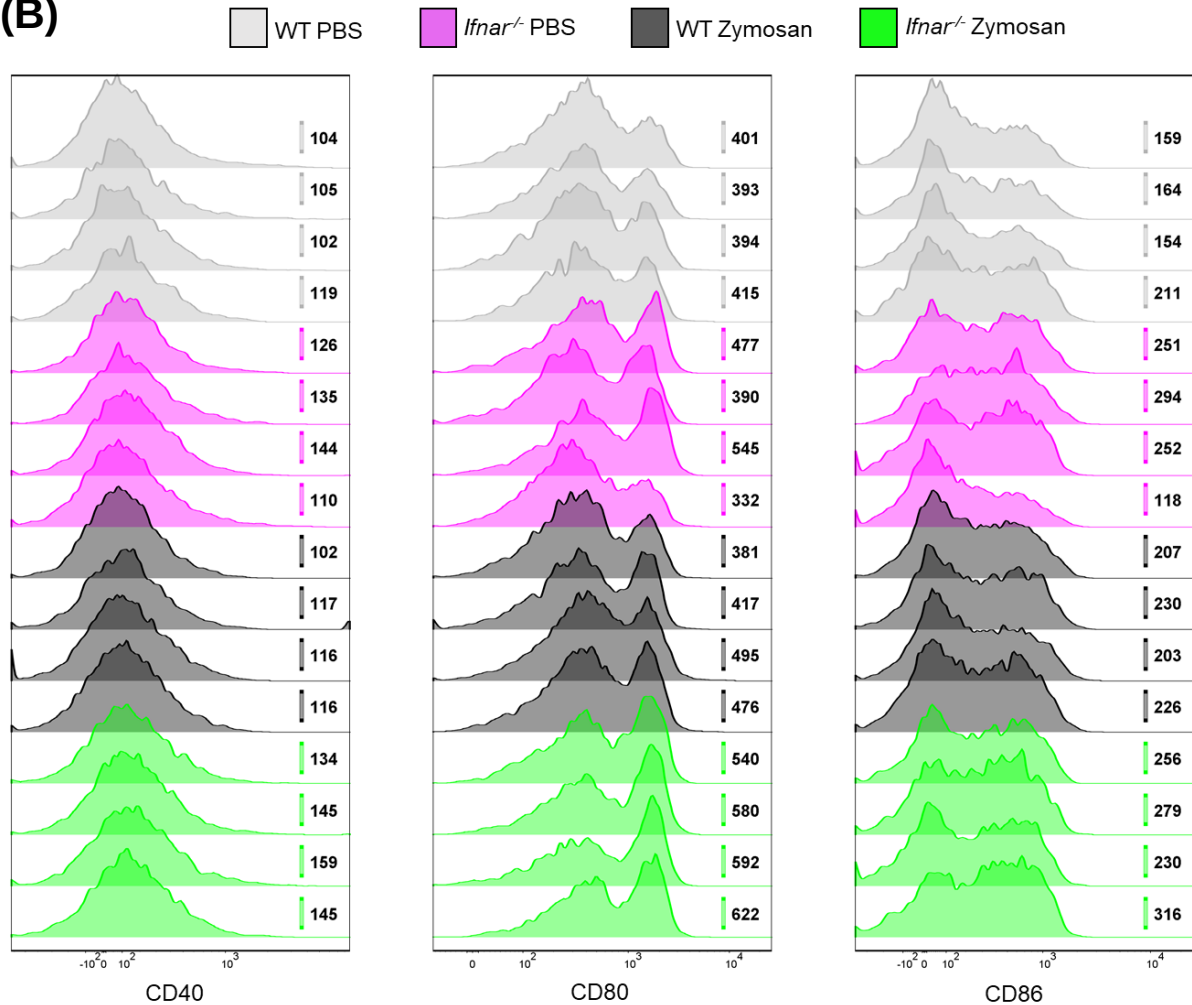
(A)**(B)**

Fig 4.25: *Ifnar*^{-/-} mice treated with intranasal Zymosan have greater numbers of total DCs expressing co-stimulatory molecules in the lungs than WT mice. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5×10^6 cells were stained for flow cytometry analysis. DCs were gated as live CD3⁺B220⁻Gr1⁺F4/80⁻CD11c⁺MHCII^{hi}. (A) Number of DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). Horizontal bars are mean for each group (n=4).

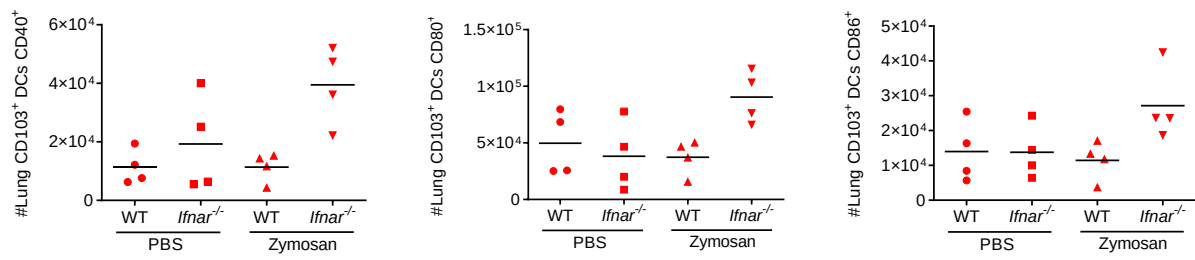
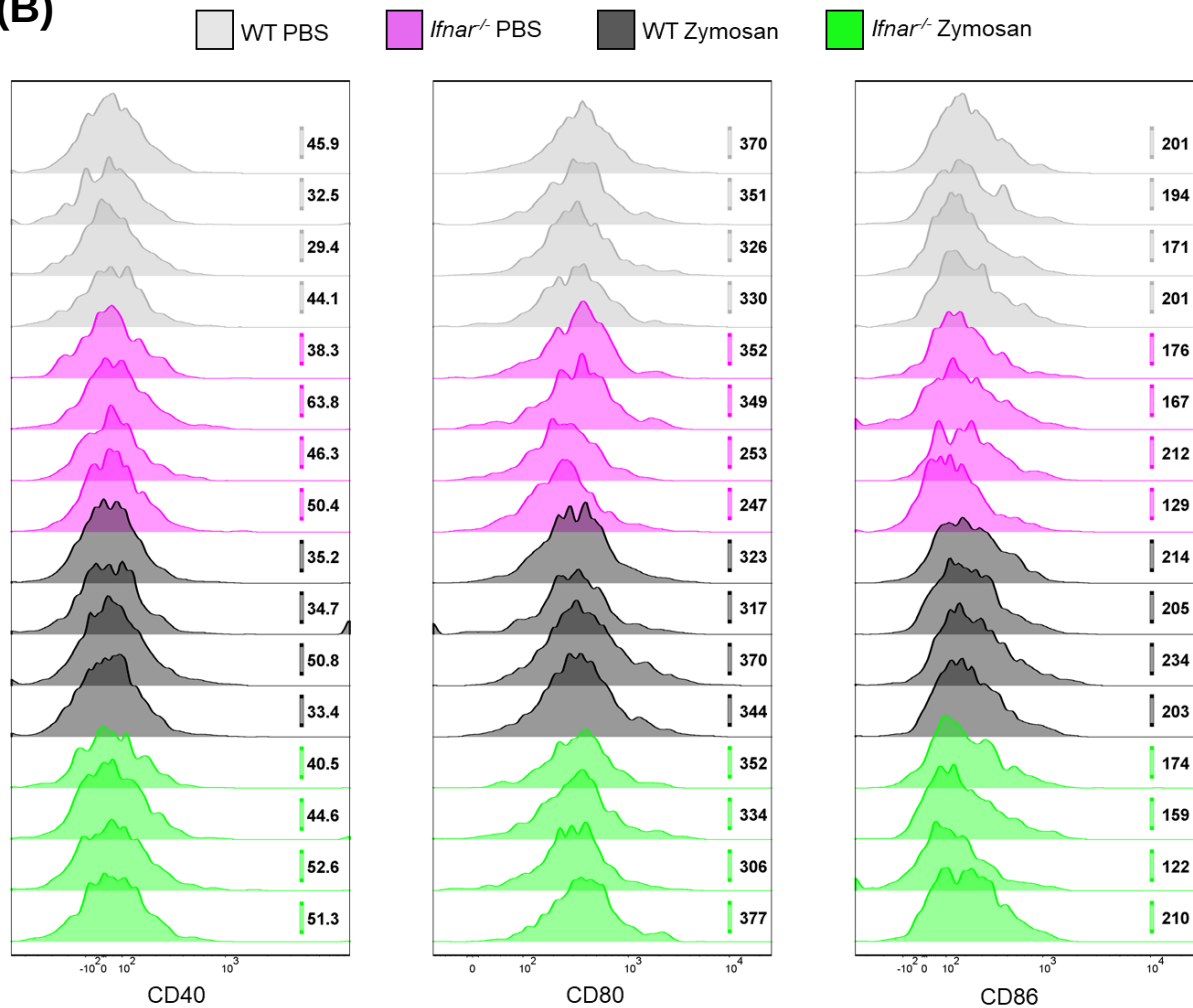
(A)**(B)**

Fig 4.26: *Ifnar*^{-/-} mice treated with intranasal Zymosan have greater numbers of CD103⁺ DCs expressing co-stimulatory molecules in the lungs than WT mice. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5 × 10⁶ cells were stained for flow cytometry analysis. CD103⁺ DCs were gated as live CD3⁺B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺. (A) Number of CD103⁺ DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for CD103⁺ DC expression of CD40 (left), CD80 (middle) and CD86 (right). Horizontal bars are mean for each group (n=4).

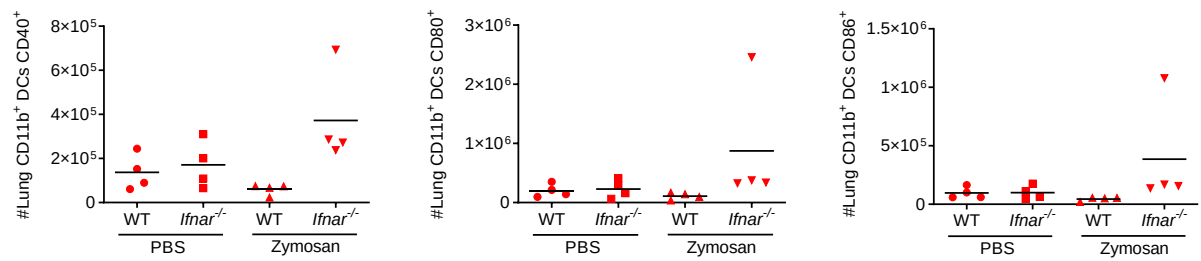
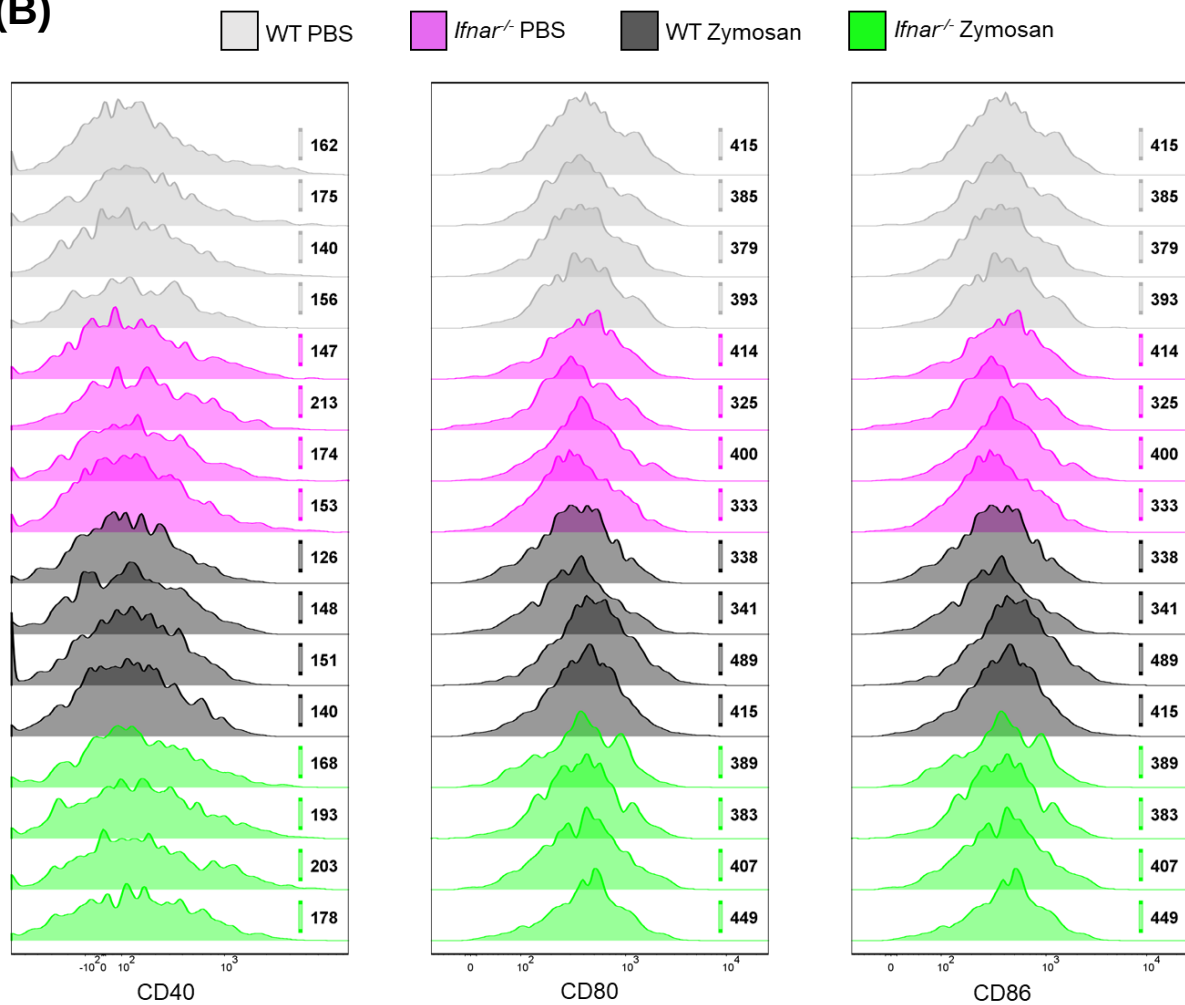
(A)**(B)**

Fig 4.27: *Ifnar*^{-/-} mice treated with intranasal Zymosan have greater numbers of CD11b⁺ DCs expressing co-stimulatory molecules in the lungs than WT mice. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and lung tissues were processed into single-cell suspensions. Cells were counted and 5×10^6 cells were stained for flow cytometry analysis. CD11b⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. (A) Number of CD11b⁺ DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for CD11b⁺ DC expression of CD40 (left), CD80 (middle) and CD86 (right). Horizontal bars are mean for each group (n=4).

4.15 IFNAR deficiency does not impair DC trafficking to lung-draining lymph nodes after intranasal Zymosan challenge

In addition to the lungs, it was important to analyse whether IFNAR deficiency affected DC populations in lung-draining mediastinal lymph nodes, as these are a key site of T cell priming during respiratory infections. Indeed, during intranasal infection with *Cryptococcus neoformans*, DCs uptake fungal spores, become activated in the lungs and migrate to the mediastinal lymph nodes [444]. Therefore, in the same *in vivo* studies with WT and *Ifnar*^{-/-} mice, mediastinal lymph nodes were isolated 12h after intranasal Zymosan challenge and analysed by flow cytometry.

While Zymosan administration significantly increased the total number of DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}) in the nodes, this response was not affected by IFNAR deficiency (Fig. 4.28 A-B). Similarly, Zymosan led to an increase in the number of CD103⁺ DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺) in the nodes, but IFNAR deficiency did not cause a significant defect in this response (Fig. 4.29 A, C). Regarding CD11b⁺ DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺), Zymosan triggered a marked increase in the mediastinal lymph nodes and this was not impaired in *Ifnar*^{-/-} mice (Fig. 4.29 B-C).

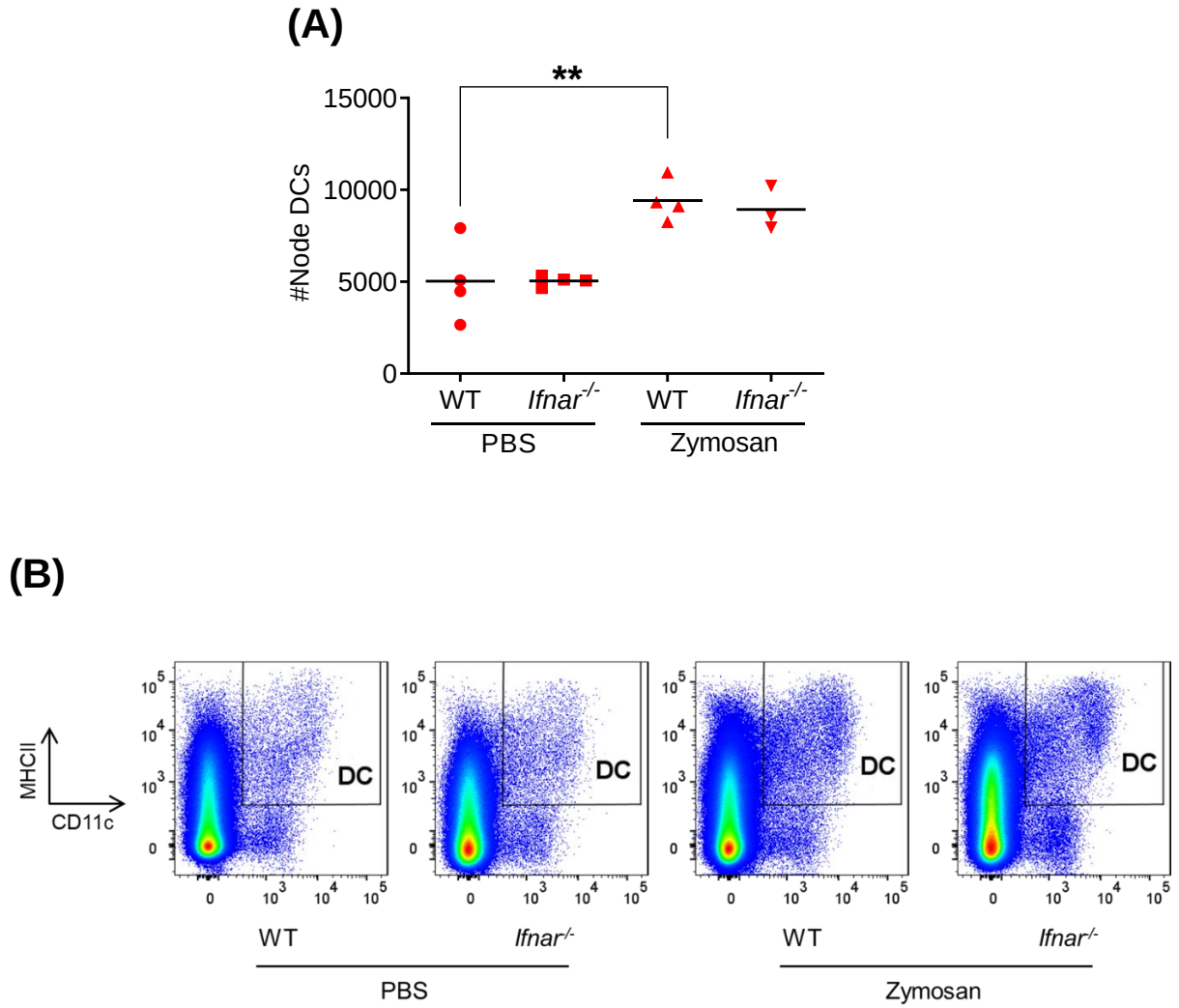


Fig 4.28: Total DC numbers in mediastinal lymph nodes were comparable in WT and *Ifnar*^{-/-} mice following intranasal Zymosan challenge. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. DCs were gated as live CD3⁺B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}. (A) Total lymph node DC counts. (B) Representative DC dot plots. **, $p < 0.01$; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group ($n=4$).

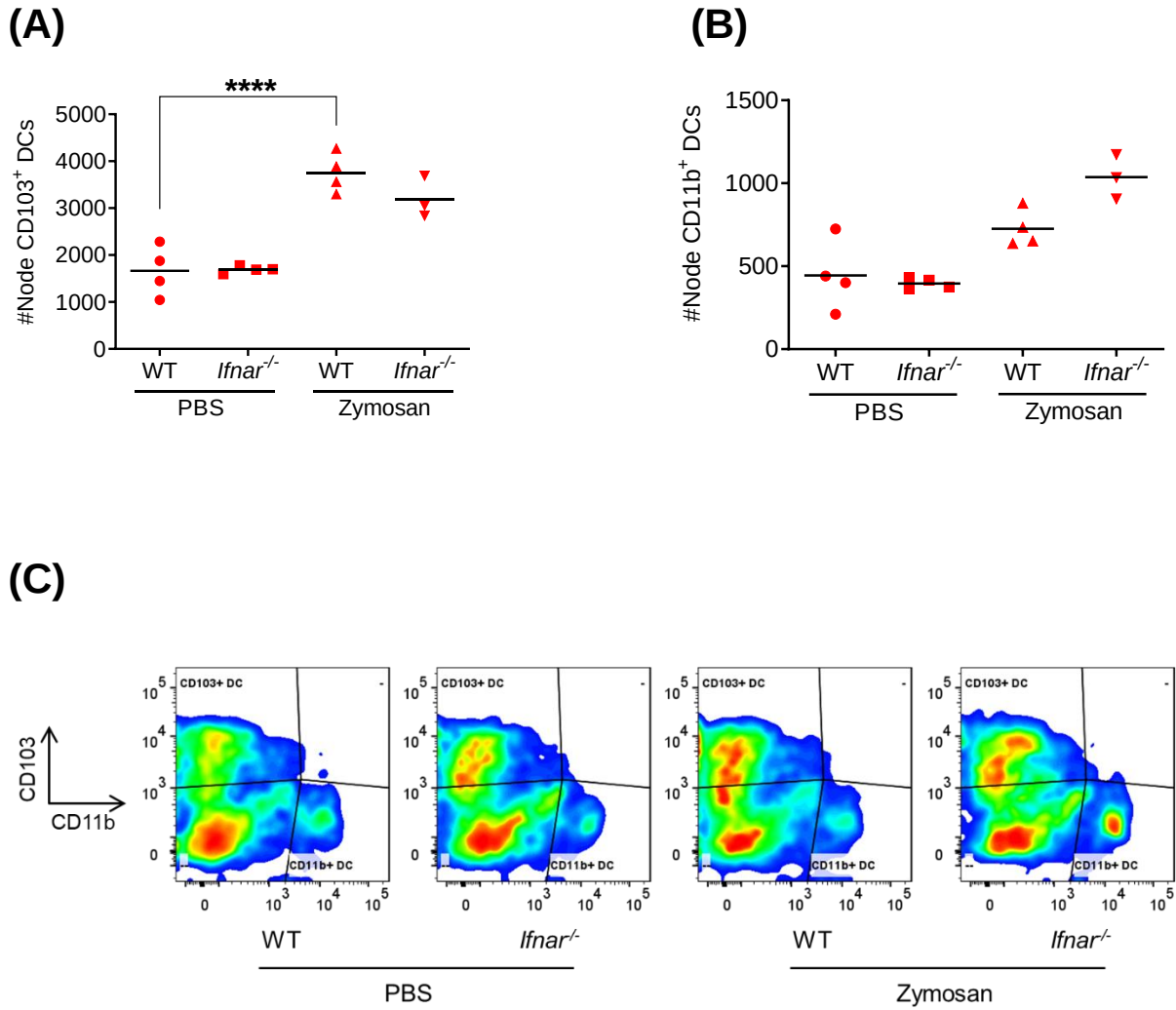


Fig 4.29: CD103⁺ and CD11b⁺ DC numbers in mediastinal lymph nodes were comparable in WT and *Ifnar*^{-/-} mice following intranasal Zymosan challenge. WT and *Ifnar*^{-/-} were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. CD103⁺ DCs (A, C) were gated as live CD3⁺B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺ cells, while CD11b⁺ DCs (B, C) were gated as live CD3⁺B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. (A) Total lymph node CD103⁺ DC counts. (B) Total lymph node CD11b⁺ DC counts. (C) Representative CD103⁺ and CD11b⁺ DC dot plots. ****, $p < 0.0001$; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group (n=4).

4.16 *Ifnar*^{-/-} mice administered with Zymosan nasally have reduced numbers of total DCs and CD103⁺ DCs expressing co-stimulatory molecules in the mediastinal lymph nodes

DCs are key for priming protective T cell responses during fungal infection [16]. Upon recognition of fungal PAMPs, DCs upregulate co-stimulatory molecules such as CD40, CD80 and CD86 on DCs, which directly interact with T cell co-stimulatory receptors, including CD40L and CD28 [4, 290]. Oral administration of Neoral® reduced expression of these co-stimulatory molecules on CD103⁺ DC in the mediastinal lymph nodes. Subsequently, the iCD103 DC model demonstrated that inhibition of IFNAR signalling was important for reducing maturation. Therefore, it was important to determine whether IFNAR was essential for the upregulation of co-stimulatory molecules *in vivo*, on DCs trafficking to the mediastinal lymph nodes following intranasal Zymosan challenge. Thus, DC populations in the mediastinal lymph nodes were analysed for surface expression of CD40, CD80 and CD86 by flow cytometry using the same panel as before (Fig. 3.1).

For total DCs (live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}), *Ifnar*^{-/-} mice had reduced numbers of CD40⁺, CD80⁺ and CD86⁺ cells compared to WT mice treated with Zymosan (Fig. 4.30 A). Moreover, histograms and MFI values indicated that the intensity of CD40 expression was reduced in *Ifnar*^{-/-} mice treated with Zymosan, while CD80 and CD86 intensity was comparable with WT mice (Fig. 4.30 B). Regarding CD103⁺ DCs, *Ifnar*^{-/-} mice had significantly lower numbers of cells positive for CD40, CD80 and CD86 in the mediastinal nodes (Fig. 4.31 A). In addition, histograms and MFI values for each mouse highlight that CD40 and CD86 expression was lower in Zymosan-treated *Ifnar*^{-/-} mice, while CD80 expression was comparable to WT mice (Fig. 4.31 B). Unlike CD103⁺ DCs, IFNAR deficiency did not reduce the number of lymph node CD11b⁺ DCs positive for CD40, CD80 or CD86 after Zymosan treatment (Fig. 4.32 A). However, histograms and MFI values suggested that

the expression of CD80 and CD86 was reduced in *Ifnar*^{-/-} mice administered with Zymosan (Fig. 4.32 B).

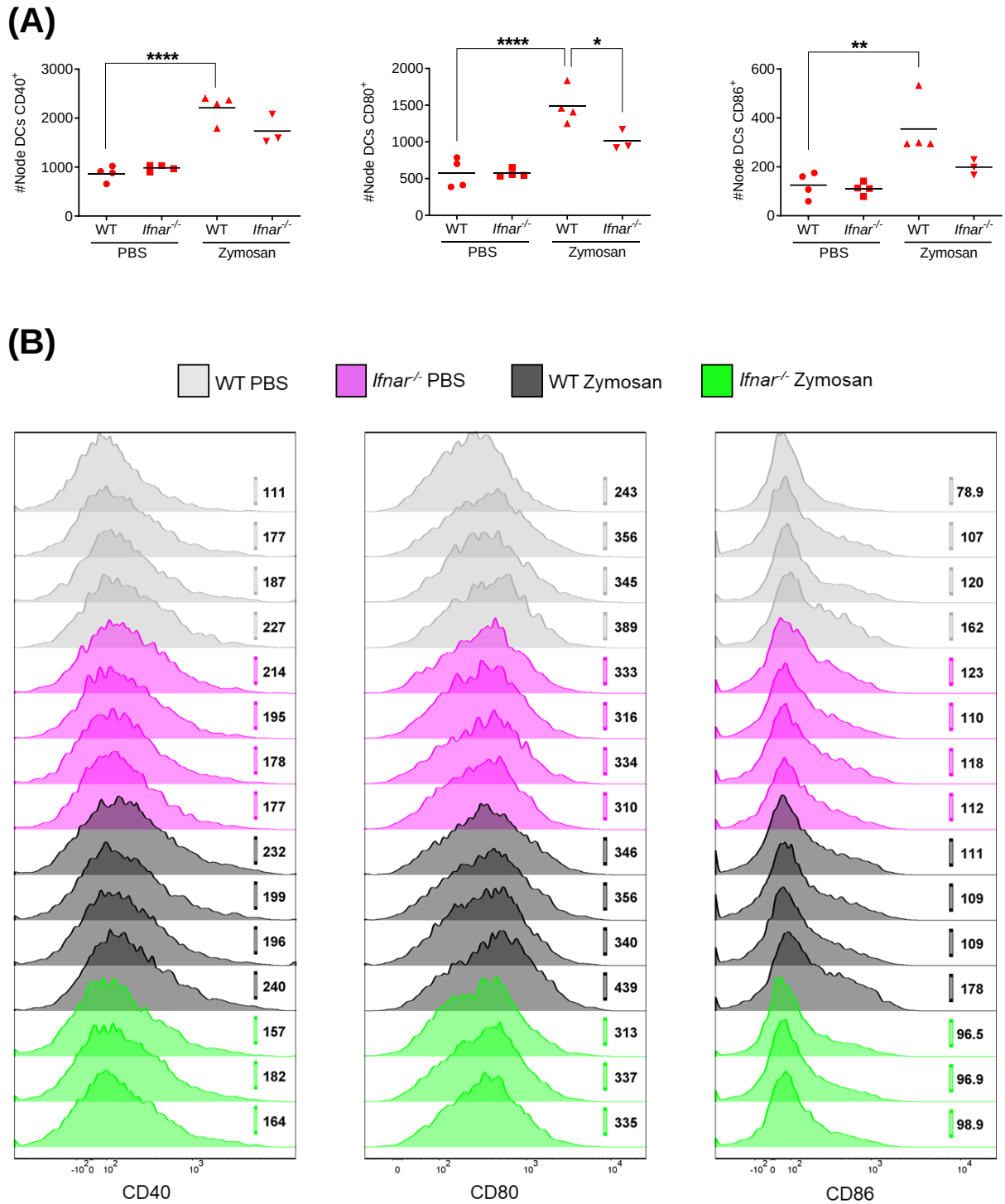


Fig 4.30: *Ifnar*^{-/-} mice administered with Zymosan nasally, have reduced numbers of DCs expressing CD40, CD80 and CD86 in the mediastinal lymph nodes and reduced expression intensity of CD40. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}. (A) Number of DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for DC expression of CD40 (left), CD80 (middle) and CD86 (right). *, p<0.05; **, p<0.01; ****, p<0.0001; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group.

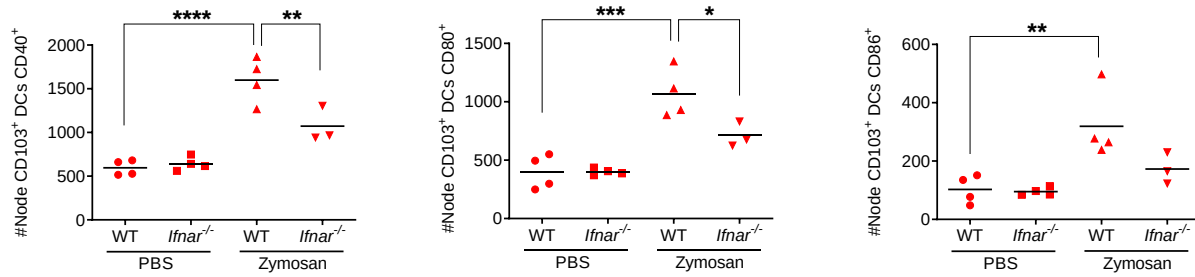
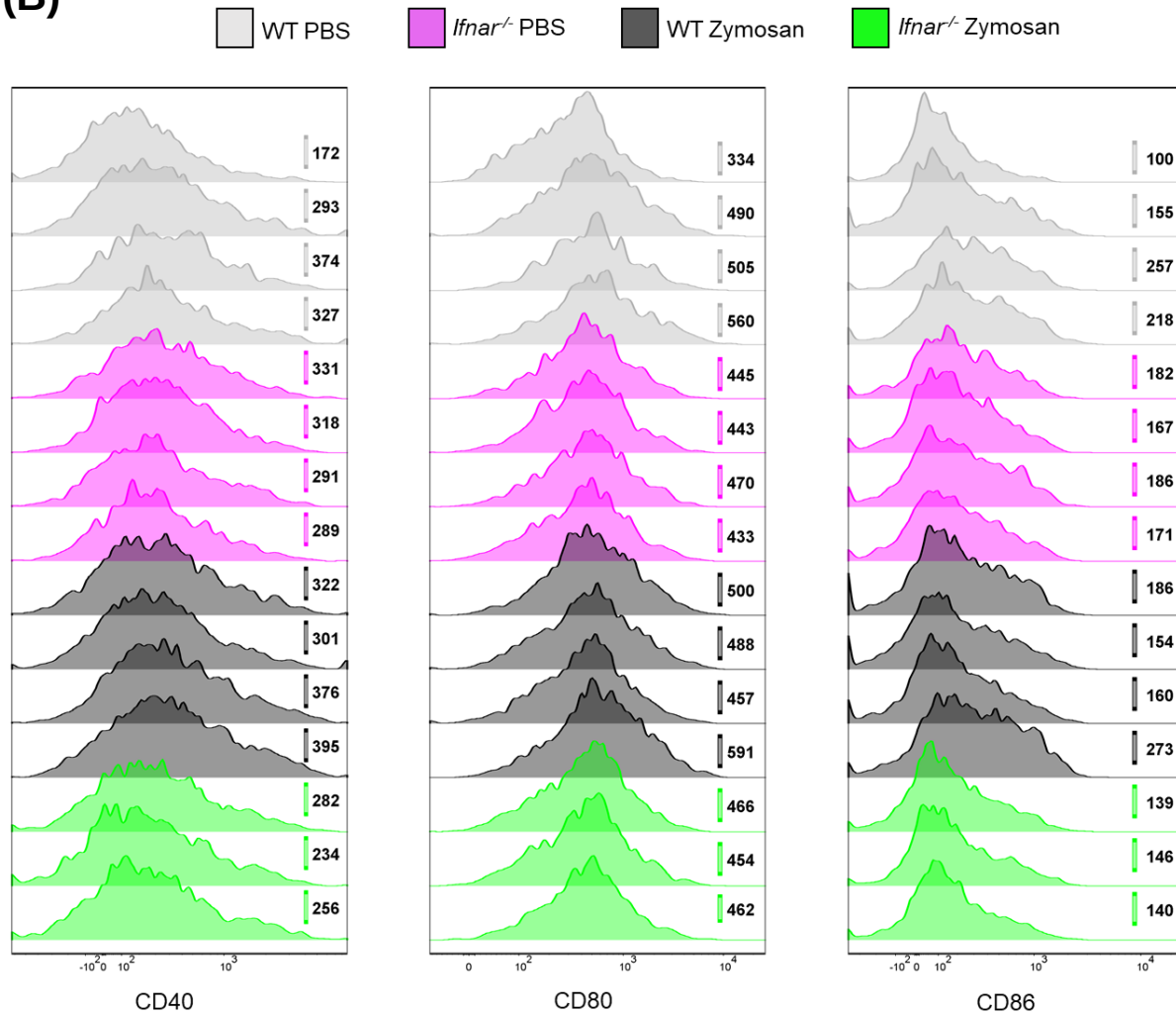
(A)**(B)**

Fig 4.31: *Ifnar*^{-/-} mice administered with Zymosan nasally have reduced numbers of CD103⁺ DCs expressing CD40, CD80 and CD86 in the mediastinal lymph nodes and reduced expression intensity of CD40 and CD86. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. CD103⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD103⁺. (A) Number of CD103⁺ DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for CD103⁺ DC expression of CD40 (left), CD80 (middle) and CD86 (right). *, p < 0.05; **, p < 0.01; ****, p < 0.0001; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group.

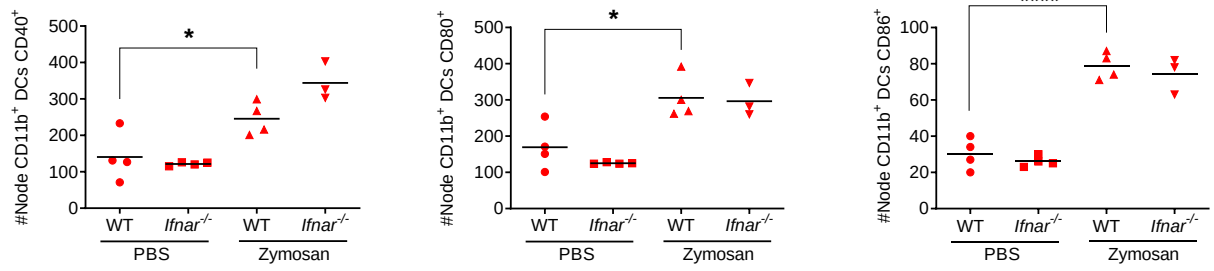
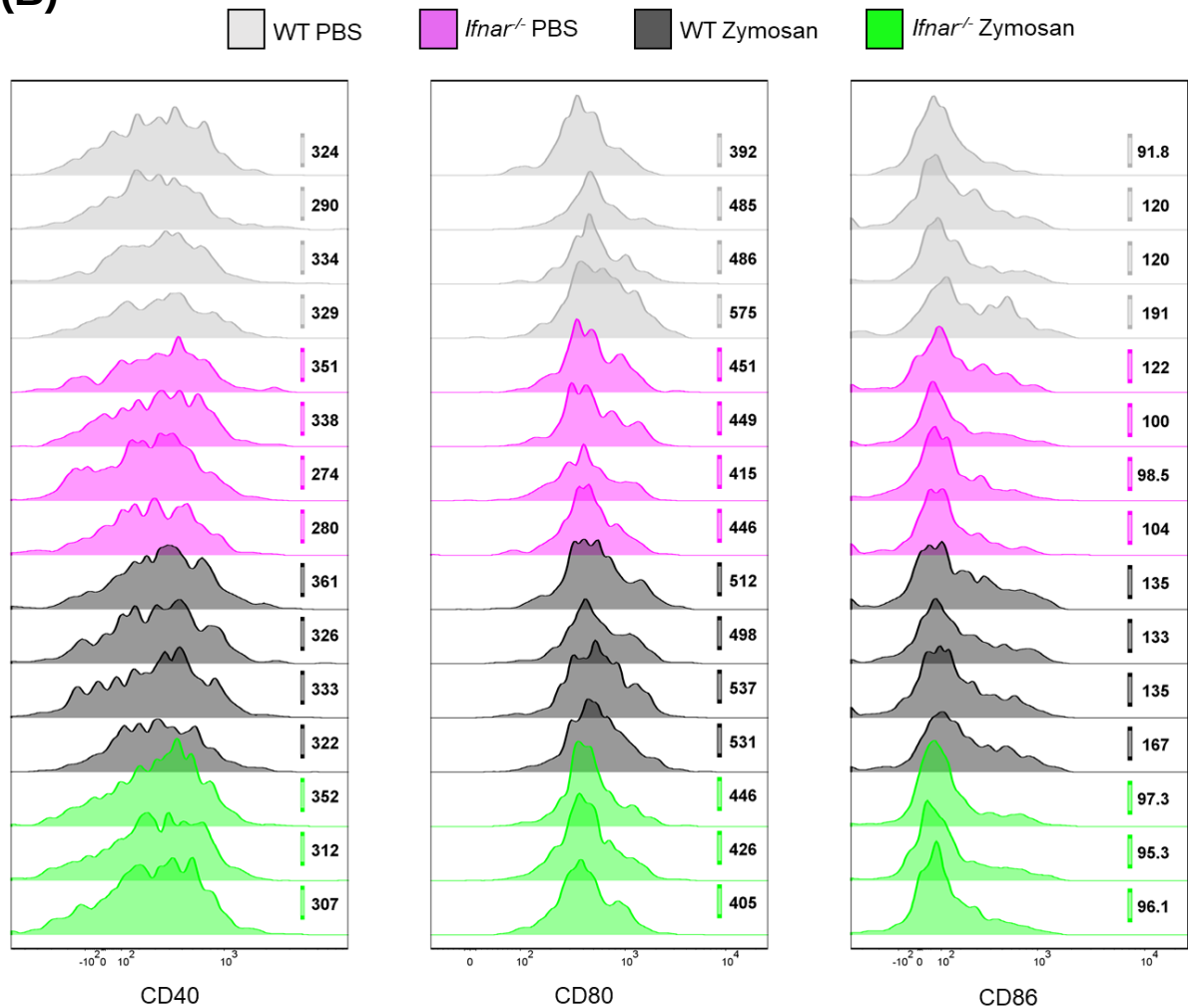
(A)**(B)**

Fig 4.32: Compared to WT mice, *Ifnar*^{-/-} mice treated with Zymosan nasally have comparable numbers of CD11b⁺ DCs expressing CD40, CD80 and CD86 but reduced expression of CD80 and CD86. WT and *Ifnar*^{-/-} mice were challenged intranasally with Zymosan or PBS for 12 h. Mice were sacrificed and mediastinal lymph nodes were processed into single-cell suspensions. All node cells were stained for flow cytometry analysis. CD11b⁺ DCs were gated as live CD3⁻B220⁻Gr1⁻F4/80⁻CD11c⁺MHCII^{hi}CD11b⁺. (A) Number of CD11b⁺ DCs positive for CD40, CD80 and CD86. (B) Histograms for each mouse with corresponding MFI values for CD11b⁺ DC expression of CD40 (left), CD80 (middle) and CD86 (right). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; One-way ANOVA, Tukey's multiple comparison test. Horizontal bars are mean for each group.

4.17 Discussion

This work provides insight into the mechanisms by which CsA modulates antifungal immune responses in CD103⁺ DCs. This may aid current understanding of why this drug increases the risk of fungal infections in patients [366, 368, 391, 393-401]. Indeed, BATF3-dependent CD103⁺ DCs are now recognised as important mediators of protection against fungal infections, such as *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Candida albicans* [152, 232, 248].

Inhibition of iCD103 DC cytokine secretion by CsA has significant implications for fungal susceptibility. CsA has long been known to impair calcineurin activity and subsequently inhibit NFAT [3]. As such, it was expected that direct inhibition of NFAT with 11R-VIVIT would impair IL-2 secretion by iCD103 DCs. However, it was less certain whether NFAT was also needed for other Zymosan-induced cytokine responses in these cells. Regarding the literature, previous studies have found that NFAT can bind to the promoters for TNF α and IL-12 [356], while NFAT inhibition was found to reduce IL-12p70 secretion in GM-CSF-generated BMDCs [130]. Interestingly, NFAT activity in iCD103 DCs was not limited to inducing IL-2, as it was also important for secretion of TNF α , IL-12p70, IL-6 and IL-23. These cytokines mediate key antifungal roles that include phagocyte mobilisation and activation, as well as priming of IFN γ ⁺ Th1 and IL-17⁺ Th17 cells [4, 152, 232, 262]. In addition to cytokines, DCs contribute to fungal immunity by upregulating co-stimulatory molecules, such as CD40, CD80 and CD86, as well as migrating to draining lymph nodes through upregulation of migratory markers such as CCR7 [4, 161, 288, 290]. These changes are essential for the capacity of DCs to prime adaptive T cell responses, highlighting the significance of CsA reducing the expression of these molecules in iCD103 DCs. Mechanistic studies herein highlight that this may be through blockade of NFAT activity, due to 11R-VIVIT treatment impairing iCD103 DC upregulation of CD40, CD80 and CD86. CCR7 inhibition was also

observed, however, this was less pronounced and may also occur through additional mechanisms.

CsA is known to bind to cyclophilin D in the MPT pore, inhibiting the release of mitochondrial DAMPs such as ROS into the cytosol [3]. This is potentially significant as mitochondrial DAMPs are known to trigger multiple mediators of DC activation, including inflammasomes, MAVS and the cGAS-STING pathway [74, 113, 377, 385]. In iCD103 DCs, CsA was found to increase the level of mitochondrial ROS and this may be due to prevention of their release into the cytosol. Regardless, specific quenching of mitochondrial ROS with the targeted antioxidant MitoTempo failed to inhibit a number of responses that are impaired by NFAT inhibition. Indeed, MitoTempo inhibited Zymosan-induced IL-23 secretion but not TNF α , IL-12p70 and IL-6, while the reduction of co-stimulatory molecule expression was minimal compared to that caused by NFAT inhibition. Together, these studies suggest that CsA inhibition of Zymosan responses in iCD103 DCs has a major dependency on calcineurin/NFAT inhibition and a minor dependency on targeting of the MPT pore.

Recent studies have confirmed that type I IFNs, which are classically associated with viruses, are essential for protection against fungal infections [150-152, 435, 436, 439]. A number of these studies demonstrate this using IFNAR-deficient mice, which rapidly succumb to infection with *Candida albicans* and *Histoplasma capsulatum* [152, 435]. Type I IFNs are likely to aid fungal clearance through multiple mechanisms, since IFNAR is expressed on a wide range of immune cells, as well as non-haematopoietic populations, including epithelial cells [136]. As a key example during fungal infection, type I IFNs were required for optimal IL-12-dependent induction of Th1 cells, which produce IFN γ to activate killing mechanisms within macrophages [152, 436]. IFNAR deficiency was also found to exacerbate fungal infection due to excessive induction of Th17 cells and neutrophil recruitment, leading to tissue damage at the site of infection [152, 436]. One particular study found that during fungal

infection in the lungs, CD103⁺ DCs were a dominant source of type I IFNs [152]. Therefore, the impact of CsA on iCD103 DC secretion of type I IFNs was a key route of investigation. Significantly, CsA potently inhibited Zymosan-induced type I IFN secretion by iCD103 DCs. 11R-VIVIT and MitoTempo assays highlight that this response was more dependent on NFAT than mitochondrial ROS.

Type I IFNs are well-known to promote DC activation [136, 153, 160]. This occurs through a positive feedback loop, whereby autocrine and paracrine IFNAR signalling triggers further secretion of type I IFNs [136, 467]. The feedback loop was confirmed in iCD103 DCs by using *Ifnar*^{-/-} cells, which produced significantly less type I IFNs than WT cells in response to Zymosan. Despite this, it remained essential to confirm that CsA treatment impaired Zymosan-induced IFNAR signalling in iCD103 DCs. This was demonstrated by significant inhibition of STAT1 phosphorylation in cells stimulated with Zymosan but not IFN β . Moreover, STAT1 phosphorylation was entirely dependent on IFNAR. This confirmed that CsA was reducing Zymosan-induced IFNAR signalling by reducing the levels of type I IFNs in the extracellular space. After confirming this, *Ifnar*^{-/-} mice were used to assess the impact of impaired IFNAR signalling on Zymosan-induced activation. IFNAR deficiency led to widespread defects in Zymosan-induced iCD103 DC activation, as highlighted by impaired secretion of TNF α , IL-12p70, IL-6 and IL-23, as well as defective upregulation of CD40, CD80, CD86 and CCR7.

Oral administration of Neoral® impaired antifungal responses in CD103⁺ DCs in the mediastinal lymph nodes, and CsA impaired the critical IFNAR signalling pathway in iCD103 DCs. Therefore, it was decided to assess whether IFNAR was essential for CD103⁺ DC activation *in vivo* after intranasal Zymosan treatment. In the lungs, IFNAR deficiency was associated with greater numbers of DCs expressing co-stimulatory molecules, with comparable expression intensity to WT mice. It was speculated that this may be due to activated DCs in WT mice migrating out of the lungs and into lymphatics. Indeed, in the

mediastinal lymph nodes, although the overall DC numbers were unchanged in *Ifnar*^{-/-} mice, the number of total DCs and CD103⁺ DCs expressing CD40, CD80 and CD86 was significantly lower after Zymosan treatment. Of the cells that reached the nodes, histograms and MFI values indicated that *Ifnar*^{-/-} mice treated with Zymosan had reduced expression of CD40 on total DCs, as well as CD40 and CD86 on CD103⁺ DCs. Therefore, IFNAR may be required for trafficking of mature CD103⁺ DCs into mediastinal nodes and optimum upregulation of co-stimulatory molecules in response to fungal PAMPs, which is likely to impact the ability of these cells to prime adaptive immune responses. As such, both the iCD103 DC and intranasal Zymosan model highlight the importance of IFNAR in CD103⁺ DC responses to fungal PAMPs. These findings complement prior studies demonstrating that CD103⁺ DCs are important for immunity to fungal infection, by producing type I IFNs that confer protection [152, 232]. One interesting question regards the link between Zymosan uptake in the lung tissue and DC activation and migration. This could be addressed using fluorescently-labelled Zymosan, to determine whether a high proportion of CD103⁺ DCs reaching the nodes have internalised Zymosan, while assessing whether these cells also express co-stimulatory molecules at the highest intensity. It is also possible that a proportion of Zymosan can drain to the lymph nodes and activate DCs *in situ*. Furthermore, since IFNAR-deficient mice had high numbers of DCs expressing co-stimulatory molecules in the lungs, it would be useful to perform a Zymosan challenge and assess responses at an earlier time point than 12h. This would help to determine whether IFNAR deficiency disrupts the early kinetics of DC activation in the lungs and subsequent migration of activated cells through the lymphatics. Another intriguing question is whether mice lacking IFNAR exclusively on DCs are more susceptible to fungal infection, as this would support the significance of CsA impairing type I IFN secretion and subsequent IFNAR signalling in iCD103 DCs. In addition, it would be useful to sort DCs from the lymph nodes of IFNAR-deficient mice, after

administration of Zymosan, and assess their ability to prime CD4 T cell responses and compare this to WT mice.

In summary, this work has determined a number of key mechanisms by which CsA is likely to modulate CD103⁺ DC responses to fungal PAMPs. Zymosan-induced activation of iCD103 DCs was significantly dependent on NFAT and had a minor dependency on mitochondrial ROS. Moreover, CsA was found to inhibit Zymosan-induced type I IFN secretion and IFNAR signalling. This was significant due to the clear dependency of iCD103 DCs on IFNAR signalling for activation. Moreover, IFNAR was required for the influx of mature CD103⁺ DCs expressing co-stimulatory molecules into the draining lymph nodes upon intranasal Zymosan challenge. Given the major role of CD103⁺ DCs and the importance of type I IFNs in antifungal immunity [150-152, 232, 248, 435, 436, 439], these mechanistic studies may help to explain why patients receiving CsA are more susceptible to opportunistic fungal infections [366, 368, 391, 393-401]. Understanding these mechanisms, along with those yet to be discovered, may assist in reducing the incidence of infection and increasing the therapeutic benefit of CsA.

Chapter 5: General Discussion

5.1 General Discussion

CsA is a fungal metabolite that was discovered by Sandoz Pharmaceuticals. It was initially intended for use as an antibiotic but showed poor efficacy. Despite this, Sandoz continued to assess its ability to modulate the immune system. This was a pivotal decision as CsA soon revolutionised the field of transplant medicine [468]. A key trial published in the *Lancet* found that CsA improved the survival rate of cadaveric renal transplant patients compared to azathioprine and steroids (72% vs 52%) [321]. This aided its clinical approval for transplantation in the early 1980s. In early animal studies, CsA reduced inflammation in Freund's complete adjuvant-induced arthritis, supporting its potential application to other clinical settings [316]. Mechanistic work found that the drug inhibits T cell activity of calcineurin and subsequently NFAT, a key transcription factor for expression of IL-2 [323]. This has enabled CsA to be applied clinically to T cell-mediated diseases such as RA, SLE and colitis, demonstrating high efficacy in reducing disease severity [324, 325, 469]. In recent clinical trials, CsA was found to improve the survival rate of patients hospitalised with severe COVID-19 and one study suggested this was associated with reduced systemic concentrations of inflammatory cytokines [470, 471]. More novel applications for CsA are likely to emerge in the future.

While the efficacy of CsA is clear, a number of side effects have been reported, chief among them nephrotoxicity and opportunistic infections [326]. Indeed, the risk of infection is highlighted on the patient leaflet for Neoral®, a commercially available oral CsA solution. Of particular interest to this research is the increased risk of opportunistic fungal infections caused by CsA [366, 368, 391, 393-401]. Most fungal infections are asymptomatic due to protection provided by the immune system. Severe or “invasive” cases of infection – where the fungi spread to multiple organs – predominantly occur in patients that are immunocompromised or immunosuppressed. Invasive fungal infections are estimated to cause over 1.5 million deaths

per year, due to high mortality rates [250]. One meta-analysis found that mortality rates for invasive infections with *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* can be estimated as 30-95%, 46-75% and 20-70% respectively [250]. In 2021, invasive fungal infections were prominent in the media due to the “black fungus” epidemic in India. This was caused by ubiquitous moulds triggering mucormycosis, a condition characterised by black necrotic lesions arising in the sinuses, lungs, skin, intestines and brain [472]. The condition has an estimated mortality rate of 54% (<https://www.cdc.gov/fungal/diseases/mucormycosis/statistics.html>). Unfortunately, many survivors in India required invasive surgeries, to remove necrotic tissue and prevent systemic dissemination and these procedures left permanent scarring. For example, many patients’ eyes were removed due to the infection. Mucormycosis cases were excessive during the COVID-19 pandemic, where high viral case numbers in India caused overcrowding and poor hygiene in hospitals. Importantly, mucormycosis cases were infrequent in immunocompetent patients but prominent in those receiving steroids, which cause a broad suppression of the immune system comparable to CsA [472]. The black fungus epidemic provided a stark reminder of the dangers posed by invasive infections. It highlighted the need to continuously improve the current mechanistic understanding of how CsA increases susceptibility to fungi.

The inhibition of T cell responses by CsA is likely to contribute to fungal susceptibility [432]. Indeed, Th17 cell-derived IL-17 and subsequent IL-17R signalling is essential for protection against *Candida albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii* infections [291-295]. IL-17 triggers granulopoiesis and recruitment and activation of neutrophils that directly kill fungi with ROS, RNS and NETs [280-282, 285, 291, 301]. Moreover, Th1 cells are needed to produce IFN γ during fungal infection, which prevents systemic dissemination, by activating intracellular killing mechanisms in macrophages, such as ROS and RNS [272-275, 281, 296-298, 301].

While T cell inhibition is the best known immunosuppressive mechanism, CsA also modulates innate immune cells including neutrophils, macrophages and DCs [3]. Of note, neutrophils and macrophages – not helper T cells – are directly responsible for fungal clearance, as well as antifungal cytokine secretion, highlighting the significance of CsA targeting these cells. This was demonstrated in *ex vivo* assays, where CsA impaired neutrophil killing of *Candida albicans* and *Aspergillus fumigatus* [366, 368]. This is likely due to impaired production of NETs and ROS [367, 369]. In macrophages, CsA may impair antifungal production of RNS, as it was found to inhibit NFAT binding to the iNOS promoter [364]. After phagocytosing *Aspergillus fumigatus*, Macrophages secrete TNF α to increase neutrophil recruitment and this is dependent on calcineurin and NFAT, meaning CsA may impair this response in macrophages [262].

The discovery of IL-2 secretion by DCs, along with its inhibition by CsA was a pivotal landmark [329]. This gave rise to multiple studies assessing the impact of CsA on DCs. Regarding antifungal responses, CsA inhibited Zymosan-induced IL-12p70 secretion in GM-CSF-generated BMDCs [130]. Since calcineurin expression in DCs was required for optimal clearance of *Aspergillus fumigatus*, it is highly likely that CsA targeting DCs is a key factor in susceptibility to infection [433]. Despite this, previous studies did not assess the impact of CsA on specific DC subsets that are important for antifungal immunity. One important subset is the BATF3-dependent CD103⁺ DC. These cells are known for constitutively migrating from peripheral tissues to draining lymph nodes, driven by high CCR7 expression, to enable T cell priming [178, 216, 217, 236]. Induced responses include Treg cells to maintain tolerance in mucosal tissues, and CD8⁺ T cell that protect against viruses and cancers [178, 218, 240]. However, it was particularly interesting to this research that CD103⁺ DCs protect against *Candida albicans*, *Aspergillus fumigatus* and *Histoplasma capsulatum*, and this is also likely the case for other fungal species [152, 232, 248]. CD103⁺ DCs were required for priming of

protective Th17 cells during aspergillosis through secretion of IL-2 and IL-23 [232]. In addition, protection against *Histoplasma capsulatum* was dependent on secretion of type I IFNs and IFNAR signalling and CD103⁺ DCs were the dominant producer of type I IFNs during infection [152]. Thus, it was highly significant that Neoral® – a commercial oral CsA formulation – was found to impair the ability of these cells to migrate to lung-draining lymph nodes after treatment with Zymosan. Neoral® also reduced the expression of co-stimulatory molecules in CD103⁺ DCs. Together, this suggested that CsA, despite being delivered by the oral route, may impair antifungal immunity in the lungs. This has significant implications for patients, as fungal spores typically enter the respiratory tract with each inhalation, totalling $\sim 10^3$ - 10^{10} inhaled spores per day [473]. Impairing the ability of DCs to interact with and prime naïve T cells in the draining lymph nodes is likely to increase susceptibility to infection.

Investigating the impact of CsA on CD103⁺ DCs is technically challenging, as differentiated primary cells are only present in mucosal tissues and draining lymph nodes. Isolation and processing of these tissues, followed by sorting, leads to low and variable yields [198]. This highlights the need for an *in vitro* model that can provide an accurate representation of this type of DC. This issue was resolved by Mayer *et al* publishing a protocol for generating CD103⁺ DCs by culturing murine bone marrow with GM-CSF and Flt3L [198]. The Lavelle lab was interested in this “iCD103 DC” protocol due to the high yield and purity of CD103⁺ DCs and their clear functional overlap with these cells *in vivo*. Indeed, iCD103 DCs are entirely dependent on BATF3 and, compared to GM-CSF DCs, more efficiently prime CD8⁺ T cells by cross-presentation, and migrate to skin-draining lymph nodes following subcutaneous injection [198]. Optimising this model in the Lavelle lab was a key objective for this research but it has also been applied to other projects testing the ability of vaccine adjuvants to promote cellular immunity, for which CD103⁺ DCs are essential in a number of *in vivo* settings [178, 240]. CsA had a broad impact on iCD103 DC antifungal responses,

including reduced secretion of the cytokines IL-2, TNF α , IL-12p70, IL-6 and IL-23, as well as impaired upregulation of co-stimulation molecules (CD40, CD80 and CD86) and the chemokine receptor CCR7. Subsequent mechanistic studies suggested that this may be occurring through CsA inhibiting NFAT, which was important for all responses tested. These findings have significant implications for fungal susceptibility, as all these responses are known to contribute to protection against infection.

CD103⁺ DCs mediate a number of antifungal responses *in vivo* but it was particularly interesting to assess type I IFNs and IFNAR signalling [152, 232, 248]. Indeed, IFNAR signalling is a recently identified mediator of protection against fungal infection, including *Candida albicans* and *Histoplasma capsulatum*, *Aspergillus fumigatus* and *Cryptococcus neoformans* [151, 152, 435, 438]. Moreover, patients suffering recurrent *Candida albicans* infections exhibited defective expression of type I IFN pathway genes [436]. The role of type I IFNs may vary depending on the fungal species and route of infection. However, reported roles include driving secretion of the chemokines CXCL1, CXCL2, CCL3, CCL4 and cytokines such as IL-1 β and IL-12p70; enhancing recruitment of neutrophils, DCs, T cells, NK cells and B cells to the site of infection; increasing neutrophil and DC production of ROS [150, 152, 438, 439]. Therefore, type I IFNs make a significant contribution to antifungal immunity. During *Histoplasma capsulatum* infection in the lungs, CD103⁺ DCs were the dominant source of type I IFNs, expressing higher levels than CD11b⁺ DCs, pDCs, monocytes and alveolar macrophages [152]. This raised the key question of whether CsA affected antifungal type I IFN secretion and IFNAR signalling in iCD103 DCs. Inhibition of both IFN β secretion and IFNAR signalling were two of the most significant findings of this research. This provides novel insight into how CsA modulates DC responses to Zymosan. Indeed, experiments on IFNAR-deficient cells highlighted that IFNAR is essential for a number of Zymosan responses, including cytokine secretion and co-stimulatory molecule and CCR7

expression. CD103⁺ DC activation and trafficking *in vivo* was also impacted by IFNAR deficiency, as observed in the intranasal Zymosan model. Indeed, it was interesting that IFNAR-deficient mice treated with intranasal Zymosan had reduced numbers of CD103⁺ DCs expressing co-stimulatory molecules in the draining lymph nodes, while the intensity of CD40 expression on these cells was also reduced. This is likely to disrupt normal priming of antifungal T cell responses in the draining lymph nodes by CD103⁺ DCs. Therefore, if drugs such as Neoral® impair type I IFN secretion at the site of a fungal infection – as the iCD103 DC data would suggest – these findings may help to explain why CsA patients are more susceptible to severe disease.

Altogether, this work has highlighted how CsA modulates fungal PAMP-induced responses in CD103⁺ DCs, a subset that has recently emerged as important for protection against infection. Key findings include inhibition of DC trafficking to draining lymph nodes and chemotaxis *in vitro*, impaired upregulation of CCR7 and co-stimulatory molecules and reduced secretion of inflammatory cytokines. These effects can at least in part be attributed to inhibition of NFAT and also secretion of type I IFNs and IFNAR signalling, which was necessary for optimal CD103⁺ DC activation (Fig. 5.1). Overall, this work provides new insights into how CsA predisposes patients to opportunistic fungal infections. This may assist in reducing the incidence of infection, thereby increasing the therapeutic benefit of CsA.

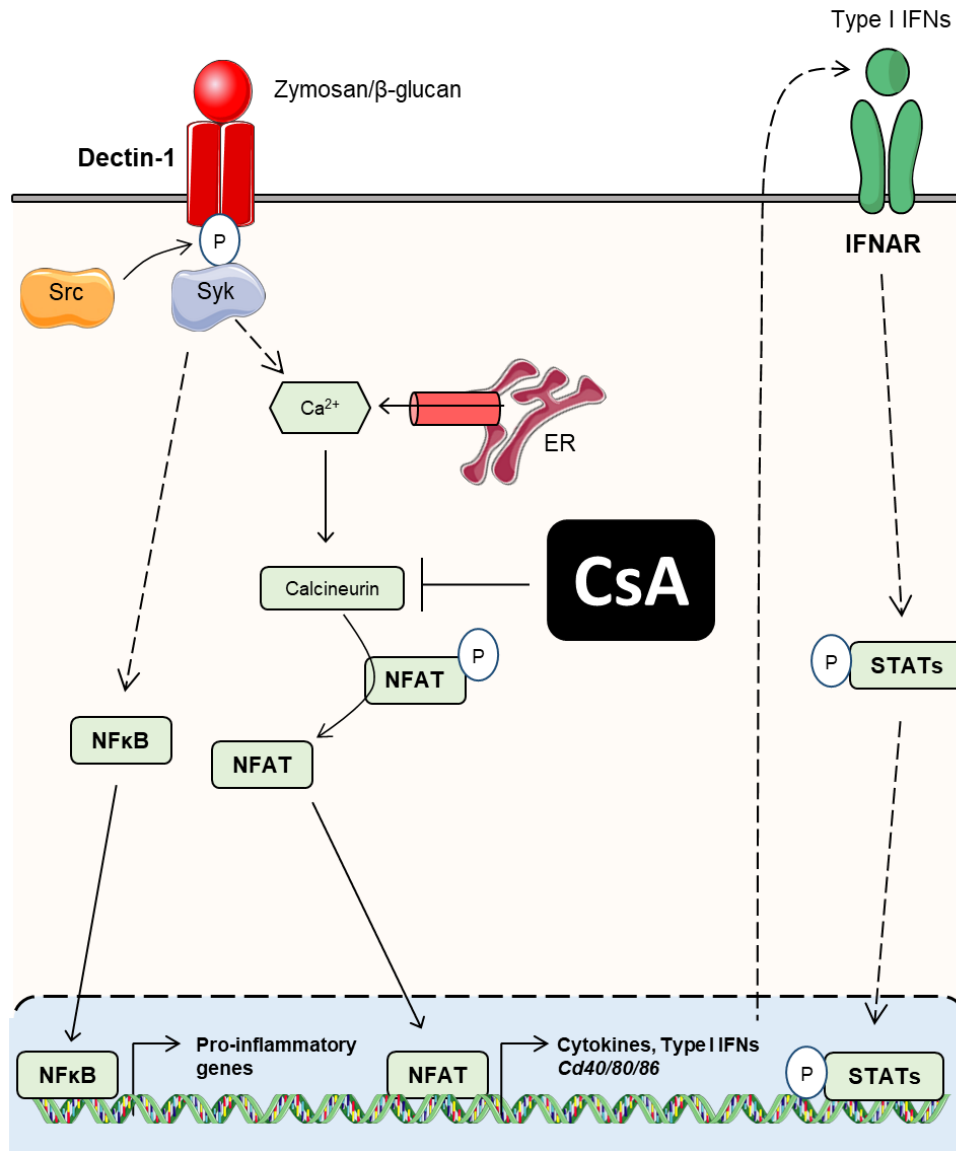


Fig 5.1: Proposed mechanisms by which CsA modulates CD103⁺ DC responses to fungal PAMPs. Zymosan/β-glucans are a prominent fungal PAMP bound by Dectin-1 on the DC surface. Bound Dectin-1 recruits Src and Syk family kinases. This induces a NFκB pathway but also a Ca²⁺ influx from the ER. This Ca²⁺ flux will activate calcineurin, a phosphatase that activates the transcription factor NFAT. In the nucleus, NFκB and NFAT will collectively transcribe immune genes, including cytokines, co-stimulatory molecules and type I IFNs. CsA inhibits this process by targeting calcineurin, thereby impairing the activation of NFAT. This directly reduces CD103⁺ DC activation. In addition, the impaired secretion of type I IFNs will enhance the inhibitory effect of CsA, as DCs significantly depend on IFNAR signalling – primarily mediated by STATs – for activation. Abbreviations: CsA, Cyclosporine A; IFN, interferon; IFNAR, IFN receptor; NFAT, nuclear factor of activated T cells; STAT, signal transducer and activator of transcription.

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