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In vitro vaccine immunogenicity analysis
using Tuberculosis and Dengue infection as
models.

Adam Sparrow

PhD Thesis

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Abbreviations

APC	Antigen presenting cells
BMDC	Bone marrow derived dendritic cell
J774	Macrophage cell line
YC NaMa	Yellow carnauba wax combined with sodium myristate (vaccine delivery system)
Liposome P/S	Liposome formed from phosphatidylserine (vaccine delivery system)
R10	Complete RPMI media
PFA	Paraformaldehyde
EtOH	Ethanol
PBS	Phosphate buffered saline
MFI	Mean Fluorescence Intensity
Ki67	Proliferation marker
CPG DNA	CpG oligodeoxynucleotides (TLR 9 agonist)
PGN	Peptidoglycan (TLR 2 agonist)
Poly I:C	Polyinosinic:polycytidylic acid (TLR 3 agonist)
LPS	Lipopolysaccharide (TLR 4 agonist)
MPL	Monophosphorylated lipid A (TLR 4 agonist)
FP1	Fusion Protein 1, composed of MTB antigens Ag85b, HBHA and Acr.
D-PIGS	Dengue polymeric immunoglobulins (anti-Dengue vaccine candidate)
DERICS	Dengue, Ebola recombinant immune complexes (anti-Dengue vaccine candidate)
Anti Acr IgA ICM	IgA based immune complex mimic (anti TB vaccine candidate)
Anti Acr IgG ICM	IgG based immune complex mimic (anti TB vaccine candidate)

Abstract

This study has used *in vitro* models to test the immunogenicity of novel vaccines against TB and Dengue Fever. Initially murine dendritic cells were used to model the immunogenicity of novel vaccines candidates and an array of potential adjuvants. Three vaccine candidates, Spore FP1, YC NaMa FP1 and Liposome FP1 were found to be immunogenic, increasing expression of DC activation markers and production of proinflammatory cytokines.

Further vaccine testing was carried out using *in vitro* models based on human tonsillar mononuclear cells (TMCs). These cells were first characterised and then used to establish immunogenicity models for TB and dengue fever. In this model, the BCG vaccine elicited IFN γ , IL-17 responses and proliferative responses. The novel vaccine candidates Spore FP1, YC NaMa FP1, Liposome FP1, IgA ICM were all immunogenic, generating IFN γ responses. The Dengue vaccine candidate DERICS elicited IFN γ and IL-4 responses in CD4 $^{+}$ T cells. Another candidate, D-PIGS, generated IL-4, IFN γ and TNF α responses.

This study developed a mycobacterial infection model using TMCs. This model examined the activation of monocytes and their ability to eliminate mycobacteria. Novel TB vaccines were applied to this model. The BCG vaccine activated monocytes and promoted the clearance of intracellular mycobacteria. Similarly, the novel vaccine candidates Spore FP1, YC NaMa FP1 and Liposome FP1 were also able to activate monocytes and enhance their ability to clear mycobacterial infection.

In the course of this study, it was discovered that GFP BCG directly interact with human B cells. Further investigation revealed that BCG binds to the cell surface but is not internalised by B cells. This interaction is mediated by antibodies and has a profound suppressive effect on B cells. This finding may have implications for our understanding of the humoral response following BCG vaccination. These studies demonstrate that human TMC *in vitro* models can play a useful role in early vaccine development.

1. Introduction

1.1 Tuberculosis

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB). TB is an airborne disease associated with poverty, malnutrition and over-crowding (Cruz-Knight and Blake-Gumbs 743-756; Fogel 527-531). It is estimated that between 1.7 and 2 billion people are now infected with MTB (Houben and Dodd e1002152). 1.6 million people died of TB in 2021 (World Health Organisation). The majority of these deaths were reported in low and middle income countries. The HIV epidemic has contributed to the incidence of TB, with HIV patients 30x more likely to develop active TB (Cruz-Knight and Blake-Gumbs 743-756). There is a downwards trend in the incidence of TB, from 184 cases per 100,000 in the year 2000, compared to 134 per 100,000 in 2021 (World Bank). Much of this progress is due to improving standards of sanitation and a lowering burden of poverty and malnutrition. This progress is however offset by the large proportion of the population which is still latently infected with TB. Treatment of TB involves a prolonged course of antibiotics. The standard regimen is a 6 month course of rifampicin and isoniazid with pyrazinamide and ethambutol. Moxifloxacin, rifabutin, fluoroquinolone, 4-aminosalicylic acid and streptomycin are also available as antibiotics against Tuberculosis. The incidence of drug resistant TB is increasing, with 450,000 cases in 2021 (World Health Organisation).

1.2 Immune responses against tuberculosis

The immune response against tuberculosis requires a complex interaction of different immune cells. The most significant immune responses against TB are mediated by macrophages, dendritic cells (DCs) and T cells. MTB is able to colonise and proliferate inside naïve macrophages and DCs (Collins and Kaufmann 1-9). Type 1 activation of macrophages by IFN γ and TNF α allows MTB to be eliminated from macrophages (Cooper et al. 2243-2247; Heldwein and Fenton 937-944). Activation of macrophages and effective specific responses to MTB require co-ordination between macrophages, DCs and T lymphocytes. This co-ordination is achieved through presentation of TB antigens by APCs to T cells and cytokine production (Wolf et al. 105). IFN γ and IL-17 generated by T helper lymphocytes have both been associated with protection (Coulter et al. 1252; Fletcher 319-325). IFN γ is needed for protection against TB. T helper cells, positive for TNF α , IL-2, IFN γ and IL-17 in BAL have also been identified as correlates of protection against TB in non human primates (NHPs) (Dijkman et al. 1).

Macrophages and DCs play an important role as APCs presenting antigens via MHCII to CD4 $^{+}$ T helper cells (Hickman, Chan, and Salgame 4636). DCs represent a biological bottleneck in the development of protective responses against TB (Griffiths et al. 13894). Virulent MTB and BCG are both able to limit antigen presentation by DCs (Bakhru et al. 53-61). This inhibits the development of MTB antigen specific CD4 $^{+}$ T cell responses (Grace and Ernst 357-364). MTB impairs the function and development of DCs. MTB is able to block antigen presentation by DCs by producing ESXH (Mehra et al. e1003734; Portal-Celhay et al. 16232) This bottleneck can be overcome using primed exogenous dendritic cells. The MTB serine hydrolase Hip 1 limits maturation of DCs, with reduced expression of pro-inflammatory cytokines, including TNF α , IL-6, IL-1 β and IL-12. Expression of CD40, CD86 and MHC II is also abrogated by Hip1. This leads to suboptimal antigen presentation, and diminished Th1 and Th17 responses (Madan-Lala et al. 4263-4272).

Targeting new TB vaccines against DCs may prove to be a useful strategy. Improving DC function leads to accelerated T cell activation, and enhanced protection against TB (Griffiths et al. 13894). Vaccine

antigens have been successfully targeted to DCs using IgG2a specific for the DC surface molecule Clec9A. This targeting led to enhanced antigen presentation by DCs, culminating in greater antibody responses and protection against influenza infection (Park et al. 31). DCs have also been used as targets for novel anti TB vaccines. Targeting DC-SIGN on DCs using an antibody bound to Ag85b led to enhanced protection with more poly functional, antigen specific CD4+ T cells (Velasquez et al. 471). Dendritic cell function has also been enhanced using BCG combined with an IFN- β adjuvant. This approach activated IRF-3, leading to DC maturation, IL-12 expression and enhanced T cell responses (El-Sahrigy et al. 15-21; Giacomini et al. 462-468).

1.3 Vaccination against tuberculosis using BCG

The current vaccination strategy against TB uses the Bacillus Calmette Guerin (BCG). This is a live attenuated strain of bovine TB. This strain was first made available in 1927 (Calmette). Several strains of BCG are now in existence. These include BCG Glaxo, Bulgaria, Denmark, Pasteur, Phipps. Each of these strains is associated with different immune responses and differing levels of protection. The current vaccination strategy uses single intradermal doses given to neonates. It is estimated that more than 120 million children are vaccinated with BCG each year (UNICEF). The efficacy of BCG vaccination depends, in part, upon the ability of the bacteria to induce a CD4+ T effector memory response which confers protection against TB (Kaveh et al. e21566; Kaveh, Garcia-Pelayo, and Hogarth 6911-6918). BCG elicits Th1 responses and Th17 responses, serum anti TB IgG and leads to the expression of TNF α and IFN γ by T cells (White et al. 992-1003).

BCG vaccination is more than 70% effective in mycobacteria naïve infants. It protects against pulmonary, disseminated and meningeal forms of TB in children (Roy et al. g4643; Mangtani et al. 470-480; Colditz et al. 29-35). This protection can endure for as long as 20 years (Barreto et al. 1171-1173). Efficacy is more variable in adolescents and adults, with waning protection against pulmonary TB (Fine 1339-1345). Studies have demonstrated that BCG has variable efficacy, BCG confers 80% protection

against pulmonary TB in UK. However, BCG has been observed to offer no protective immunity against pulmonary TB in Malawi. This discrepancy in protection may be due to prior exposure to mycobacterial species (McShane). TB is a major public health problem in Malawi. The national TB prevalence survey completed in 2014 showed a TB incidence of 1014/100,000 (Kanyerere et al. 101-107). No studies have shown geographical differences in the efficacy of BCG in infants who are naïve to mycobacteria. The current vaccination strategy is not sufficiently protective to enable eradication of TB. The live BCG vaccine is also unsuitable for patients who are HIV positive or otherwise immunocompromised due to the high risk of disseminated BCG disease (Rezai et al. 413-416). As a result, extensive work has been done to improve, boost and replace the BCG vaccine. There are currently 15 anti TB vaccine candidates undergoing human clinical trials (Li, J. et al., 2020). These are shown in table 1.1.

Table 1.1 Current vaccine candidates against tuberculosis

Vaccine Candidate	Vaccine Type	Phase	BCG Boost
VPM 1002	Live attenuated	phase 3	N
MTB VAC	Live attenuated	phase 2a	N
BCG re-vaccination	Live attenuated	phase 2a	Y
rBCG30	Live attenuated	Phase 1	N
MIP	Killed mycobacterial	phase 3	N
Vaccae	Killed mycobacterial	phase 3	N
DAR 901	Killed mycobacterial	phase 2b	Y
RUTI	Killed mycobacterial	phase 2a	N
MVA 85 A	Viral vector	Phase 2b concluded.	Y
TB FLU 04L	Viral vector	Phase 2a	Y
Ad5 Ag85A	Viral vector	phase 1	Y
chAdOx 185A/ MVA85A	Viral Vector	Phase 1	Y
ID93 + GLA SE	Fusion Protein	phase 2a	Y
M72 + AS01E	Fusion Protein	phase 2b	N
H56: IC 31	Fusion Protein	phase 2b	Y
H4: IC 31	Fusion Protein	Phase II concluded	Y

Table 1.1 The current vaccine candidates against TB have been be grouped in to four categories; Live attenuated vaccine candidates, killed mycobacterial vaccine candidates, viral vector vaccine candidates and vaccines based on fusion proteins and adjuvants. These have been further divided into vaccines which would replace (prime) or boost the BCG vaccine (Kaufmann, Hussey, and Lambert 2110-2119).

1.4 Correlates of immunity

Early selection of suitable TB vaccine candidates depends upon a clear understanding of the correlates of immunity. Unfortunately, the correlates of immunity for TB have not been fully elucidated. There is substantial evidence for an important protective roles played by IFN γ and IL-17. Multifunctional CD4 $^{+}$ T cells generating IFN γ , IL-2 and TNF α have also been investigated as potential correlates of immunity. These responses alone, whilst important, may not be sufficient for protection against TB. The MVA85A booster vaccine was an immunogenic vaccine formulation which elicited enhanced IFN γ responses when combined with BCG. In spite of this, enhanced protection against TB disease was not observed in human clinical trials (Tameris MD et al. 2013). This finding suggests that further tuberculosis vaccine development requires suitable laboratory based studies.

1.5 Measuring mycobacterial load

The ability of a vaccine to reduce mycobacterial load remains the most appropriate metric of efficacy. In human clinical trials, clinical manifestations of TB, radiography and microbiological samples can be used to determine the protection conferred by vaccines. QuantiFERON TB responses have also been used as a measure of vaccine efficacy (Andrews et al., 2017) (Nemes et al., 2018). The Quantiferon assay is an ELISA assay which detects the release of IFN γ by PBMCs in response to TB antigen. Mycobacterial growth inhibition assay (MGIA) using ex vivo samples can also be used to evaluate the generation of protective antimycobacterial responses (Tanner et al., 2021) (Pepponi et al., 2017). Mycobacterial load assay (MBLA) provides a RT PCR molecular test to quantify and detect live mycobacteria (Sabiiti et al., 2020).

Laboratory based TB vaccine development relies upon animal models. The effect of novel vaccines on mycobacterial load can be assessed using murine TB infection models. CFU counts taken from lungs and spleens can be used to determine pulmonary load and measure protection against disseminated TB. Further development may take place in hamster and non human primate models. These models have several limitations. They are expensive, labour intensive and require Category III facilities. In

addition the immune responses observed in animals may not directly correspond to those seen in humans. This study sought to develop an *in vitro* assay using human primary tissue which could be used to test the effect of novel vaccines on antimycobacterial immune responses. Tonsil models have previously been used to assess the immunogenicity of novel vaccines against Influenza (Wagar et al., 2021a), Dengue virus (Kim, Mi Young, Copland, Nayak, Chandele, Ahmed, Zhang, Diogo, Paul, Hofmann, & Yang, 2018a) and Rabies (Wagar et al., 2021a). This study uses tonsil cells to investigate the immunogenicity of novel vaccines against TB.

1.6 Novel vaccine formulations

Initially, this study investigated three novel TB vaccine formulations; Spore FP1, YC NaMa FP1 and Liposome FP1. These were based upon a recombinant molecule FP1, formed from three mycobacterial antigens. This recombinant FP1 was combined with a delivery system consisting of *Bacillus subtilis* spores, YC NaMa or Phosphatidylserine liposomes.

1.7 Vaccine delivery system candidates - *B. subtilis* spores

B. subtilis is a rod forming, Gram positive bacterium. It is well characterised and has been used as an antigen delivery system for several novel vaccine candidates (Ferreira, Ferreira, and Schumann 113-124; Potocki et al. 151). *B. subtilis* spores were shown to be relatively well tolerated and elicit weak immunogenic responses against themselves. Instead, immune responses were directed against antigens adsorbed onto the spore surface. In low doses, *B. subtilis* spores are able to enhance T cell proliferation in response to specific antigens (Barnes et al. 1538-1547). Peptidoglycan from *B. subtilis* acts as a TLR 2 agonist (de Souza et al. e87454). TLR 2 activation of APCs elicits production of IL-12 and IL-10. This then enhances the generation of Th2 humoral responses and blocks Th1 responses (Re and Strominger 7548-7555). Exopolysaccharide (EPS) from *Bacillus subtilis* binds to TLR 4 leading to pro-inflammatory signalling through MyD88 and IRF-3 (Jones, Paynich, and Knight). EPS causes naïve

macrophages to develop into anti-inflammatory M2 macrophages which are tolerogenic. EPS also inhibits the proliferation of CD4+ and CD8+ T cells through a mechanism reliant on TGF β and PD-L1 (Paynich, Jones-Burrage, and Knight 2689-2698). *B. subtilis* spores have been used in combination with fusion protein 1 (FP1) to form a mucosal vaccination strategy against Tuberculosis. Spore FP1 has been tested as a boost to BCG and as a homologous prime vaccine. Spore FP1 vaccination resulted in enhanced immune responses. Significantly, this strategy led to a combined Th1 and Th17 response, with enhanced INF γ production. Antigen specific secretory IgA was detected in lung lavages and antigen specific IgG was generated in sera. Spore FP1 confers equivalent protection against MTB infection when used as a boost to the BCG vaccine (Copland et al. 346; Reljic et al. 4071-4080).

1.8 Vaccine delivery system candidates - YC NaMa

YC NaMa is composed of yellow carnauba wax combined with sodium myristate. Yellow carnauba wax is derived from palm oil. It is used for a range of different purposes, from foodstuffs to cosmetics.

YC NaMa is currently being used as a nanoparticle delivery system for vaccine candidates. It has been used in conjunction with GP140 against HIV and against TB in combination with Ag85B (Stylianou et al. 440-449; Arias et al. 1258-1269). YC NaMa has been combined with Fusion Protein 1, (FP1), composed of Acr, HBHA and Ag85b. YC NaMa FP1 offered protection against MTB infection in a murine study (Hart et al. 822-833). Mice treated with YC NaMa FP1 developed Ag85B and Acr specific antibody responses. Antigen specific serum IgG and mucosal IgA exceeded the levels generated by BCG immunisation. YC NaMa FP1 generated a mixed Th1 and Th17 response. This combined Th1 and Th17 response is hypothesised to be a correlate of protection against TB.

1.9 Vaccine delivery system candidates - Liposome P/S

Phosphatidylserine is present in cell surface membranes of all nucleated human cells, but not red blood cells. It is synthesized from phosphatidylcholine and phosphatidylethanolamine (Christie). Phosphatidylserine is present in the inner membrane of cells, where it is fixed into position by the enzyme Flippase. It plays a significant role in facilitating infections and suppressing anti-tumour responses (Birge et al. 1962). During apoptosis, phosphatidylserine is exposed to the external side of the cell membrane by the enzyme Scramblase (Hankins et al. 35-47). Phosphatidylserine is commonly used as a marker for apoptosis, but not necrosis. Phagocytes recognise exposed phosphatidylserine on the cell surface as an “eat me” signal, prompting efferocytosis. Phosphatidylserine is recognised by the TIM and TAM receptors. The TAM receptors; Tyro 3, Axl and Mer are receptor tyrosine kinases which act as homeostatic regulators and control phagocytosis. T cell immunoglobulin mucin domain-1 (TIM) receptors play an important role in controlling immune responses on APCs and T cells. Dengue Virus is able to gain entry into cells through the TIM and TAM receptors (Meertens et al. 544-557). Previous work showed that phosphatidylserine binding to TAM receptors leads to signalling which enhances efferocytosis by epithelial cells and PD-L1 expression when combined with Gas-6 or ProS (Kasikara et al. 753-764). Phosphatidylserine is also recognised by RAGE, which generates pro inflammatory responses through Nf- κ B and upregulates engulfment of apoptotic cells through Rac1 (Kim, S. et al. e0174603).

In this study phosphatidylserine was formed into liposomes (Liposome P/S). Liposomes have been used to adjuvant a novel dengue vaccine candidate (Swaminathan et al. 34215). The uptake of liposomes by APCs can be enhanced using IGF-1 (Han et al. 570). Liposome P/S has been combined with Ag85b and ESAT6 and administered to C57BL/6 mice as a BCG boost vaccination against TB. This strategy led to enhanced protection compared to BCG alone (Diogo et al., 2019a). The mechanism by which liposome FP1 enhances protection has not previously been determined and is explored in this study.

1.10 Fusion protein FP1

FP1 is a fusion protein consisting of three antigens derived from *Mycobacterium tuberculosis*: Acr, Ag85B, and heparin binding hemagglutinin (HBHA). These antigens have disparate functions and are expressed at different times during TB infection. Combining them is intended to generate a broader immune response offering greater protection against TB. FP1 is an integral part of three TB vaccine candidates; Spore FP1, YC NaMa FP1 and Liposome FP1. The fusion protein FP1 has been designed to generate protective immune responses against tuberculosis and target TB during its growth phase, its dissemination and then during its latency.

1.11 Ag85B

Ag85B (Rv1886c) is a mycolyl-transferase surface protein expressed on the surface of tuberculosis and BCG. It is known to play an important role in TB infection and is a component of multiple TB vaccine candidates currently in development (Cai et al. 450-456; Hess and Kaufmann 165-173; Horwitz and Harth 1672-1679; Kaufmann et al. 301-320; Olsen et al. 6148-6150; Teixeira et al. 930-935). Expression levels of Ag85B vary between strains and alter over the course of infections. Ag85B is typically expressed on the surface of active, replicating mycobacteria but it is not essential for mycobacterial survival. It is known to play a role in enhancing infection of macrophages by MTB (Prendergast et al. 2608-2615). Expression of Ag85B diminishes once MTB enters into a latent state (Rogerson et al. 195-201). Ag85B is strongly recognised by human PBMCs and it elicits strong immunodominant responses (Malin et al. 1677-1685). Vaccination using Ag85B has been shown to induce IFN γ producing T cells which offer protective immunity against TB (Kamath et al. 1702-1707). A sub unit vaccine which includes Ag85B should provide protection against replicating MTB, but would not necessarily drive responses against latent TB.

1.12 Acr

Acr (Hsp_x RV2031c) is a 16-kDa α -crystallin heat shock protein required for *in vivo* persistence of MTB. Acr is also necessary for growth of MTB in macrophages (Yuan et al. 9578-9583). Acr is upregulated in response to low oxygen levels, nitric oxide, once the bacilli enter into the stationary phase (Cunningham and Spreadbury 801-808; Hu and Coates 1380-1387; Garbe, Hibler, and Deretic 460-465).

BCG vaccination does not generate strong specific T cell responses to Acr or other latency antigens. Combining Acr with BCG vaccination has shown greater protection against TB and a decline in persistent MTB (Spratt, Britton, and Triccas 61-65; da Costa et al. e112848). Recombinant Acr combined with BCG is effective, but the presence of a mycobacterial host or vaccine component is important. The immunogenic and protective qualities of Acr are lost when the antigen is recombinantly generated using a non-mycobacterial host organism. Recombinant Acr generated in *E. coli* does not protect against mycobacterial challenge. The protective properties of Acr are then regained once the recombinant antigen is incubated with mycobacterial cell lysate. It was hypothesised that this indicated that Acr is not intrinsically protective, but that rather it acts as a chaperone for a different mycobacterial antigen (Taylor et al. 945-954). A vaccine which uses the Acr antigen may be most effective in conjunction with a mycobacterial vaccine component or as a booster to the BCG vaccine.

Acr has been shown to have disparate effects on DCs depending on their activation status. Naïve dendritic cells which encounter Acr have impaired maturation (Siddiqui et al. 1436-1445). Active DCs which are exposed to Acr show enhanced secretion of pro inflammatory cytokines. These cells also activate T cells more efficiently and direct naïve T helper cells to Th1 and Th17 phenotypes, culminating in restriction of MTB intracellular growth (Amir et al. 624). The addition of Acr to the FP1 fusion protein is intended to generate responses which target latent TB.

1.13 HBHA

Heparin binding hemagglutinin (HBHA) is a 29kDa protein expressed on the surface of MTB. It is important for adhesion of MTB to non-phagocytic cells such as epithelial cells (Pethe et al. 190; Menozzi, F. D. et al. 993-1001; Esposito et al. 339-344). It is also responsible for inducing apoptosis in macrophages (Choi, J. A. et al. e957). HBHA has been implicated as a key mediator of extra pulmonary dissemination by MTB (Balasubramanian et al. 168-178; Menozzi, Franco D. et al. 1-9; Pethe et al. 190). HBHA is differentially expressed during infection and is most associated with early stationary phase during infection (Delogu et al. 3006-3011). HBHA is predominantly expressed by MTB present in lung tissue and is not associated with disseminated MTB present in the spleen (Shi, North, and Gennaro 2420-2424; Timm et al. 14321-14326). Immunoglobulin M (IgM) reacts with HBHA and may be an important factor in preventing MTB dissemination. Sera from TB patients with anti HBHA IgM neutralises the adherence and entry of MTB into epithelia cells (Shin et al. 869-875). Post translational methylation and glycosylation impacts the immunogenic and functional properties of HBHA. (Menozzi, F. D. et al. 12625-12630; Temmerman et al. 935) This methylation is not present on recombinant forms of HBHA formed by *E.coli*. In spite of this HBHA has been used to effect in novel vaccine formulations. Mouse models have demonstrated that vaccines based upon HBHA derived from mycobacterial sources can be protective against MTB challenge (Parra et al. 6799-6805; Temmerman et al. 935). A boost vaccination of BCG with intranasal HBHA and cholera toxin (VAC7P.972) enhanced Th1 immune response and diminished extra-pulmonary dissemination of MTB from the lungs of challenged animals (Fukui, Umemura, and Matsuzaki). These studies show the value of using HBHA as a component of novel sub unit vaccines.

1.14 TLR agonists as adjuvants

TLR agonists can be used to activate DCs and prime T cells and B cells. They can also generate antimicrobial responses, inflammation and cytokine production. TLR agonists have been used as adjuvants to enhance the protective properties of vaccines. YF-17D yellow fever vaccine activates several TLR receptors on DCs, leading to a wide range of innate and adaptive immune responses which provides effective protection (Querec et al. 413-424). For novel vaccines, a range of TLR agonists are available as potential adjuvants. The most significant of these are described here.

Peptidoglycan is an agonist of TLRs 1 and 2 and NOD 2. TLR 1 and 2 signalling leads to MyD88 signalling and activation of the inflammasome through NF- κ B signalling (Wang Q. et al. 2001).

Polyinosinic-polycytidylic acid (Poly I:C) is a TLR 3 and RIG I agonist (Alexopoulou L. et al 2001). TLR3 signalling uses TRIF as an adaptor molecule leading to activation of IRF3 and the IFN I pathway (Yamamoto M et al. 2003). This leads to activation of myeloid dendritic cells (Akazawa T. et al 2007). TLR3 signalling is able to mediate cross-priming of antigen-specific CD8⁺ T cells by DCs (Quinn K. M. et al. 2013).

Lipopolysaccharide (LPS) and Monophosphoryl lipid A (MPL) (*Salmonella enterica*) are both TLR 4 agonists. Glucopyranosyl Lipid A (GLA) is also a TLR 4 agonist (Martin M. et al. 2003). Agonist binding to TLR 4 leads to MyD88 dependent signalling, inflammasome activation and Nf- κ B activation through TIRAP. It also leads to IRF 3 signalling through TRIF (Lu Yong Chen et al. 2008).

Flagellin is a TLR5 agonist signalling using the adapter protein MyD88 leads to the production of Th1 pro inflammatory cytokines and activation of Nf- κ B. TLR 5 signalling is suppressed by the adapter protein TRIF (Choi, Y. J., Im, Pothoulakis, and Rhee 21382-21390). In human monocytes and DCs, TLR 5 binding is associated with the secretion of TNF α and pro inflammatory cytokines (Miao et al. 275-288).

CPG DNA is synthetic DNA strand which act as a TLR 9 agonist. It activates MyD88 and the Myddosome (Schnare M et al. 2000). CPG DNA signals using TRAF 3 leading to activation of mitogen activated protein kinase (MAPK) pathway and IRF 7 (Hacker H. et al. 2000). This activation generates pro inflammatory cytokines (Takauji R. et al. 2002).

Using these TLR agonists, it is possible to selectively enhance certain immune responses. With TLR 1/2/5 signalling it is possible to initiate pro inflammatory responses alone through MyD88 signalling. TLR 3 and TLR 9 signalling lead exclusively to activation of the IFN I pathway. Combined signalling using IRF 3 and MyD88 is required for the activation of DCs (Shen, H. et al. 1849-1858).

1.15 Dengue Fever

This study developed an *in vitro* model to test the immunogenicity of novel vaccines for Dengue Fever. Dengue Fever is caused by the Flavivirus Dengue virus (DENV). It is spread by the *Aedes aegypti* mosquito. Infected humans are the main reservoir for the disease. It is estimated that 390 million people are infected each year and that 96 million of these develop clinical symptoms (World Health Organisation). Dengue Fever manifests clinically as a severe fever with joint pains, headaches and nausea. The symptoms typically last for up to 7 days. Dengue Haemorrhagic fever (DHF) and Dengue Shock Syndrome (DSS) are complications which may arise during infection. These complications are typified by uncontrolled bleeding, leaking plasma and respiratory failure. Whilst appropriate medical care and rehydration can significantly reduce the mortality rates of infected patients, there is no direct treatment for Dengue fever.

Mononuclear phagocytes and DCs are the primary hosts for replicating DENV. Receptors such as DC SIGN and the mannose receptor CD206 are used by the virus to gain entry into cells (Liu, P. et al. 218-231; Miller et al. e17). DENV is able to infect lymphocytes, hepatocytes and endothelial cells. This infection contributes to high levels of pro inflammatory cytokines. DHF and DSS are typically accompanied by a cytokine storm with monocytes, T lymphocytes, macrophages and endothelial cells

generating pro inflammatory cytokines and chemokines (Costa et al. 1950-1961; Green and Rothman 429-436; Kalayanarooj et al. 313-321; Schnittler and Feldmann 967-972). Type I and II IFN responses are essential for control of infection (Johnson and Roehrig 783-786). STAT 2 and STAT1 signalling are both able to limit viral replication (Perry et al. e1001297). Neutralising antibodies generated during infection are decisive in providing long lasting protection against symptomatic infection. Non neutralising antibody responses can contribute to the pathology of the disease by binding to dengue epitopes and enhancing the ability of virions to enter host cells via Fc receptors (Rothman 532).

Dengvaxia has been approved in 20 countries as a vaccine against Dengue virus. Dengvaxia is only used in areas where the prevalence of dengue fever is greater than 70%. Dengvaxia is unsuitable for seronegative patients, as vaccination of seronegative patients with Dengvaxia led to more severe manifestations of Dengue Fever in these patients. There are four principal serotypes of DENV. New vaccines should protect against each of these distinct serotypes. Because of the limitations of the Dengvaxia vaccine, new vaccine candidates against Dengue virus are required. There are currently 5 vaccine candidates against Dengue Fever in human clinical trials. These are shown in table 1.2, below.

Table 1.2 Vaccine candidates against Dengue Virus

Vaccine Candidate	Vaccine Type	Phase
TDENV PIV	Formalin inactivated	Phase I
DenVax Live	Live attenuated	Phase II
TV003/TV005	Live attenuated	Phase II
V180	Recombinant Subunit vaccine	Phase I
DIME 100	DNA vaccine	Phase I

The pre membrane and envelope are major constituents of three of these vaccines. The rationale for this is that the Env protein of Dengue is the principal target of dengue neutralising antibodies during infections (Clements et al. 2705-2715; Govindarajan et al. 4105-4116; Collier et al. 7267-7275). The envelope protein of flaviviruses is able to combine with the pre membrane protein C-prM and form immature virions which are immunogenic but not infectious (Ferlenghi et al. 593-602; Stiasny et al. 8142-8147; Hsieh et al. 33149-33160). Antibodies against EDIII are serotype specific and less prone to eliciting antibody dependent enhancement of infection. This makes EDIII a promising antigen for novel dengue vaccines (Shukla et al., 2020).

The consensus envelope domain III (CEDIII) has been incorporated into several novel dengue vaccines now in development. In a mouse model, CEDIII based vaccines have been shown to generate DENV specific IFN γ and IL-4 CD4 $^{+}$ T cell responses (Chen et al., 2015). A vaccine formulation based on CEDIII encapsulated in nanospheres led to DC maturation (Nantachit et al., 2017)

The Env protein CEDIII also forms a key part in the D-PIGS vaccine; Anti-Dengue Virus poly-immunoglobulin G scaffold (D-PIGS). This vaccine uses a CEDIII fused to IgG. This IgG is then aggregated into a polymeric structure. These polymeric IgGs induced stronger immune activation than the monomeric form in transgenic mice expressing a humanised Fc γ RI/CD64. This enhanced immunogenicity indicates that polymeric IgGs interact more efficiently with low-affinity Fc γ receptors (Webster et al.). Tonsil tissue can be used as a relevant platform for Dengue Poly IGs (D-PIGS) (Kim, M. Y. et al.). The D-PIGS vaccine has previously been shown to induce neutralising antibodies in mice (Kim, M. Y. et al.). D-PIGS induced immune activation in transgenic mice expressing a humanised Fc γ RI/CD64 (Webster et al.).

CEDIII is also used as an antigen in the novel vaccine candidate, Dengue Recombinant Immune Complex Scaffolds (DERICS). This is composed of the CEDIII antigen bound to the binding epitope of the anti Ebola monoclonal antibody 6D8. Vaccination of mice with DERICs induces anti CEDIII specific IgG. These antibodies were capable of neutralising the Dengue 4 serotype (Kim MY. et al. 2015).

Both D-PIGS and DERICS have been shown to be immunogenic and capable of eliciting neutralising antibodies in a mouse model. Further work is now needed to bring them to a clinical setting and demonstrate their effectiveness in humans.

1.16 *In vitro* modelling of novel vaccine candidates using human tonsil tissue

Palatine tonsils are lymphoid organs situated on the mucosal surface at the rear of the throat. Tonsillectomy is a relatively common procedure which is carried out for a range of conditions. Ex vivo tonsil samples have previously been used to model infections of mucosal tissue (Mahallawi et al. 5331-5339; Maher et al. 631-642). Tonsillar cells have been used previously to develop infection models for HHV6, Influenza, EBV and HIV (Grivel et al., 2003a) (Gotoh et al., 2011a) (Maher, Diane M., Zhang, Schacker, & Southern, 2005a) (Wagar et al., 2021a).

Several groups have developed HIV infection models using human tonsils. These studies demonstrated binding of HIV to tonsil cells and infection of tonsillar lymphocytes (Maher, Diane M., Zhang, Schacker, & Southern, 2005b). The protective effect of the intact mucosal surface was also revealed (Maher, Diane et al., 2004). A HIV tonsil infection model led to the discovery of a novel antiviral function in neutrophils which suppresses HIV. Confocal microscopy of HIV infected tonsils showed that neutrophils destroy HIV infected cells in a contact dependant manner (Reif et al., 2021). A tonsil HIV infection model has also been used to examine the production of cytokines by tonsillar cells in response to acute HIV infection. The expression levels of IFN γ , IL-4, IL-12a, IL-10a, IL-1b, IL-2, IL-15, IL-6, IL-8, TGF β and TNF α were all measured and, disappointingly, were all found to be unaltered by HIV infection (Audigé et al., 2004).

Tonsil tissue explants have been used to model infection with Epstein-Barr virus (EBV). EBV is a γ -herpesvirus. Recombinant EBV expressing a GFP tag was used to develop an EBV infection model. Infected tonsil cells could be visualised by the expression of GFP. Viral replication was measured using RT PCR of EBV DNA. Using this model, it was found that EBV replication principally occurred in B cells.

Acyclovir treatment suppressed EBV replication in the tonsil model. This showed that tonsil models can be used to investigate novel anti-viral agents. No antibody responses were generated against EBV in the tonsil model. In spite of this, the EBV tonsil system was an effective model for infection which has value for the screening of novel anti-viral agents (Gotoh et al., 2011b).

Human tonsils have also been used to model infection of Human Herpes Virus 6 (HHV6). Infection was measured by RT-PCR and by flow cytometry, similarly to the EBV model. HHV6 is thought to be immunosuppressive, but this cannot be modelled in PBMCs. HHV6 infection in the tonsil model revealed insights into HHV6 behaviour and its suppressive effects. The virus replicated preferentially in non-naïve CD4⁺ T cells and significantly depleted their population. Flow cytometry revealed that the virus induced a 179% increase in the expression level of CD4 by infected cells. Simultaneously, the infected cells showed a 99.3% reduction in CD3 expression. The study found that production of the chemokine RANTES increased 6 fold during HHV6 infection (Grivel et al., 2003b).

It is notable that none of these infection studies found any evidence of tonsil tissue generating new antibodies against the novel infection.

Two studies used tonsils to develop an Influenza infection model. This model did reveal information about the generation of new antibody responses in tonsils following vaccination. Live attenuated influenza vaccine (LAIV) was administered to children in the weeks before their scheduled tonsillectomy. In these studies, children generated virus-specific IgA and B cell responses. IFN γ responses in CD4⁺ and CD8⁺ T cells were also found along with IL-13, IL-4, GM-CSF and IL-2 responses. The model used in these studies elicited antibody responses, however, it required the participants to be vaccinated weeks prior to their surgery. This makes it unsuitable for the development of novel vaccine candidates which have not previously been safety tested in humans.

Cells derived from human palatine tonsils have been used as a platform for testing novel vaccine candidates (Zhang, Q. et al. e1002175; Mahallawi et al. 5331-5339; Aljurayyan et al. 10.1128/JVI.00114-18. Print 2018 Jun 1; Gray et al. 705-717; Mahallawi et al. 5331-5339; Zhang, Q. et

al. 1194-1202) Tonsil tissue has most often been used as a source of mononuclear cells. Tonsil tissue contains a high proportion of B cells and T cells with a smaller proportion of macrophages and epithelial cells (Boyaka et al. 2023-2035). It is possible to preserve tonsil tissue structure during tissue culture. Blocks of tonsil tissue retain their architecture, including B cell follicular germinal centres and lymphoid follicles. Maintaining these structures can generate experimental data which is more relevant to *in vivo* mucosal immunology (Giger et al. 179-190).

Human tonsils may be a relevant platform for infections of TB and the development of novel mucosal vaccines against it. Transmission of tuberculosis frequently occurs across the mucosal surfaces of the lungs. There is also increasing interest in delivering TB vaccines via the respiratory mucosal route (Li et al. ; Kaushal et al. 8533; Reljic et al. 4071-4080; Lai et al. 356-360). It is important to test novel mucosal vaccines using an *in vitro* platform which is relevant to mucosal tissue. The mucosal lymphatic tissue found in tonsils may represent a good model. In this study, tonsil mononuclear cells (TMCs) were used as an *in vitro* model for T cell responses to TB vaccine candidates. To date, tonsil cells have not been used to develop an infection model for Tuberculosis. This study uses tonsil cells to establish a mycobacterial infection model. Using human tonsils for this purpose has several advantages; tonsil cells are a good source of relevant human lymphoid tissue, tonsil *in vitro* models can be used for high throughput screening of vaccine candidates and most significantly, the tonsil *in vitro* model provides a way to reduce the use of animals in vaccine research.

Aim

The aim of this project is to develop *in vitro* models which can be used to determine the immunogenicity of novel vaccine candidates.

Objectives

APCs

Determine the effect of vaccine candidates on the function and activation of dendritic cells.

Establish whether TLR agonists can be used as adjuvants to enhance vaccine immunogenicity.

Tonsil cell cultures

Establish an *in vitro* model using tonsil tissue which can be used to test vaccine immunogenicity.

Determine the effect of vaccine candidates on Intracellular cytokine production and the development of CD4+ and CD8+ T cell responses.

Determine the effect of vaccine candidates on antibody production and B cell responses.

Hypothesis

Using *in vitro* models, it will be possible to determine which vaccine candidates are immunogenic and lead to the development of protective immune responses.

2. Materials and Methods

Protocols for Chapter 1 – Immunogenicity of TB vaccine candidates in BMDCs

2.1 Preparation of Murine Bone Marrow Dendritic Cells (BMDC)

Murine BMDCs were used as a model for antigen presenting cells. They were obtained using the following method. Schedule 1 was performed on 6-12 week old C57/BL6 mice. Limbs were pinned back with needles and legs, abdomen and torsos sprayed with 70% ethanol containing hydrogen peroxide. Using sterile scissors, a cut was made from the lower abdominal flank above the pelvis, to capture both major bones of the leg, including the tip of the femur. Legs were removed and placed on ice. Under sterile conditions, the skin was stripped back from the legs and paws removed. Legs were placed in complete RPMI 1640 media (R10 media) for 5 minutes to soften tissue. Muscle tissue was manually removed from bones, epiphyses removed and 500µl of R10 media flushed through the bone marrow in a brisk manner using a needle. This was repeated until bone marrow was removed. Exudate was collected into 50ml falcon tubes containing R10 media and centrifuged at 400 RCF for 10 minutes without brake, at 4 °C. 5ml of ACK lysis buffer added to cells for 2 minutes to lyse erythrocytes. After washing, cells were resuspended in R10 media with 20ng/ml GMCSF. Cells were incubated for 48 hours. Media was then replaced and the cells were incubated for a further 72 hours. On day 6, non-adherent and loosely adherent cells were collected. R10 was pipetted across the cell monolayer while the flask was tilted. Collected cells were washed and counted using trypan blue exclusion. Cells were then frozen in FBS with 10% DMSO and stored in liquid nitrogen for later use. For phenotypic analysis by flow cytometry, the following fluorochromes were used.

Antibody	Fluorochrome	Clone
α CD11c	PerCP Cy5.5	N418
α MHC II	BV510	M5/ 114.15.2
α CD11b	PE	M1/70)
Viability	APC Cy7	Efluor 780 (Thermofisher)

2.2 Vaccine delivery system analysis using murine BMDCs.

The immunogenicity of three vaccine delivery systems was tested. These delivery systems were *B. subtilis* spores, YC NaMa and Liposome P/S. They were applied to murine BMDCs and cell surface markers and pro inflammatory cytokine production were monitored. 1×10^5 BMDCs were added to 96 well round bottomed plate in 100 μ l of R10 media and left to settle for 1 hr. BMDCs were stimulated with 100ng/ml LPS. Liposome PS/PS was added to BMDCs in a ratio of 5:1, 2:1 and 1:1. This corresponded to a concentration of 5×10^5 , 2×10^5 and 1×10^5 liposomes per ml. YC Nama was added to wells at concentrations of 10 μ g/ml, 1 μ g/ml and 0.1 μ g/ml. Spores of *B. subtilis* were added to wells at a density of 2×10^4 , 2×10^3 and 2×10^2 spores per well. This corresponded to multiplicities of infection (MOI) of 5:1, 50:1 and 500:1, BMDCs to *B. subtilis* spores. Cells were incubated in a final volume of 200 μ l at 37°C, 5% CO₂, 95% humidity for 48 hours until the endpoint of the study.

2.3 Cytokine analysis; Enzyme linked immunosorbent assay (ELISA)

ELISAs were performed to determine the expression of IL-6, TNF alpha, IL-10 and IL-1 β . Ready To Go ELISA kits from Affymetrix were used according to the manufacturer's instructions. Anti-mouse capture antibody (eBioscience) was diluted 1/250 in 1 x coating buffer (eBioscience). 100 μ ls were incubated overnight in wells of a 96 well ELISA plate (Maxisorp). The plate was washed 3 times. Wash buffer was composed of 1 x PBS with 0.05% Tween. Wells were blocked with assay diluent (eBioscience) and incubated for 1 hour. The plate was then washed 3 times. Cytokine standards (eBioscience) were prepared in 1x assay diluent (eBioscience) and 100 μ ls added to wells. A standard curve was used. Plate incubated at room temperature for 2 hours. The plate was washed 5 times. Detection α -mouse cytokine antibody was diluted 1/250 in assay diluent (eBioscience). 100 μ ls were added and incubated for 1 hour at room temperature. Plate washed 5 times. 100 μ ls of Avidin HRP (eBioscience) diluted 1/250 in assay diluent was added. The plate was incubated for 30 minutes and

washed 5 times. 100 µls of substrate solution was added. After 1 minute, the colour developed and 100 µls of 0.2 M sulphuric acid was added to wells. The plate was then read in a TECAN Sunrise absorbance microplate reader at 450nm.

2.4 Cell Surface Marker Flow Cytometry Analysis.

To determine the effect of vaccine formulations on murine BMDC activation, expression of cell surface markers was measured. At the end point of the study, plates were removed from incubator and the plate spun at 400 RCF for 5 minutes. 100 µl removed and frozen at -20 °C for later supernatant analysis. 200 µl of PBS were added to each well. Plate was spun at 400 RCF for 5 minutes. Supernatant was discarded and the plate patted dry. 50 µl of viability dye was added to wells apart from unstained control wells. Plate incubated in fridge for 1 minutes. 250 µls of FACS buffer (PBS + 0.1% BSA and 0.05% NaN₃) added to wells. Plate spun at 400 RCF for 5 minutes. Supernatant discarded and plate patted dry. The fluorochromes were diluted 1/100. 25µl of master mix containing each fluorochrome was placed into wells. The fluorochromes which were used are shown in supplemental table 10.1. Plate was incubated in the dark at 4 °C for 30 minutes and then washed. 250µl of FACS buffer (PBS + 0.1% BSA and 0.05% NaN₃) were added to wells and flow cytometry was performed.

2.5 Intracellular cytokine analysis; flow cytometry

It was appropriate to use intracellular staining to determine expression levels of IL-1β and IL-12 in murine BMDCs. The effect of vaccine delivery systems on expression of IL-1β and IL-12 was tested. 1x10⁵ BMDCs were added to round bottomed wells of a 96 well plate in 100 µl of R10 media and left to settle for 1 hr. Brefeldin A was added to wells at a concentration of 5 µg/ml to prevent export of cytokines outside of the cells. Duplicate wells were stimulated with 100ng/ml LPS. The appropriate concentration of liposome P/S, YC NaMa and Spores were based on the results shown in figure 2, 3

and 4. Liposome P/S was added to duplicate wells in a ratio of 5:1 compared to the murine BMDCs. This corresponded to a concentration of 5×10^5 liposomes per well. YC Nama added to duplicate wells at concentrations of 10 $\mu\text{g}/\text{ml}$. 2×10^4 Spores of *B. subtilis* were added to duplicate wells. This gave an MOI of 5:1, BMDCs to spores. Additional R10 media was added to wells to give a final volume of 200 μls . Cells were incubated at 37 °C, 5% CO₂, 95% humidity for 48 hours until the endpoint of the study. After 18 hours, expression of IL-12 and IL-1 β was determined by intracellular staining and subsequent flow cytometry. Plates were spun at 400 RCF for 5 minutes. The supernatant was discarded and 200 μls of PBS added to wells. Plate was spun at 400 RCF for 5 minutes and the supernatant was discarded. 25 μls of viability dye master mix added to wells. Viability dye master mix contained the viability dye Efluor 780 and an Fc Block (Trustain), which prevented nonspecific binding. Each plate was incubated for 15 minutes at 4°C. 200 μls of FACS buffer (PBS + 0.1% BSA and 0.05% NaN₃) was added to wells. The plate was spun at 400 RCF for 5 minutes and supernatant discarded. 4% PFA fixative (Biolegend) 100 μls was added and cells were resuspended to prevent doublets from forming. The plate was incubated at 4°C for 30 minutes before being spun at 600 RCF for 5 minutes and the supernatant removed. In order for the intracellular cytokines to be stained, the cells were first lysed by adding 200 μls of permeabilisation buffer (PBS containing 0.5% saponin), to all wells. The plate was spun at 600 RCF for 5 minutes and supernatant discarded. Antibody master mix was prepared in permeabilisation buffer containing 0.5% saponin. Antibody master mix contained IL-12 antibody clone 15.6 bound to PE (Biolegend) and IL-1 β (pro form) antibody clone NUTEN3 bound to APC. 25 μls of antibody master mix was added to wells and cells were incubated for 30 minutes at 4 °C. Cells were washed in permeabilisation buffer, then FACS buffer (PBS + 0.1% BSA and 0.05% NaN₃) and then resuspended in 250 μls of FACS buffer. Flow cytometry was performed using a FACS CANTO flow cytometer.

2.6 Apoptosis and Efferocytosis Assay

Apoptosis of APCs plays an important role in limiting MTB. Efferocytosis is a key function of dendritic cells which enables them to remove apoptotic cells. Cellular contents from dying cells can then be processed and presented other cells. This assay measures the ability of murine dendritic cells to phagocytose dead cells. It is used to determine the effect of three vaccine delivery systems on efferocytosis by DCs (Spores, YC NaMa and Liposomes). The study will also give information about whether these vaccine delivery systems induce apoptosis of DCs. Murine BMDCs were seeded into U bottomed wells on a 96 well plate, with 1×10^5 cells added into each well. The cells were left to settle for 1 hour before vaccine stimulations were added to the cells. Liposome PS/PS was added to murine BMDCs at a concentration of 5×10^5 per well. This represented a 5:1 ratio of liposomes and BMDCs. YC NaMa was added to wells at a concentration of $10 \mu\text{g/ml}$. 1×10^4 CFU *B. subtilis* spores were added to wells at an MOI of 5:1 BMDCs to spores. Cells were incubated for 30 minutes. Apoptotic cells were generated by heat-treating BMDC cells at 1×10^6 cells/ml in a falcon tube. The cells were maintained at 60°C for 120 minutes. $50 \mu\text{l}$ s of heat-treated cells were added to wells. This gave a 2:1 ratio of live cells to heat treated cells. $25 \mu\text{l}$ s of PBS was added to wells that were not stimulated with liposome. This acted as a control for the PBS buffer in which the liposome PS/PS was present. R10 media was added to wells to give a final volume of $250 \mu\text{l}$ s in each well. Cells were incubated for 18 hours at 37°C , 5% CO_2 , 95% humidity. After 18 hours, the cells were prepared for analysis by flow cytometry using the following method; the plate was spun at 400 RCF for 5 minutes. The supernatant was discarded and the plate patted dry. $200 \mu\text{l}$ s of PBS was added to each well. The plate was spun at 400 RCF for 5 minutes and the supernatant was removed. This step was then repeated. A master mix was created which contained Propidium iodide (Biolegend) diluted 1/100 and Annexin V (Biolegend) diluted 1/200 in Annexin V Binding Buffer (Biolegend). $100 \mu\text{l}$ s of master mix was added to each well except for unstained control wells. The plate was stored in the dark for 15 minutes. $100 \mu\text{l}$ s of Annexin 5 binding buffer was added to all wells. The cells were analysed using a FACS Canto flow cytometer.

2.7 TLR agonist adjuvant study.

This assay tested the hypothesis that combining vaccine delivery systems with TLR agonists would enhance their immunogenicity. 1×10^5 murine BMDCs were added to round bottomed wells of a 96 well plate in 100 μ l of R10 media and left to settle for 1 hr. TLR agonists were then added to duplicate wells. The TLR agonists used for this assay, and their concentrations are shown in supplemental table 10.2. Liposome P/S was added to duplicate wells in a ratio of 5:1 compared to the BMDCs. YC NaMa was added to duplicate wells at concentrations of 10 μ g /ml. 2×10^4 CFU Spores of *B. subtilis* were added to duplicate wells giving an MOI of 5:1 BMDCs to Spores. Additional R10 media was added to wells to give a final volume of 200 μ l. Cells were incubated at 37 °C, 5% CO₂, 95% humidity for 48 hours until the endpoint of the study. Cytokine expression and cell surface marker expression were determined using the previously described protocols.

2.8 Antigen presentation and T cell proliferation assay

Co-cultures of vaccine pulsed BMDCs and naïve T cells were used to establish the effect of vaccine formulations on antigen presentation and T cell proliferation. Murine BMDCs were seeded at 1×10^5 cells per well in a 96 well plate in R10 media and left to settle for 1 hour. LPS was added to selected wells at 100ng/ml. FP1 was added at a concentration of 5 μ g/ml. 2×10^4 *B. subtilis* spores were added to cells in each well giving an MOI of 5:1 cells to spores. Poly I:C was added at a concentration of 10 μ g/ml. Wells were made up to a final volume of 200 μ ls with R10 media cells were incubated for 24 hours at 37 °C, 95 % humidity, 5% CO₂. After 24 hours, the cells were spun at 400 RCF for 5 minutes and the supernatant was removed. The cells were then washed with R10 media and spun at 400 RCF for 5 minutes and the supernatant was removed. This washing step is important as it means that the DCs are pulsed with vaccine formulation, but that the T cells will not be directly stimulated. The wells were then refilled with R10 media containing suspended splenocytes. Murine splenocytes were

generated from C57BL/6 mice. Spleens were cut and passed through a 40 µM strainer. ACK lysis buffer was added for 2 minutes to lyse erythrocytes. The cells were then washed. Splenocytes were then suspended in R10 media and added to wells at concentrations of 1×10^6 cells per well, 5×10^6 cells per well and 1×10^6 cells per well. 1 µg/ml of LEAF anti CD3 Clone 145-2cl (Biolegend) and LEAF anti CD28 Clone 37-51 (Biolegend) was added to selected splenocytes. This acted as a positive control for T cell proliferation. Cells were incubated for 72 hours at 37°C, 95 % humidity, 5% CO₂. Cells were then processed for cell surface flow cytometry using the previously described protocol for cell surface markers. Expression of the T cell markers CD90.2, CD4 and CD8 was measured, along with the proliferation marker Ki67. The antibodies and fluorochromes used for this experiment are shown in supplemental Table 10.3.

2.9 Animal Work Ethics Statement

St. George's University of London Ethics Committee approved the work with animals undertaken in this project. This work was conducted under an approved Home Office animal project license (70/7490). C57BL/6 mice sourced from Charles River UK, were used in this study. The mice were female and aged between 5 and 12 weeks. They were kept in pathogen free conditions. This work was carried out in accordance with the Animals (Scientific Procedures) Act 1986.

2.10 *In Vivo* Antigen Recall Assay

Two research questions were addressed using this assay. Firstly, whether spore FP1 Poly I:C by itself induces immune responses in mice. Proliferation of T cells and production of IL-17 and IFN γ were examined. The second research question was whether vaccine responses could be boosted with the addition of pulsed syngeneic DCs. Antigen specific responses generated by Spore FP1 Poly I:C were measured using the following protocol. C57BL/6 mice were divided into three groups, each containing

3 animals. Group 1 received 3 doses at 2 week intervals of PBS. Group 2 received 3 doses of *B. subtilis* Spore FP1 Poly I:C vaccine formulation at 2 week intervals. Group 3 received 3 doses of *B. subtilis* spore Poly I:C FP1 vaccine formulation with BMDCs which had previously been pulsed with vaccine formulation for 24 hours. 3 weeks after the last inoculation animals were culled and spleens, sera and bone marrow were collected. Splenocytes were added to 96 well plates at a density of 5×10^5 cells per well. Cells were stimulated with the recall antigen FP1 at $5 \mu\text{g/ml}$ or soluble CD3 and CD28 at a concentration of $1 \mu\text{g/ml}$. cells were incubated at 37°C , 5% CO_2 and 95% humidity for 3 days. Cells were then processed for cell surface flow cytometry using the same protocol as previously described for the *in vitro* antigen presentation study in section 2.8. The antibodies used are listed in supplemental table 10.3.

2.11 BCG infection supernatants PD-L1 study

The effect of BCG infection on the expression of PD-L1 on APCs was unknown. In order to establish the effect of BCG on PD-L1 expression, J774 cells and murine BMDCs were used as models for macrophages and DCs. Cells were infected with BCG at an MOI of 1 or stimulated with 100ng/ml LPS or stimulated with filtered supernatant from J774 cells infected with BCG at an MOI of 1 or with filtered supernatant from uninfected J774 cells. 1×10^6 J774 cells and 1×10^6 BMDCs from C57BL/6 mice were infected with BCG at a MOI of 1 for 24 hours. Supernatants were collected and passed through a $40 \mu\text{M}$ strainer. These supernatants were then pipetted into fresh cell cultures containing 1×10^6 J774 cells or 1×10^6 BMDCs. Triplicate wells were prepared. Cells were incubated for 24 hours at 37°C , 5% CO_2 and 95% humidity. The cells were then processed for cell surface flow cytometry using the previously described protocol. Rat $\alpha\text{PD-L1}$ bound to BV 421 (Biolegend) was used in conjunction with APC Cy7 viability marker Efluor 780.

2.12 BCG B7 family study

This study determined the effect of BCG on expression levels of CD80, CD86, PD-L1 and PD-L2 on murine macrophages and DCs. 1×10^6 J774 cells and 1×10^6 BMDCs from C57BL/6 mice were infected with BCG at a MOI of 0.1, 1 or 2. Triplicate wells were prepared. LPS was used as a positive control at 100 ng/ml. Cells were cultured for 24 hours. Cells were incubated for 24 hours at 37 °C, 5% CO₂ and 95% humidity. The cells were then processed for cell surface flow cytometry using the previously described protocol. Expression of B-7 family markers CD80, CD86, PD-L1 and PD-L2 was determined. The antibodies used for this assay are listed in supplemental table 10.4.

Protocols for Chapter 2 - Characterisation of tonsil mononuclear cell cultures

2.13 Human tonsil samples

Human palatine tonsils were all sourced from eligible patients at St. George's Hospital NHS Trust. Human tonsil tissue was obtained as part of a clinical study entitled "Vaccine immunogenicity using tuberculosis and dengue fever as models" (IRAS project ID 239627). This study is run by Professor Reljic's group in collaboration with the ENT department at St Georges Hospital NHS Trust. Ethical approval for this project was provided by the SGUL JREO and the South Central Oxford-C NRES Committee. A total of 18 patients were recruited, consisting of participants with an age range of 2-80 years. Written informed consent was obtained from adults and from legal guardians of children under 16 in accordance with the Human Tissue Act (*Human Tissue Act 2004*). The inclusion criteria encompassed patients undergoing tonsillectomy, adenoidectomy or uvulopalatopharyngoplasty as treatment for breathing problems, obstructive sleep apnoea or inflamed tonsils. Samples from patients with an immunodeficiency, cancer of the tonsils or a known TB, Dengue Fever or HIV infection were excluded from the study.

2.14 Isolation of tonsillar mononuclear cells

Tonsillar mononuclear cells (TMCs) were isolated from fragments of patient palatine tonsil samples using a FICOLL gradient followed by centrifugation at 700 RCF for 30 minutes. TMCs were cryopreserved in freezing media consisting of 10% dimethyl sulfoxide (DMSO) (Sigma) and 90% heat inactivated Foetal Bovine Serum (FBS) (Sigma) at a concentration of 1×10^7 cells/ml. Samples were frozen overnight at -80°C then transferred to liquid nitrogen for storage.

Vials were removed from liquid nitrogen and thawed rapidly in a 37°C water bath when needed. TMCs were immediately transferred from vials into 50ml Falcon tubes filled with sterile Dulbecco's PBS (Sigma, D8537) and centrifuged at 400 RCF for 5 minutes. Supernatant was removed and the TMCs were resuspended in 5 ml of RPMI 1640 media supplemented with FBS, 2mM L-glutamine (both from Sigma) and 100 U/ml of penicillin and streptomycin (Gibco). For experiments which used BCG GFP, 250 $\mu\text{g}/\text{ml}$ of hygromycin B (ChemCruz, Lot F1316) were used instead of streptomycin as this strain was susceptible to streptomycin but contained a hygromycin resistance gene.

2.15 Phenotype of TMCs

Tonsil mononuclear cells (TMCs) were seeded in triplicate to 96 well plates at a density of 1×10^5 cells/well. These were spun at 400 RCF for 5 minutes and washed with PBS. The TMCs were stained with antibodies against CD3, CD4, CD8, CD11b and CD14 and a viability dye. These cells were then analysed by flow cytometry using a BD FACS Canto II.

To investigate the long term culture of TMCs, TMCs were added to triplicate wells in 96 well plates at a density of 1×10^5 cells/well and grown in 200 μl s of R10 media for 12 days. These cells were then stained with the TMC phenotype antibody panel shown in table 2.1. The antibodies were diluted 1/100 and applied for 30 minutes. Following this, the cells were washed and analysed by flow cytometry.

The expression of CD45ra by CD4⁺ T cells and CD8⁺ T cells can be used to identify naïve and memory cells. TMCs were added to triplicate wells in 96 well plates at a density of 1×10^5 cells/well and grown

in 200µl of R10 media for 12 days. These cells were stained for 30 minutes with the TMC memory antibody panels shown in tables 2.2 and 2.3, diluted 1/100. The percentage of CD45ra+ CD4+ T cells and CD45ra+ CD8+ T cells was recorded by flow cytometry.

2.16 Intracellular staining protocol for human cells

Intracellular staining was used for flow cytometry experiments which investigated proliferation or production of cytokines. Prior to intracellular staining, Brefeldin A was added to cells at 5 µg/ml for 24 hours. To fix cells, paraformaldehyde (PFA) was added at a concentration of 4% for 30 minutes at 4°C. The plates were spun at 400 RCF and the supernatant discarded. Cells were then washed with PBS containing 0.1% saponin. PBS with 0.1% saponin was added to wells for 15 minutes to lyse cells. The plates were spun at 400 RCF and the supernatant discarded. Cells were stained with antibody panels for 30 minutes. Each antibody panel consisted of conjugated antibodies diluted 1/100 in PBS with 0.1% saponin. The concentration of saponin in the lysis buffer was reduced to 0.1% in this protocol as the human cells are less robust than mouse cells. Flow cytometry was performed using a Cytoflex S system.

2.17 TB immunogenicity panel

A TB immunogenicity panel was developed to measure intracellular expression of Ki67, IFN γ , IL-17 and IL-2 by CD4+ and CD8+ T cells. To find suitable positive controls for this panel, TMCS were seeded in triplicate to 96 well plates at a density of 1×10^5 cells/well and grown in 200µl of R10 media for 5 days. A combination of PMA and ionomycin was added to triplicate wells. PMA was added at a concentration of 10 ng/ml and Ionomycin at a concentration of 500ng/ml. After 5 days, the plates prepared using the protocol for intracellular staining in section 2.16. The TMCs were stained with the TB immunogenicity panel shown in table 2.4 and analysed by flow cytometry.

2.18 Positive controls for T cells and B cells

Flow cytometry was used to measure intracellular cytokines within T cells and B cells following stimulation with a selection of chemicals. TMCs were seeded at 1×10^5 cells/well in 96 well plates with 200ul of R10 media. Cells were stimulated with 100 $\mu\text{g}/\text{ml}$ of Resiquimod, 10ng/ml PMA and 500ng/ml of Ionomycin, PHA or Con A for 5 days. After 5 days, the plates were prepared as per the protocol for intracellular staining in section 2.16. The TMCs were stained with the “lymphocyte intracellular cytokine flow cytometry panel” for 30 minutes. This is shown in tables 2.5 and 2.6. The cells were then analysed by flow cytometry and the levels of IFN γ , IL-2, IL-4, IL-10, IL-12 and TNF α were quantified.

2.19 Lymphocyte activation markers flow cytometry panel

Flow cytometry was used to measure activation markers on T cells and B cells. TMCs were seeded at 1×10^5 cells/well in 96 well plates with 200ul of R10 media. Cells were stimulated with 100 $\mu\text{g}/\text{ml}$ of Resiquimod, 10ng/ml PMA and 500ng/ml of Ionomycin, PHA or Con A for 5 days. After 5 days, the plates were washed and the TMCs were stained for 30 minutes with the “Lymphocyte activation markers flow cytometry panel” which is listed tables 2.7 and 2.8. After staining, the cells were washed and analysed using a Cytoflex S system. T cells and B cells were identified and CD40, CD69, CD80 and CD86 levels were quantified.

2.20 Mycobacterial antigen recall

TMCs from all 18 patients were stimulated with PPD. This was done to identify whether any of the patients had previously been infected with TB. 1×10^5 TMCs/well were seeded into triplicate wells in 96 well plates. These were incubated for 6 days with 5TU of PPD. After 6 days, the cells were spun at 400RCF for 5 minutes, washed with PBS and stained using the 'Mycobacterial antigen recall' antibody panel in table 2.9. Flow cytometry was used to identify CD4+ and CD8+ T cells. Activated CD4+ and CD8+ T cells expressing IFN γ , Ki67 or CD69 were identified.

2.21 Suitability of the tonsil model for a COVID 19 infection model

Whole tonsil cells and TMCs were seeded into triplicate wells on 96 well plates at a density of 1×10^6 cells/ml. The cells were spun at 400 RCF for 5 minutes, washed with PBS and stained with a primary antibody anti ACE 2 for 30 minutes. This was mouse anti ACE2 antibody from ProteinTech diluted 1/100 in PBS. After 30 minutes, the cells were spun at 400RCF for 5 minutes and stained with the ACE 2 receptor flow cytometry panel for a further 30 minutes. The ACE2 receptor flow cytometry panel contained antibodies for the detection of CD4+ T cells, CD8+ T cells, CD19 B cells, monocytes and myeloid cells (Table 2.10). This panel also contained a secondary detection antibody bound to FITC. This was Anti mouse IgG FITC "Poly 4060" from Biolegend. After 30 minutes, the cells were spun at 400 RCF, the supernatant removed and the cells were resuspended in PBS. These cells were then analysed by flow cytometry. Cells expressing ACE 2 receptors were FITC+. The MFI of FITC was measured by flow cytometry, along with the percentage of FITC+ cells.

Table 2.1. TMC Phenotype Flow Cytometry Panel

Antigen	Fluorochrome	Clone
CD3	FITC	UCHT1
CD19	PE Cy7	HIB19
CD4	PerCP Cy5.5	RPA-T4 (RUO)
CD8	BV510	HTT8a
CD11b	APC	M1/70
CD14	PE	M5E2
Viability	APC Cy7	Efluor 780 viability dye

Table 2.2. Identification of memory and naïve B cells flow cytometry panel.

Antigen	Fluorochrome	Clone
CD19	PerCP Cy5.5	HIB19
CD45ra	PE Cy7	HI100
Viability	APC Cy7	Efluor 780 viability dye

Table 2.3. Identification of memory and naïve T cells flow cytometry panel.

Antigen	Fluorochrome	Clone
CD3	FITC	UCHTI
CD4	PerCP Cy5.5	RPA-T4 (RUO)
CD8	BV510	HTT8a
CD45ra	PE Cy7	HI100
Viability	APC Cy7	Efluor 780 viability dye

Table 2.4. TB immunogenicity flow cytometry panel

Antigen	Fluorochrome	Clone
CD3	FITC	UCHTI
CD4	PerCP Cy5.5	RPA-T4 (RUO)
CD8	BV510	HTT8a
IFN γ	BV421	B27
IL-17	APC Cy7	BL168
IL-2	PE	MQ1-17H12
Ki67	APC	Ki-67

Table 2.5. B Lymphocyte intracellular cytokine flow cytometry panel

Antigen	Fluorochrome	Clone
CD19	PE Cy7	HIB19
IL-4	BV605	MP4-25D2
IFN γ	BV421	B27
IL-12	APC Cy7	C11.5
IL-10	PE	JES3-9D7
IL-2	FITC	MQ1-17H12
TNF α	PE Cy7	Mab11

Table 2.6. T Lymphocyte intracellular cytokine flow cytometry panel

Antigen	Fluorochrome	Clone
CD3	FITC	UCHT1
CD4	PerCP Cy5.5	RPA-T4 (RUO)
CD8	BV510	HTT8a
IL-4	APC Cy7	MP4-25D2
IFN γ	BV421	B27
IL-10	PE	JES3-9D7
TNF α	PE Cy7	Mab11

Table 2.7. B Lymphocyte activation markers flow cytometry panel

Antigen	Fluorochrome	Clone
CD19	PerCP Cy5.5	HIB19
CD69	PE	FN50
CD40	PE Cy7	HB14
CD80	APC	2D10
CD86	BV421	IT2.2
Viability	APC Cy7	Efluor 780 viability dye

Table 2.8. T Lymphocyte activation markers flow cytometry panel

Antigen	Fluorochrome	Clone
CD3	FITC	UCHT1
CD4	PerCP Cy5.5	RPA-T4 (RUO)
CD8	BV510	HTT8a
CD69	PE	FN50
CD40	PE Cy7	HB14
CD80	APC	2D10
CD86	BV421	IT2.2
Viability	APC Cy7	Efluor 780 viability dye

Table 2.9. Mycobacterial antigen recall

Antigen	Fluorochrome	Clone
CD3	FITC	UCHTI
CD4	PerCP Cy5.5	RPA-T4 (RUO)
CD8	BV510	HTT8a
CD69	PE	FN50
IFN γ	BV421	B27
Ki67	APC	Ki-67
Viability	APC Cy7	Efluor 780 viability dye

Table 2.10. ACE2 receptor flow cytometry panel

Antigen	Fluorochrome	Clone
CD3	BV421	Biolegend OKT3
CD19	PE	Biolegend 4G7
CD4	PerCP Cy5.5	BD Pharmigen
CD8	BV510	RPA T8
CD14	APC Cy7	HCD14
Anti mouse IgG	FITC	Poly 4060 Biolegend

Protocols for Chapter 3 - *in vitro* immunogenicity models

2.22 Immunogenicity of the BCG vaccine in TMCs

TMCs were seeded at 1×10^5 cells/well in triplicate in 96 well plates. The cells were maintained in RPMI media containing 10% FBS, 2mM L-Glut and 100 U/ml of Penicillin. The TMCs were infected with BCG Pasteur at an MOI of 1:10, 1:1 and 10:1 for 5 days. After 5 days, the cells were washed with PBS and prepared using the intracellular staining protocol described in section 2.16. The cells were fixed using 4% PFA at 4°C for 15 minutes. The cells were spun at 400 RCF and the supernatant removed. The cells were then lysed using 0.1% saponin in PBS for 30 minutes. After lysis, the cells were stained with the TB immunogenicity flow cytometry panel for 30 minutes. The antibodies in this panel were diluted 1/100 in PBS containing 0.1% saponin. Flow cytometry was used to identify CD4+ T cells and CD8+ T cells and measure the intracellular expression of IFN γ , IL-17 and IL-2. Proliferation of CD4+ and CD8+ T cells was measured using Ki67 expression. MFI values were recorded using a Cytoflex S system.

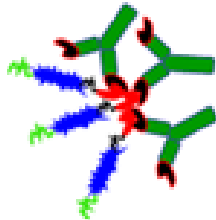
2.23 Effect of adjuvants on tonsillar lymphocytes

TMCs were seeded in 96 well plates at a density of 1×10^6 /ml. These TMCs were stimulated for 5 days with Poly I:C at 100 μ g/ml, Quil A at 50 μ g/ml, Alum hydroxide at a concentration of 100 μ g/ml, and 10 μ g/ml of peptidoglycan. PMA / Ionomycin was included as a positive control. The cells were then washed and stained according to the protocol in section 2.16. The cells were stained with the TB immunogenicity flow cytometry panel. A Cytoflex S system was used for the flow cytometry analysis.

2.24 Immunogenicity and proliferative properties of novel TB vaccines – Protocol

The TMC immunogenicity model was used to investigate novel vaccine formulations against TB. The study examined the production of IL-2, IFN γ and IL-17 by T cells. This assay also investigated the proliferation of CD4 $^{+}$ and CD8 $^{+}$ T cells. Expression levels of CD3, CD4, CD8, Ki67, IL-2, IL-17, and IFN γ were measured by flow cytometry. A range of vaccine formulations were tested. These are described below. Immune Complex Mimics (ICM) were tested in this study. These ICMs are composed of polymeric immunoglobulin combined with antigen. IgA ICM was composed of anti Acr IgA combined with FP1. This was added to cells at 10 μ g/ml. IgG ICM was composed of anti Acr IgG and FP1 and was added at 10 μ g/ml to TMCs. Heat aggregated IgG and IgA at 10 μ g/ml were used as positive controls. Quil A was added as an adjuvant at a concentration of 5 μ g/ml.

Schematic of IgA and IgG ICMs.

FP1 Antigen	 Acr Ag85b HBHA	
Anti Acr antibody (2E9)	Anti Acr IgG 	anti Acr IgA 
Monomeric FP1	FP1 + anti Acr IgG 	FP1 + anti Acr IgA 
Trimeric FP1	IgG ICM 	IgA ICM 

B. subtilis spores (dead) were added at a MOI of 5:1, 50:1 and 500:1 (Cells to spores). YC NaMa, which is made from yellow carnauba and sodium myristate was added at a concentration of 10 µg/ml. Liposome P/S was added to cells, with additional antigen in a non encapsulated form. This liposome was added at a ratio of 2:1, 1:1 and 1:5 of liposome to cells. Liposome A/E was added. This was a 50/50 ratio of phosphatidylserine liposome with encapsulated Ag85B or ESAT6. This was added in a ratio of 2:1, 1:1 and 1:5 of liposome to cells. Each of these vaccines were added in combination with FP1 or alone. Wells were also prepared which contained Spore FP1 and YC NaMa FP1 combined with Poly I:C.

Cells were stimulated with FP1 at 5 µg/ml, PMA at 10ng/ml, Ionomycin at 500 ng/ml, Poly I:C at 10 µg/ml, 5 µg/ml and 1 µg/ml. Quil A was added at 5 µg/ml, 2.5 µg/ml and 0.5 µg/ml.

To determine the immunogenicity of these candidates, TMCs were seeded at a density of 1×10^5 cells per well in 96 well U bottomed plates. To measure T cell IL-17 responses, TMCs were seeded in flat bottomed plates at 1×10^5 cells per well and then stimulated with each of the vaccine candidates. Cells were incubated at 37°C, 5% CO₂, 95% humidity. After 5 days the cells were prepared for intracellular flow cytometry using the protocol previously described in section 2.16. The cells were treated using the antibody panel shown in table 2.4. Flow cytometry was performed using a Beckton Dickinson CANTO flow cytometer and a Cytotflex S system.

2.25 Antigen recall protocol

96 well plates were seeded with triplicate wells containing 1×10^5 TMCs. The TMCs were stimulated with the BCG vaccine at a 1:1 ratio or the Liposome FP1 vaccine candidate at a concentration of 5:1 for 5 days, as before. After this, the TMCs were stimulated for a further 7 days with Acr and Ag85b recall antigens. Expression of IL-17, Ki67, IL-2 by CD4⁺ T cells and CD8⁺ T cells was measured by flow cytometry as previously described in section 2.16. The antibody panel shown in section 2.4 was also used for this study.

2.26 Tonsil cell binding study protocol

In order for vaccines to exert immunogenic effects, it is necessary for them to interact with cells. This binding study sought to determine whether novel vaccine candidates were binding to the surface of human cells. A range of new vaccine candidates were tested. Polymeric D-PIGS are polymeric structures composed of IgG fused with Dengue antigen, (cEDIII). My 42J is a formulation of D-PIGS co-expressed with a J chain to enhance polymerization. It is secreted from *N. benthamiana*. My 45J was also used, it was also composed of D-PIGS co-expressed with J chain but it was purified from the ER of *N. benthamiana* cells as opposed to the secreted form. DERICS are formulated from Polymeric 6D8 IgG fused to the Dengue antigen cEDIII. MTB5J is a formulation designed as an anti-tuberculosis vaccine candidate. It is composed of polymeric immunoglobulin scaffolds (PIGS) fused with Ag85b. Positive controls were included, which consisted of human polymeric IgG (heat aggregated) and monomeric IgG. The common feature of all these vaccines is that they contain the human IgG – Fc fragment to facilitate binding to APC IgG receptors and re used adjuvant free.

For this binding study, tonsil mononuclear cells (TMCs) were seeded at a concentration of 5×10^5 cells per well. These were washed twice with FACS buffer (PBS + 0.1% BSA and 0.05% NaN_3), then incubated for 2 hours at room temperature with 20 μl s of vaccine formulations. All vaccine formulations were used at 500 $\mu\text{g}/\text{ml}$ except My42J which was used at 200 $\mu\text{g}/\text{ml}$. Cells were washed twice with FACS buffer (PBS + 0.1% BSA and 0.05% NaN_3) and secondary antibodies added. Cells were stained with αCD14 bound to PE, αCD11b bound to APC and $\alpha\text{Human IgG}$ conjugated to FITC. Cells were incubated at 4°C in the dark for 30 minutes and then washed 2x with FACS buffer. Once cells had been resuspended in 250 μl s of FACS buffer, they were analysed by flow cytometry using a Beckton Dickinson CANTO flow cytometer.

2.27 Immunogenicity of Dengue vaccine candidates

Intracellular flow cytometry was used to measure the immunogenicity of dengue vaccine candidates in T cells. TMCs from four patients were seeded at a density of 1×10^6 cells/ml in 96 well plates. The cells were stimulated with PMA / Ionomycin for 5 days. Cells were also stimulated with four Dengue vaccine candidates. D-PIGS, DERICS, My42J and Mod – D-PIGS were added to cells at a concentration of 25 µg/ml. After 5 days, the cells were prepared using the intracellular staining protocol in section 2.16. The TMCs were then stained using the dengue immunogenicity panel.

Table 2.11. Dengue Immunogenicity Panel

Marker	Function	Fluorochrome
CD3	T cell marker	FITC
CD4	CD4+ T cell marker	PerCP Cy5.5
CD8	CD8 T cell marker	BV510
Ki67	Proliferation marker	APC
IL-4	Th2 cytokine	APC Cy7
IFN γ	Th1 cytokine	BV421
IL-10	Suppressive cytokine	PE
TNF α	Proinflammatory cytokine	PE Cy7

The cells were then analysed by flow cytometry. CD4+ and CD8+ T cells were identified. Ki67 expression was measured along with the levels of intracellular IFN γ , IL-4, IL-10 and TNF α . MFI values are shown. Data was analysed using one way ANOVA and Dunnett's multiple comparisons test. For the recall experiment, TMCs were stimulated with Dengue vaccines for 5 days as previously described. 5µg/ml of CEDIII was added to wells on day 5. The TMCs were then incubated with this recall antigen for a further 7 days until day 12. The cells were then prepared for flow cytometry as before.

Protocol for Chapter 4 – Tuberculosis Infection model developed using TMCs

2.28 TMC BCG Infection model

To develop a tonsil BCG infection model, TMCs were seeded with 1×10^5 cells/well in 96 well flat-bottomed plates with 100 U/ml of penicillin and 250 µg/ml per well of Hygromycin. Cultures of TMCs require at least two antibiotics to maintain their viability. Penicillin and Streptomycin were generally used. In these experiments, Penicillin and Hygromycin were used instead as the BCG GFP was susceptible to streptomycin but contained a hygromycin resistance gene. BCG-GFP was thawed at 37°C and washed with sterile PBS using centrifugation at 5,000 RCF for 10 minutes at 25 °C. Following this, BCG-GFP was resuspended in RPMI-1640 medium again supplemented with FBS and L-glutamine. BCG-GFP was sonicated for 120 seconds for proper dispersion of bacterial clumps prior to infection. BCG-GFP was sonicated in 30 second bursts and put on ice for 30 seconds before each subsequent burst to prevent overheating and cell death. Sonication was conducted using a Hielscher UP200St ultrasonic processor. Following sonication, BCG-GFP was centrifuged at 5,000 RCF for 10 minutes and resuspended in RPMI 1640 media. TMC cells were infected at an MOI of 4:1 for 24 hrs.

2.29 T cell immunogenicity

This assay established the effect of vaccine formulations on the production of IFN γ and IL-17 by T cells during infection with BCG. Expression levels of CD3, CD4, CD8, IL-17, and IFN γ were measured by flow cytometry. A range of vaccine formulations were tested. These are described below.

B. subtilis spores (dead) were added at a MOI of 5:1. YC NaMa was added at a concentration of 10 µg/ml. Liposome P/S was added to cells at a ratio of 2:1 of liposome to cells. Each of these vaccines were added in combination with FP1 or alone. Cells were stimulated with FP1 at 5 µg/ml, PMA at 10ng/ml, Ionomycin at 500 ng/ml.

To determine the immunogenicity of these candidates, TMCs were seeded at a density of 1×10^5 cells per well in 96 well U bottomed plates. To measure T cell IL-17 responses, TMCs were seeded in flat bottomed plates at 1×10^5 cells per well and then stimulated with each of the vaccine candidates. Cells were incubated at 37°C, 5% CO₂, 95% humidity. After 5 days the cells were prepared for flow cytometry using the intracellular flow cytometry protocol described in section 2.16. The antibody panel is shown in supplemental table 10.5.

2.30 TMC BCG Infection model - Antibody panels for flow cytometry

Flow cytometry was used to assess the infection of lymphocytes by BCG. Following infection, TMCs were washed with FACS Buffer (PBS + 0.1% BSA and 0.05% NaN₃) and stained with Fc Block and Efluor 780 Viability Stain (APC Cy7). The cells were stained at 4 °C for 30 minutes with an antibody panel of CD3 BV421, CD4 PerCP Cy5.5, CD8 BV510 and CD19 PE Cy7 (Biolegend). Changes in cell interaction with BCG GFP were then measured using a CytoFLEX LX flow cytometer. Data was analysed using FlowJo software.

To assess the effect of FP1 vaccines on tonsillar monocytes during mycobacterial infection, TMCs were stimulated with vaccines for 24 hours.

TMCs were stimulated for 24 hours with BCG Pasteur, IFN γ and the novel vaccine formulations; Spore FP1, YC NaMa FP1 and Liposome FP1. 1×10^4 CFU of BCG Pasteur were added to wells (10:1 ratio of cells to BCG). IFN γ was added at a concentration of 20ng/ml. *Bacillus subtilis* spores were added at a concentration of 2×10^4 CFU/well (5:1 ratio of cells to spores). YC NaMa was added to wells at 10 μ g/ml. The recombinant antigen FP1 was added at a concentration of 5 μ g/ml. Liposome FP1 capsules were used at a concentration of 4×10^5 liposomes /well (4:1 ratio liposomes to cells).

Cells were then infected with BCG GFP at an MOI of 4:1 for a further 24 hours. Cells were then washed and stained with Fc Block and Efluor 780 Viability Stain. Monocytes were then stained with an antibody panel of CD14 PE, CD40 PE Cy7, CD80 KO525, CD86 PB450 and CCR5 PerCP Cy5.5 (Biolegend). Changes in cell surface markers were then measured by flow cytometry.

The level of nitrites in cell supernatants was measured using Greiss Reagent. 50µls of sample were mixed with 50µls of Greiss reagent in a 96 well plate. The solution was incubated at room temperature in the dark. After 5 minutes, a stop solution of 0.5M sulphuric acid was added. The absorbance of the sample at 540nm was detected using a sunrise TECAN microplate reader after 15 minutes. A standard curve was generated using 100µM sodium nitrite and absorbance values were converted to nitrite concentrations.

2.31 Infection of TMCs for confocal microscopy

For imaging work, TMCs from patient 5 were thawed at 37 °C and washed with sterile Dulbecco's PBS (Sigma, D8537) at 450 RCF for 5 minutes. TMCs were resuspended in Sigma's RPMI 1640 media prepared as mentioned above. The cells seeded onto a 96 well flat-bottomed plate (Falcon, ref:353072) at $\sim 2 \times 10^6$ cells/ml. the TMCs were then infected with BCG GFP at an MOI 4:1 for 1 hour. Prior to use, BCG-GFP was sonicated for 120 seconds as described in section 2.28. After infection, the cells were stained with CD19 PE Cy7, CD4+ BV421 and CD8 BV510 for 30 minutes. GFP+ CD19+, CD4+ and CD8+ lymphocytes were sorted using a BD FACS melody. Images were captured with a Nikon AR1 confocal microscope.

Protocol for Chapter 5 - Interaction of B cells with BCG

2.32 Effect of trypsin treatment on BCG binding to B cells

2.33 Trypsin treatment after BCG infection.

To investigate the interaction between lymphocytes and GFP BCG, cells were infected with BCG GFP with a trypsin treatment prior to infection and post infection to remove surface bound BCG. Triplicate wells were seeded with TMCs at 1×10^5 cells/well. These were infected with BCG at an MOI of 4:1 for 3 hours using the protocol in section 2.28. After 3 hours, the cells were centrifuged at 400 RCF and the supernatant was removed. The cells were then treated with 100µls of 1x trypsin for 5 minutes at 37°C in the dark. After 5 minutes, the trypsin was blocked by adding 100µls of RPMI media to wells. Cells were then stained with CD19 PE-Cy7 (Clone HIB19) and prepared for flow cytometry using the using the staining protocol described earlier. Infection of B cells with BCG GFP was examined by measuring FITC MFI. Flow cytometry was performed using a Cytoflex S system.

2.34 Trypsin treatment prior to BCG infection

To investigate the effect of trypsin treatment prior to infection with BCG, triplicate wells were seeded with RAJI cells at 1×10^5 cells/well. The cells were washed three times with PBS, with plates spun at 400 RCF for 5 minutes. The cells were treated with trypsin for 10, 20 or 30 minutes at 37°C. The cells were then washed with PBS. The cells were then infected with BCG at an MOI of 4:1 in RPMI media for 3 hours. Cells were then stained for CD19 and the MFI of FITC in B cells was measured by flow cytometry as a measure of interaction with GFP BCG. Flow cytometry was performed using a Cytoflex S system.

2.35 Role of phagocytosis and macropinocytosis in BCG / B cells interactions.

The role of phagocytosis and macropinocytosis in B cell interaction with BCG was investigated by infecting TMCs with BCG GFP in the presence of Amiloride, Cytochalasin D and PMA. Amiloride inhibits macropinocytosis and was used at a concentration of 5 μ M. Cytochalasin D inhibits phagocytosis by blocking the formation of actin filaments. This was used at a concentration of 20nM. PMA is known to stimulate macropinocytosis and was used at 100ng/ml. Triplicate wells of TMCs were prepared for each condition, with 1x10⁵ cells/well. Cells were treated with cytochalasin D, PMA or Amiloride for 1 hour. These cells were then infected with BCG at an MOI of 4:1 for a further 3 hours. The cells were spun at 400 RCF for 5 minutes and the supernatant removed. These cells were then stained with CD19 – PE for 30 minutes, washed and resuspended in FACs Buffer (PBS + 0.1% BSA and 0.05% NaN₃). The cells were then analysed by flow cytometry. B cells were gated using PE MFI and the MFI of FITC in B cells was measured.

2.36 Effect of C1q on BCG binding to B cells.

The role of C1q in the binding of BCG to B cells was determined by infecting RAJI cells with BCG in the presence or absence of exogenous C1q. Triplicate wells containing 1x10⁵ RAJI cells were treated with 125 μ g/ml of C1q for 1 hour. These cells were then infected with BCG GFP at an MOI of 4:1 for three hours. The cells were spun at 400 RCF, the supernatant was removed and the cells were stained with CD19 – PE for 30 minutes, washed and resuspended in FACs Buffer (PBS + 0.1% BSA and 0.05% NaN₃). The RAJI cells were then analysed by flow cytometry and B cells were identified and the FITC MFI and % FITC + values measured.

2.37 Effect of Human Serum and Foetal Bovine Serum on BCG binding to B cells

RAJI cells were infected with BCG in the presence or absence of serum. Triplicate wells containing 1×10^5 RAJI cells were infected with BCG GFP at an MOI of 4:1. In the first experiment, wells contained RPMI media without serum or RPMI media with 10% foetal bovine serum. In the second experiment, the effect of human serum on B cell infection was tested. Wells were prepared which contained RPMI media without serum, RPMI media with 10% human serum or RPMI media with human serum which lacked immunoglobulin. (Sigma Aldrich) The cells were then infected for 3 hours with BCG GFP using the protocol outlined in section 2.29. After 3 hours of infection with GFP BCG, the cells were washed and analysed by flow cytometry. The percentage of FITC + B cells and the FITC MFI of B cells were measured.

2.38 Imaging of B cell interactions with BCG GFP

RAJI cells, tonsillar B cells and U937 cells were seeded in 96 well plates in RPMI media with Hygromycin B and 100 U/ml of Penicillin. TMCs from six different patients were used. Triplicate wells were prepared for RAJI cells, U937 cells and for each of the TMCs. BCG GFP was centrifuged at 5,000 RCF for 10 minutes, supernatant was removed and BCG were resuspended in RPMI media. These cells were infected with BCG GFP at an MOI of 10:1 for 24 hours. The cells were spun at 400 RCF and the media was removed. The cells were stained with phalloidin 647 for 30 minutes and DAPI for 5 minutes. TMCs were also stained with PE conjugated anti CD19 for 30 minutes. They were then spun at 400 RC and washed with 200µl of PBS. cells were finally resuspended in 100µl of PBS. These cells were photographed using a Nikon A1R confocal microscope. The cells were photographed at 200x magnification with 1024 resolution. Red, Far Red, Blue and Green colours were recorded for PE-CD19, Phalloidin 647 bound to actin filaments, DAPI stained nuclei and BCG GFP respectively. Z stack images were generated at 8µm intervals. 5 images were collected from every well. The Z stack images were combined into 3D models using NIS Elements Microscope Imaging Software.

2.39 Functional effect of BCG interaction with B cells - Flow cytometry panels

To determine the functional effect of BCG infection on B cells, triplicate wells containing 1×10^5 RAJI cells, TMC cells or U937 cells were infected with BCG GFP at an MOI of 4:1 for 24 hours. For flow cytometry, the cells were stained using BV510 conjugated CD19 antibody. Cells were also stained with Efluor 780 viability dye for 30 minutes. The cells were analysed by flow cytometry. Viable B cells were gated using BV510 MFI and APC Cy7 MFI. The cells were stained with HLA DR - PE Cy7, CCR7 – BV421, CD69 - PE, Ki67 - APC and IgM - PerCP Cy5.5. In addition, the levels of CD32 and IL-10 were measured using CD32 – PE and IL-10 - PE. To measure the effect of BCG on B cell expression of CD40 and CD80, cells were stained with Efluor 780 viability dye, CD19 - PE, CD40 - PE Cy7 and CD80 - BV510

Results

Chapter 3. Modelling vaccine immunogenicity using BMDCs.

Abstract

In vitro models were used to further the development of vaccine candidates against TB. The BMDC model was used to determine the immunogenic effect of three vaccine delivery systems; *Bacillus subtilis* spores, YC NaMa and Liposomes. Spores induced TNF α , IL-6, IL-12 and IL-1 β in DCs but did not impact the expression of activation markers. The YC NaMa and liposome delivery systems had no impact on the expression of activation markers or the production of cytokines by DCs.

To enhance the immunogenicity of these delivery systems, TLR agonists were proposed as adjuvants. The BMDC model was used to investigate the effect of TLR agonists on DC activation and cytokine production. This revealed suitable combinations of delivery systems and TLR agonists. Spores and YC NaMa could be combined with MPL, LPS, peptidoglycan, CPG DNA, or Poly I:C. Liposomes could be combined with LPS, CPG DNA or Flagellin. Each of these combinations resulted in increased expression of activation markers and inflammatory cytokines compared to the TLR agonists alone.

Further experiments found that BMDCs pulsed with Spore FP1 were able to induce the proliferation of naïve splenocytes. This ability was lost when Poly I:C was added as an adjuvant. DCs pulsed with Spore FP1 Poly I:C were not capable of inducing T cell proliferation. Spore FP1 Poly I:C was tested in a mouse model. No antigen specific IFN γ or IL-17 production or T cell proliferation was detected. The response was enhanced by including a syngeneic transfer of pulsed DCs. This vaccination system resulted in a 10 fold increase in antigen specific IFN γ . It also induced proliferation of CD4 $^{+}$ and CD8 $^{+}$ T cells and IL-17 production. This confirmed that DCs represent a bottleneck in the formation of novel immune responses.

It was found that overstimulation of DCs led to a negative feedback loop with upregulation of PD-L1. This phenomenon was observed in DCs stimulated with Spore FP1 Poly I:C and with BCG. This insight may help in the development of novel TB vaccines.

Aims and Objectives

- Use the murine BMDC model assess the immunogenicity of;
 - 3 delivery systems; *Bacillus subtilis* spores, YC NaMa and Liposomes.
 - The TLR agonists; CPG DNA, MPL, LPS, Poly I:C, Flagellin and Peptidoglycan.
 - Each of the delivery systems in combination with TLR agonists.
-
- Determine the effect of the delivery systems on antigen uptake and apoptosis of DCs.
 - Model the effect of Spore FP1 Poly I:C on the ability of DCs to activate naïve T cells.
 - Test the immunogenicity of Spore FP1 Poly I:C using an *in vivo* murine model.

3.1 BMDC phenotype

Murine bone marrow derived dendritic cells (BMDCs) from C57BL/6 mice were used for this work. The phenotypic characterisation of these cells is shown in figure 3.1.1. Approximately 90% of the cells were CD11b+. 80% of the cells were CD11c+. Around 25% of the cells were MHC II +. The majority of cells, around 90%, expressed Gr1. Less than 5% of cells were CD8+. The majority of DCs expressed CD11b and CD11c. The DCs expressed low levels of MHC II and high levels of Gr1. This is consistent with a population of immature DCs.

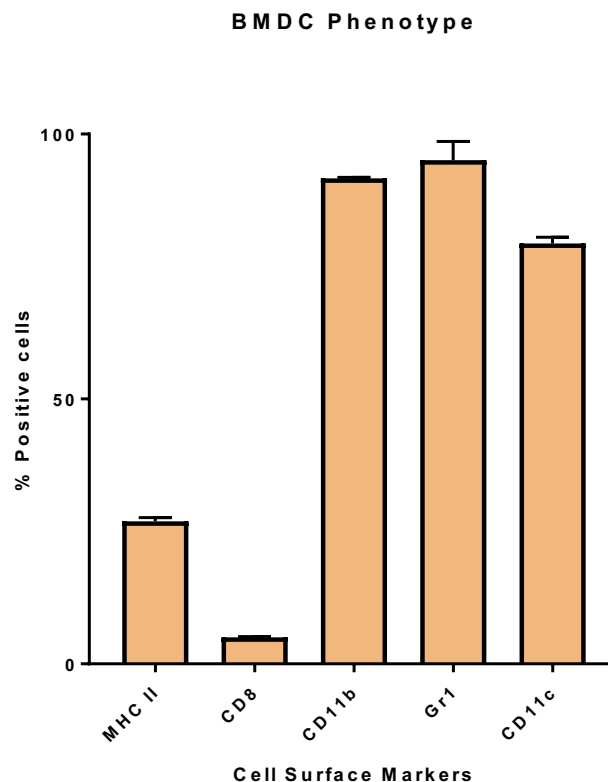


Figure 3.1.1 Phenotype of BMDCs. BMDCs were generated from the bone marrow of C57BL/6 mice. Phenotypic analysis was performed by flow cytometry in triplicate (n=3). Mean values and $\pm$ standard deviations are shown. The majority of cells were DCs expressing both CD11b and CD11c. The DCs were immature, expressing low levels of MHC II and high levels of Gr1.

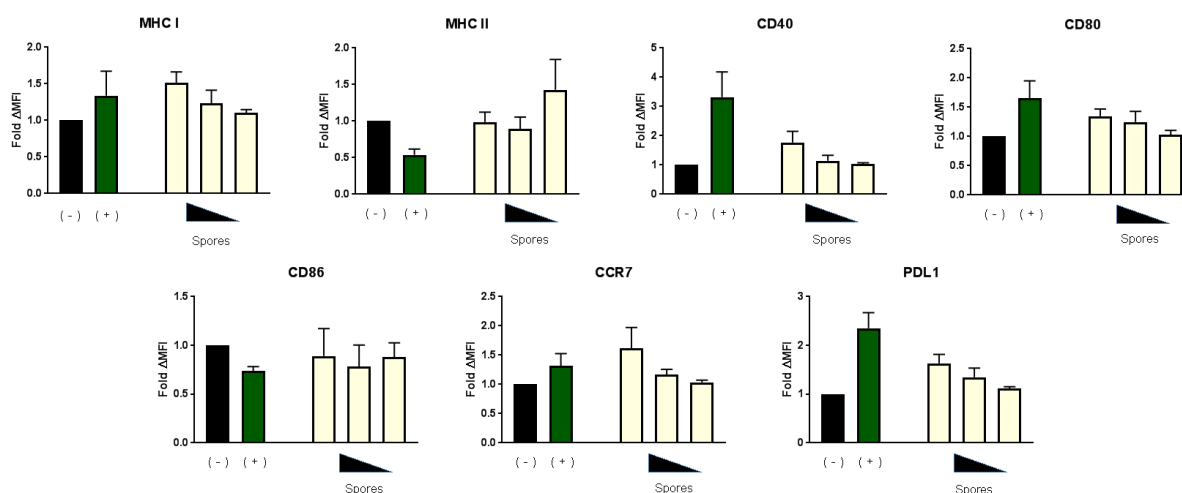
3.2 Immunogenic effects of spore vaccine delivery system

Spore FP1, YC NaMa FP1 and Liposome FP1 have all been shown to protect mice against TB infection. This experiment sought to determine whether the three vaccine delivery systems had similar immunogenic effects on DCs. BMDCs were stimulated with *B. subtilis* spores. Expression of activation markers and cytokine release was measured after 48 hours. Flow cytometry was used to measure cell surface marker expression on DCs using a gating plan shown in supplemental figure 10.1. This data was analysed using one way ANOVA and Dunnett's multiple comparisons test. Figure 3.2.1 A shows the effect of spores on cell surface markers, figure 2B shows the effect on cytokine expression.

Expression of CCR7, CD40, CD80, CD86, MHC I, MHC II and PD-L1 on DCs did not alter significantly following stimulation with spores. This result is depicted in Figure 3.2.1, section A. Cytokine responses were dissociated from the expression of cell surface markers. Stimulation with spores at an MOI of 5:1 (cells to spores) led to enhanced expression of TNF α , IL-6, IL-12 and IL-1 β . This is shown in figure 3.2.1, B. Stimulation with spores increased the mean TNF α expression by the BMDCs from below detectable levels up to 1,458 pg/ml ($p < 0.0001$). Stimulation with Spores increased IL-6 production from below detectable levels to 5,956 pg/ml ($p < 0.0001$). The percentage of IL-12+ DCs increased threefold following stimulation with spores ($p = 0.0025$). Expression of the pro-inflammatory cytokine IL-1 β also rose.

This experiment showed that pro inflammatory cytokines were upregulated following stimulation with spores, but activation markers were not upregulated. This pattern of activation is consistent with MyD88 signalling in DCs, without the involvement of IRF3 (Hoebe et al. 1223). *Bacillus subtilis* spores are known to act as TLR 2 agonists, therefore MyD88 signalling was expected (de Souza et al. e87454). Bacillus spores were expected to elicit TLR 4 signalling (Jones, Paynich, and Knight), which would generate IRF3 signalling and induce upregulation of cell surface markers, however this experiment did not find any evidence of upregulation of DC cell surface markers.

A) Effect of *Bacillus subtilis* spores on cell surface marker expression by DCs



B) Effect of *Bacillus subtilis* spores on cytokine production by DCs

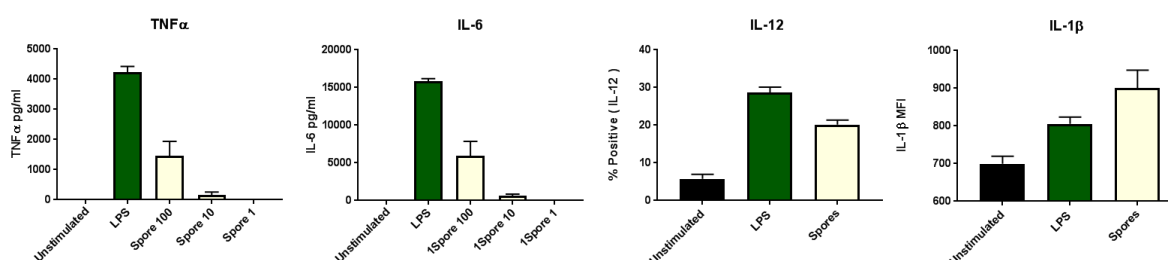


Figure 3.2.1. Immunogenicity of spore vaccine carrier system. Expression of activation markers (A) and cytokines (B) following stimulation with *B. subtilis* spores, MOI 5:1, 50:1 or 500:1, (cells to spores). 100ng/ml LPS was used as a positive control. Triplicate wells of BMDCs were stimulated for 48 hours. Cells were analysed by flow cytometry. Supernatants were collected for cytokine analysis. Mean values and $\pm$ standard deviations are shown. These graphs are representative of three repeated experiments (n=3).

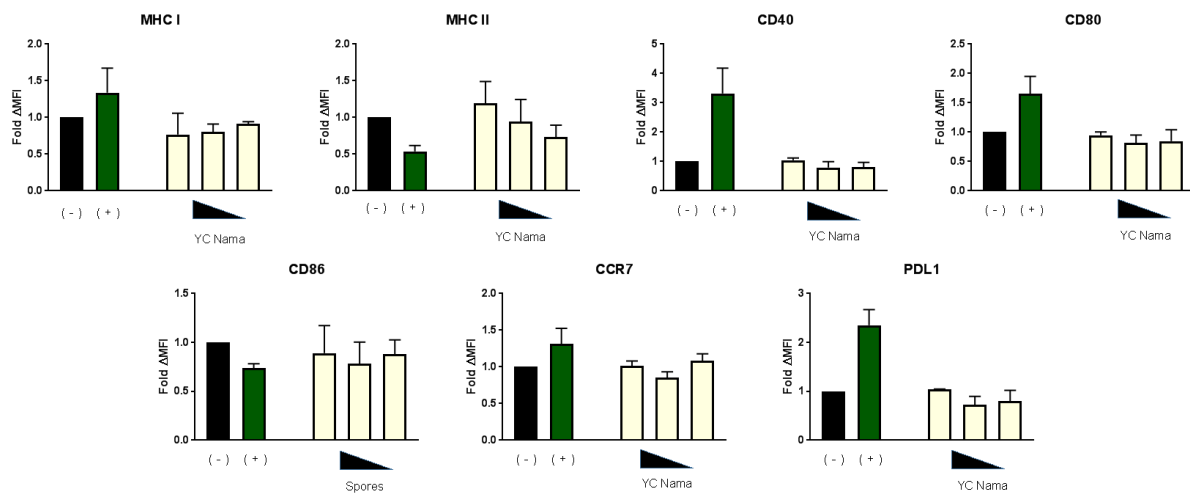
3.3 Immunogenic effects of YC NaMa vaccine delivery system

YC NaMa FP1 has been shown to protect mice against TB infection (Hart et al. 822-833). This study showed that the YC NaMa delivery system generates IRF3 signalling and upregulates activation markers on macrophages. This experiment sought to determine whether YC NaMa had similar immunogenic effects on DCs. BMDCs were stimulated for 48 hours with YC NaMa and the expression of activation markers and cytokine release was measured by flow cytometry. This data was analysed using one way ANOVA and Dunnett's multiple comparisons test. The results of this experiment are shown in figure 3.3.1.

Stimulation with YC NaMa was found to have no impact on the expression of expression of CCR7, CD40, CD80, CD86, MHC I, MHC II or PD-L1 by DCs. Stimulation with YC NaMa also had no effect on expression of pro inflammatory cytokines. Figure 3 B shows no detectable change in TNF α , IL-6, IL-12 or IL-1 β .

This finding shows that YC NaMa has disparate immunogenic effects on DCs and macrophages. In macrophages, YC NaMa has been shown to increase expression of MHC I, MHC II, CCR7, CD86 and PD-L1, but not pro inflammatory cytokine release in macrophages (Hart et al., 2018a). In contrast, this experiment revealed that YC NaMa does not have any impact on the expression of activation markers or cytokines by DCs. It is possible that YC NaMa induces IRF3 signalling in APCs. This would be consistent with YC NaMa activating macrophages but not DCs. Macrophages can be activated by IRF3 alone whereas DCs require signalling through IRF3 and MyD88 to become activated.

A) Effect of YC NaMa on cell surface marker expression by DCs



B) Effect of YC NaMa on cytokine production by DCs

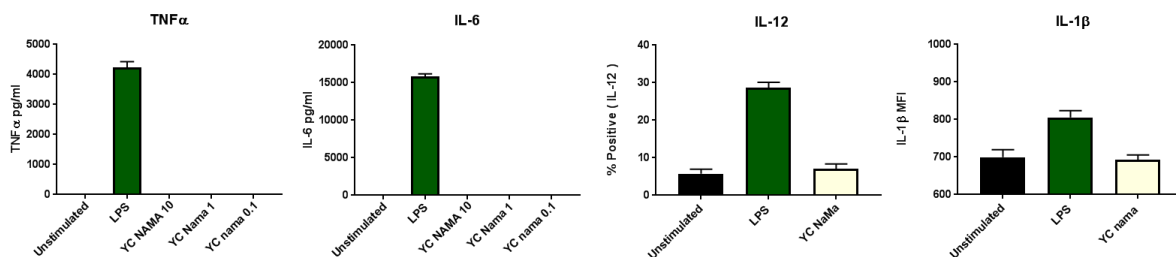


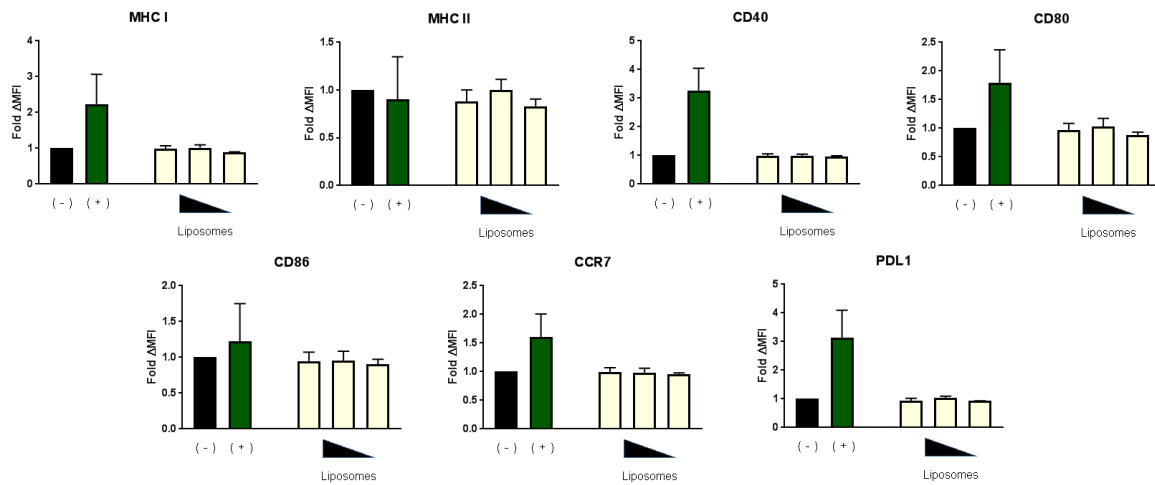
Figure 3.3.1 Immunogenicity of YC NaMa vaccine carrier system. The effect of YC NaMa on expression of cell surface markers (A) and cytokines (B) was measured. BMDCs seeded at a density of 1×10^5 cells per well. Triplicate wells were stimulated with YC NaMa at concentrations of 10 μg/ml, 1 μg/ml or 0.1 μg/ml or 100 ng/ml of LPS. Cells were incubated for 48 hours and analysed by flow cytometry. Cytokine levels in the supernatants were measured by ELISA. MFI values are shown along with standard deviations. The percentage of IL-12+ cells is also shown. These graphs represent three repeats (n=3).

3.4 Immunogenicity of Liposome P/S vaccine carrier system

The immunogenic effect of the liposome P/S delivery system was unknown. This study tested the effect of Liposomal P/S on DCs. Flow cytometry was used to measure activation marker expression and pro inflammatory cytokine release by DCs. Liposome P/S did not impact cytokine expression or the expression of activation markers either. Expression of TNF α , IL-6, IL-12 and IL-1 β remained unchanged as can be seen in Figure 3.4.1 B. Cell surface expression of CCR7, CD40, CD80, CD86, MHC I, MHC II and PD-L1 were also unchanged. This is shown in figure 3.4.1 A.

Based on these findings, there did not seem to be a common mechanism of action for spores, YC NaMa and Liposome P/S. *B. subtilis* spores activated DCs to produce pro-inflammatory cytokines, but neither YC NaMa nor Liposome P/S had any detectable effect on DCs. Further work was needed to establish the immunogenic effects of these vaccine delivery systems.

A) Effect of Liposome P/S on cell surface marker expression by DCs



B) Effect of Liposome PS on cytokine production by DCs

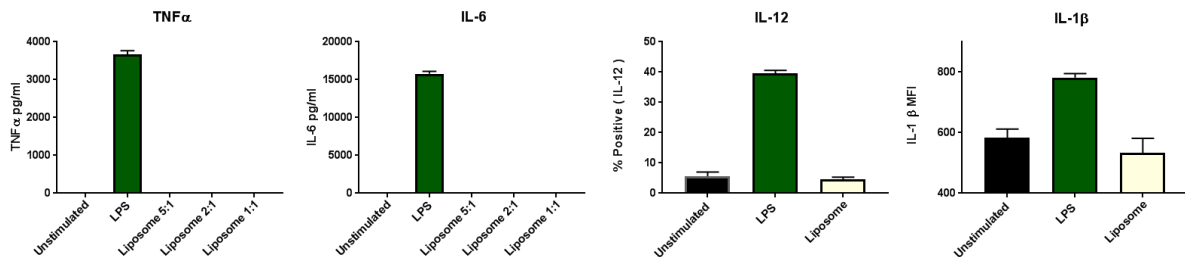


Figure 3.4.1. Immunogenicity of Liposome P/S vaccine carrier system. The immunogenic effect of Liposome P/S alone on BMDCs is shown. Expression of the cell surface markers MHC I, MHC II, CD40, CD80, CD86, CCR7 and PD-L1 was measured by flow cytometry and MFI values are shown along with standard deviations (A). Expression of pro-inflammatory cytokines TNFα and IL-6 IL-1β were measured by ELISA. MFI values are shown for IL-1β. The percentage of cells expressing IL-12 is also shown (B). This experiment was repeated three times (n=3).

3.5 Apoptosis and Efferocytosis Assay

The previous experiment showed that the Spore, YC NaMa and Liposome delivery systems have different immunogenic effects on murine DCs. This experiment sought to further characterise the effect of the vaccine delivery systems on DCs. An important function of DCs is the phagocytosis of apoptotic cells, known as efferocytosis. Phosphatidylserine, the main component of Liposome P/S, is known to be an important “eat me” expressed by apoptotic cells. Blockading phosphatidylserine with HMGB1 leads to inhibition of efferocytosis (Liu, G. et al. 4240-4246). The effect of *B. subtilis* spores and YC NaMa on efferocytosis has not previously been investigated and is therefore unknown. It was hypothesised that the three vaccine delivery systems might enhance the ability of DCs to engage in efferocytosis. If these vaccine delivery systems enhance the uptake of antigens by DCs, this might be a useful characteristic of a novel vaccine candidate.

To measure efferocytosis, live DCs were co cultured with heat treated apoptotic cells at a ratio of 2:1. The unstimulated DC population contained few apoptotic cells, whilst the heat treated cells were predominantly apoptotic. After 18 hours, apoptotic cells were gated by flow cytometry and counted. A finding of reduced numbers of apoptotic cells was indicative of efferocytosis. Once this model had been established, the effect of the delivery systems on efferocytosis was examined. The results are shown in figure 3.5.1, figure 3.5.2 and figure 3.5.3. One way ANOVA and Sidak’s multiple comparison test were used to analyse this data.

B. subtilis spores enhanced DC efferocytosis of dead cells. Combining live DCs and apoptotic cells with spores led to a fourfold reduction in the numbers of apoptotic cells present ($p<0.0001$). These results are shown in figure 3.5.1 This result shows that stimulation with *B. subtilis* spores activates dendritic cells enhancing their uptake of antigen.

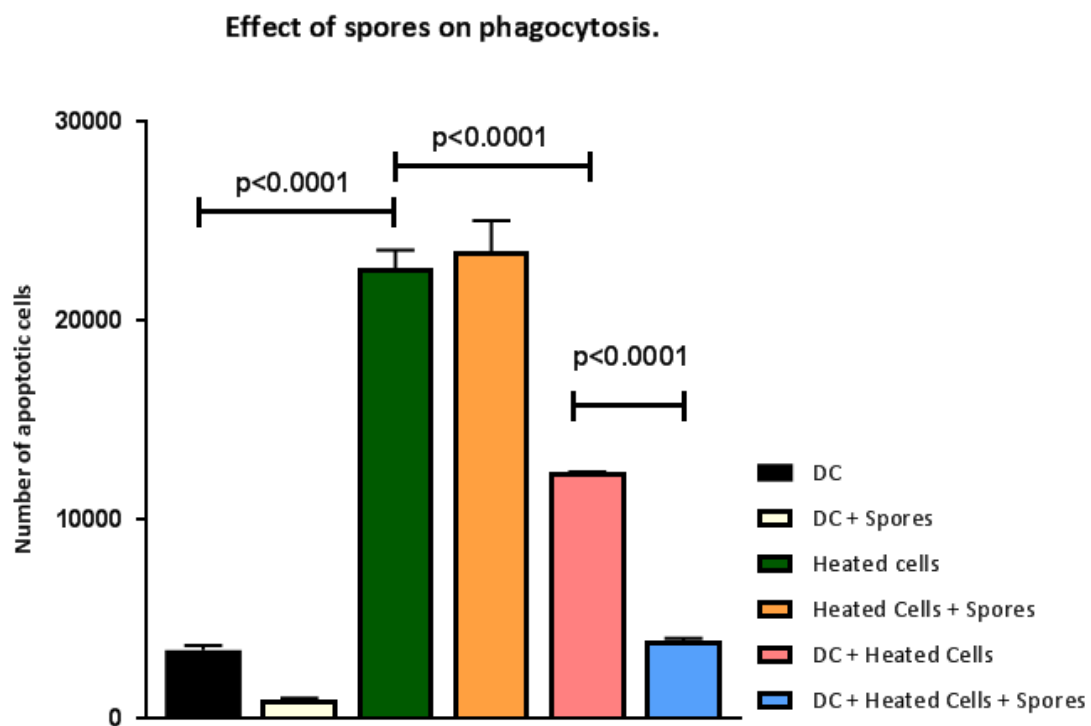


Figure 3.5.1 Spores; Apoptosis and efferocytosis assay. The effect of spores on the phagocytosis of dead cells (efferocytosis) by DCs is shown. Apoptotic cells were generated by heat treating cells for 2 hours at 70 °C. 5×10^4 of the heat treated cells were added to 1×10^5 live BMDCs. Cells were incubated for 18 hours in the presence of Spores (MOI 5:1 BMDCs to spores). Cells were stained with propidium iodide and APC Annexin V antibody. Apoptotic cells were recorded by flow cytometry. Mean values and $\pm$ standard deviations are shown. One way ANOVA and Sidak's multiple comparison test were used to analyse this data ($n=3$).

YC NaMa did not impact efferocytosis. This can be seen in figure 3.5.2. Adding YC NaMa to a coculture of live and dead cells did not alter the number of dead cells. The YC NaMa Delivery system did not alter the uptake of antigen by stimulated dendritic cells.

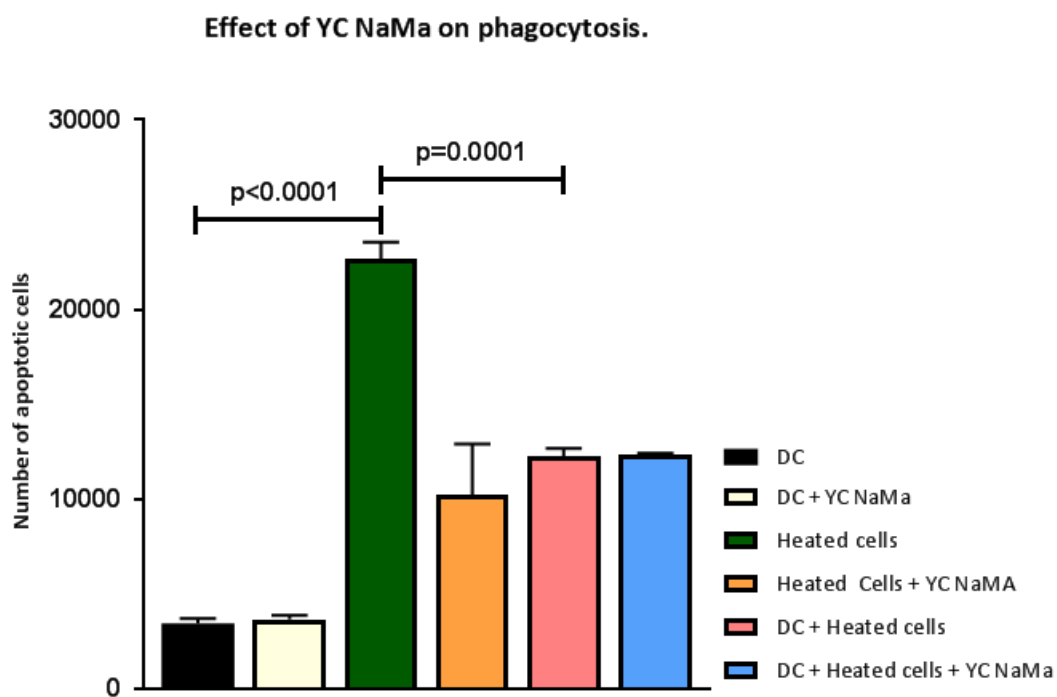


Figure 3.5.2. YC NaMa; Apoptosis and efferocytosis assay. The effect of YC NaMa on the phagocytosis of dead cells (efferocytosis) by DCs is shown. Apoptotic cells were generated by heat treating cells for 2 hours at 70 °C. 5×10^4 of the heat treated cells were added to 1×10^5 live BMDCs. Cells were incubated for 18 hours in the presence of YC NaMa (10 μ g/ml). Cells were stained with propidium iodide and APC Annexin V antibody. Apoptotic cells were recorded by flow cytometry. Mean values and $\pm$ standard deviations are shown. One way ANOVA and Sidak's multiple comparison test were used to analyse this data (n=3).

Liposome P/S inhibited efferocytosis. DCs stimulated with liposome P/S were unable to clear apoptotic cells and as a result, there was twofold increase in the number of apoptotic cells present ($p < 0.0001$). These results are shown in figure 3.5.3.

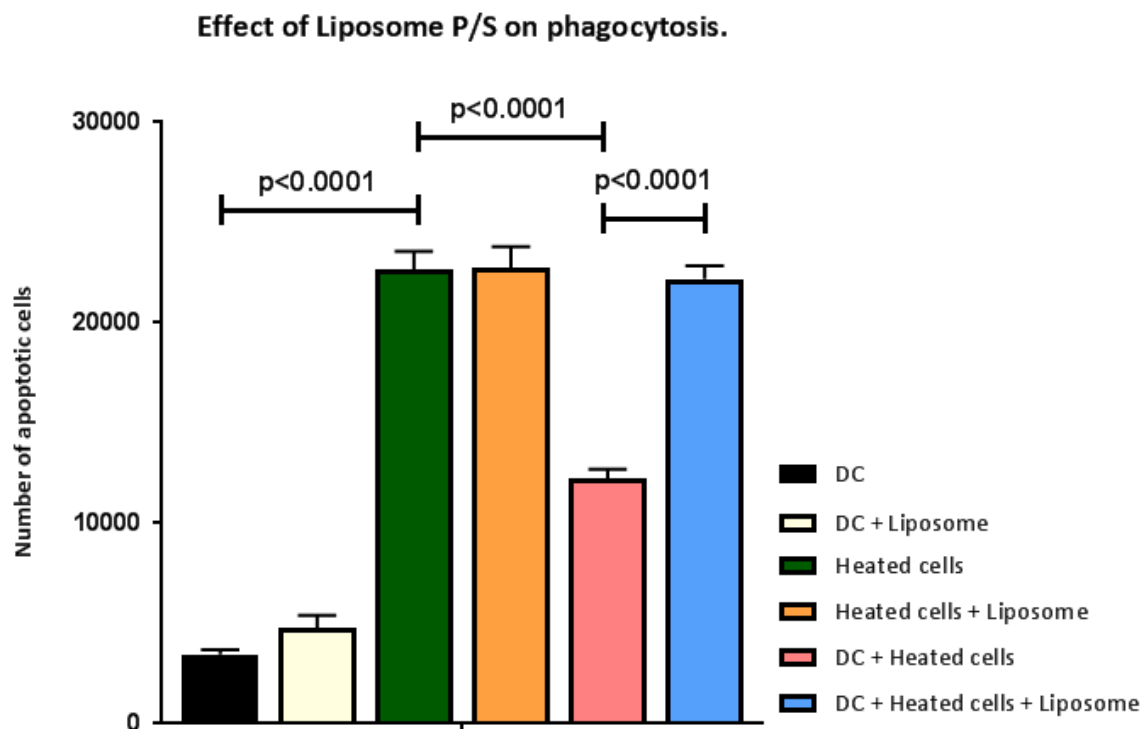


Figure 3.5.3. Liposomes; Apoptosis and efferocytosis assay. The effect of Liposome P/S on the phagocytosis of dead cells (efferocytosis) by DCs is shown. Apoptotic cells were generated by heat treating cells for 2 hours at 70 °C. 5×10^4 apoptotic cells were added to 1×10^5 live BMDCs. Cells were incubated for 18 hours in the presence of Liposome P/S (ratio of 5:1). Cells were stained with propidium iodide and APC Annexin V antibody. Apoptotic cells were recorded by flow cytometry. Mean values and $\pm$ standard deviations are shown. One way ANOVA and Sidak's multiple comparison test were used ($n=3$).

This study established that spores, YC NaMa and liposomes do not induce apoptosis in DCs. Furthermore, the efferocytosis experiment revealed that spores activate DCs, leading to enhanced antigen uptake. YC NaMa has no impact on DCs and liposomal phosphatidylserine blocks the uptake of exogenous antigen. These findings contributed to characterisation of these vaccine delivery systems, but did not determine a common mechanism of action by which the delivery systems impact DCs. The mechanism by which liposome P/S inhibited DC efferocytosis was undetermined, but is likely due to phosphatidylserine saturating the phosphatidylserine receptors.

3.6 TLR agonist immunogenicity

TLR agonists can be used to enhance the immunogenic properties of vaccines. This experiment characterised the immunogenic effect of TLR agonists on dendritic cells. Six TLR agonists were tested, LPS, MPL, Poly I:C, Peptidoglycan, CPG DNA and Flagellin. These TLR agonists induce TLR signalling through TLR 4, TLR3, TLR 2, TLR9 and TLR 5 pathways. The expression of activation markers on DCs and the production of pro inflammatory cytokines was examined. Each of the TLR agonists generated a different profile of activation in the BMDCs. This section discusses the immunogenic effects of each of the TLR agonists alone.

LPS

Figure 3.6.1 shows that LPS generated a strong immune response in the BMDCs. Cell surface expression of MHC I, CD80, CD86 and PDL-1 were all significantly raised. MHC I expression rose twofold from 4,025 MFI to 8,035 ($p=0.0001$). CD80 expression also increased twofold from 1,493 to 3,267 ($p=0.0043$). LPS increased CD86 expression threefold from 1,093 to 3,448 ($p=0.0010$). This was accompanied by a rise in PD-L1 expression from 3,427 to 5,685 MFI ($p=0.0004$). The BMDCs also reacted to LPS by producing cytokines. Figure 3.6.2 shows that levels of IL-12, IL-1 β , IL-6 and TNF α were all raised following LPS stimulation.

MPL

Another TLR4 agonist, MPL, produced a slightly more muted response. BMDCs upregulated expression of MHC I and PDL-1 in response to MPL in figure 3.6.1. MHC I expression increased from 4,025 to 7,044 ($p<0.0001$). PD-L1 expression increased from 3,427 to 5,441 MFI ($p=0.0005$). The BMDCs stimulated with MPL did not upregulate CD40, MHCII, CD80 or CD86. The BMDCs upregulated intracellular expression of IL-6 and TNF α in response to MPL. Unlike LPS stimulation, MPL stimulation did not result in upregulation of IL-12 or IL-1 β by DCs.

Peptidoglycan

Stimulation with PGN activated DCs, leading to upregulation of MHC I, The B-7 molecules CD80 and CD86 as well as the inhibitory molecule PDL-1. This is depicted in figure 3.6.1. MHC I expression rose from 4,025 up to 6,013 MFI ($p=0.0056$). CD80 levels increased from 1,493 to 2,699 ($p=0.0019$). CD86 expression increased threefold from 1,093 to 3,480 ($p=0.0008$). The expression of PD-L1 was raised on BMDCs from 3,427 to 5,458 MFI with PGN stimulation ($p=0.0010$). Figure 3.6.2 shows that PGN stimulation elicited a proinflammatory cytokine response from the DCs. IL-1 β and TNF α levels were both significantly raised.

Poly I:C

Poly I:C stimulation increased the expression of MHC I and PD-L1 on murine BMDCs. This is depicted in figure 3.6.1. MHC I levels increased from 4,025 to 6,852 MFI ($p<0.0001$). PD-L1 levels increased from 3,427 to 4,971 ($p=0.0012$). Poly I:C also upregulated the expression of IL-1 β and IL-6 by BMDCs as can be seen in figure 3.6.2.

CPG DNA

The TLR9 agonist, CPG DNA, significantly raised the expression levels of MHC I, CD80 and PD-L1. In figure 3.6.1, the levels of MHC I doubled from 4,025 to 8,260 MFI ($p=0.0017$). CD80 levels also doubled, from 1,493 to 3,809 ($p=0.0024$). This was accompanied by a near doubling of the expression levels of PD-L1, from 3,427 to 6,376 MFI ($p=0.0018$). CPG DNA also enhanced the production of IL-12, IL-1 β , IL-6 and TNF α by BMDCs, which is depicted in figure 3.6.2.

Flagellin

In contrast, Flagellin had no observable effect on the expression of activation markers or cytokines by BMDCs. This can be seen in figures 3.6.1 for activation markers and figure 3.6.2 for intracellular cytokines.

None of these TLR stimulations alone induced upregulation of MHC II or CD40 by BMDCs.

These results show the immunogenic effects of different TLR agonists on dendritic cells. This provides useful insights which allow us to rationally design novel vaccine candidates which will activate dendritic cells.

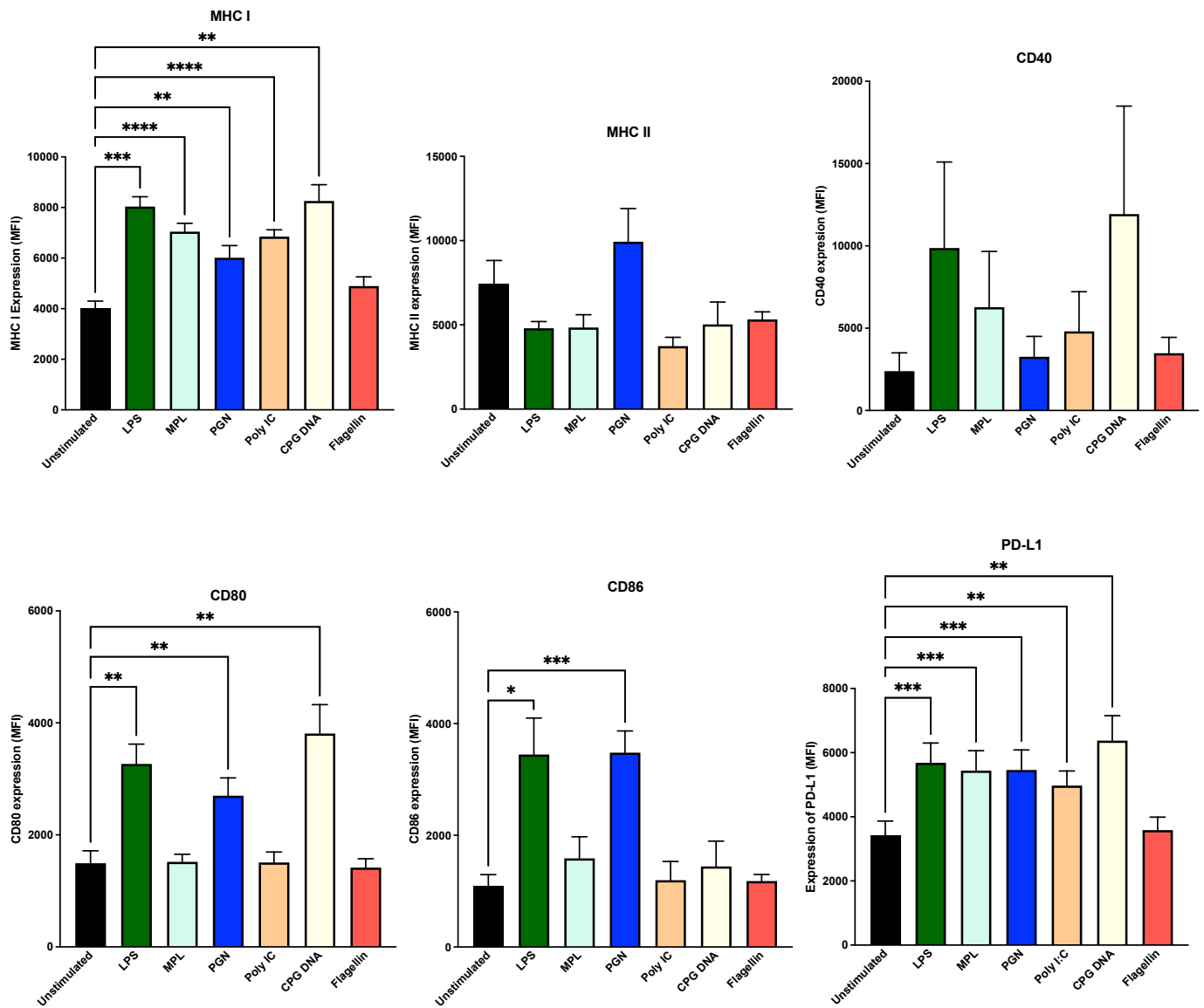


Figure 3.6.1. Effect of TLR agonists on cell surface marker expression by BMDCs. Changes in cell surface marker expression induced by TLR agonists are depicted. Duplicate wells of 1×10^5 BMDCs were stimulated for 48 hours with LPS, MPL, Peptidoglycan, Poly I:C, CPG DNA or Flagellin. Cell surface expression of MHC I, MHC II, CD40, CD80, CD86 and PD-L1 were measured by flow cytometry. MFI values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . are shown (n=3).

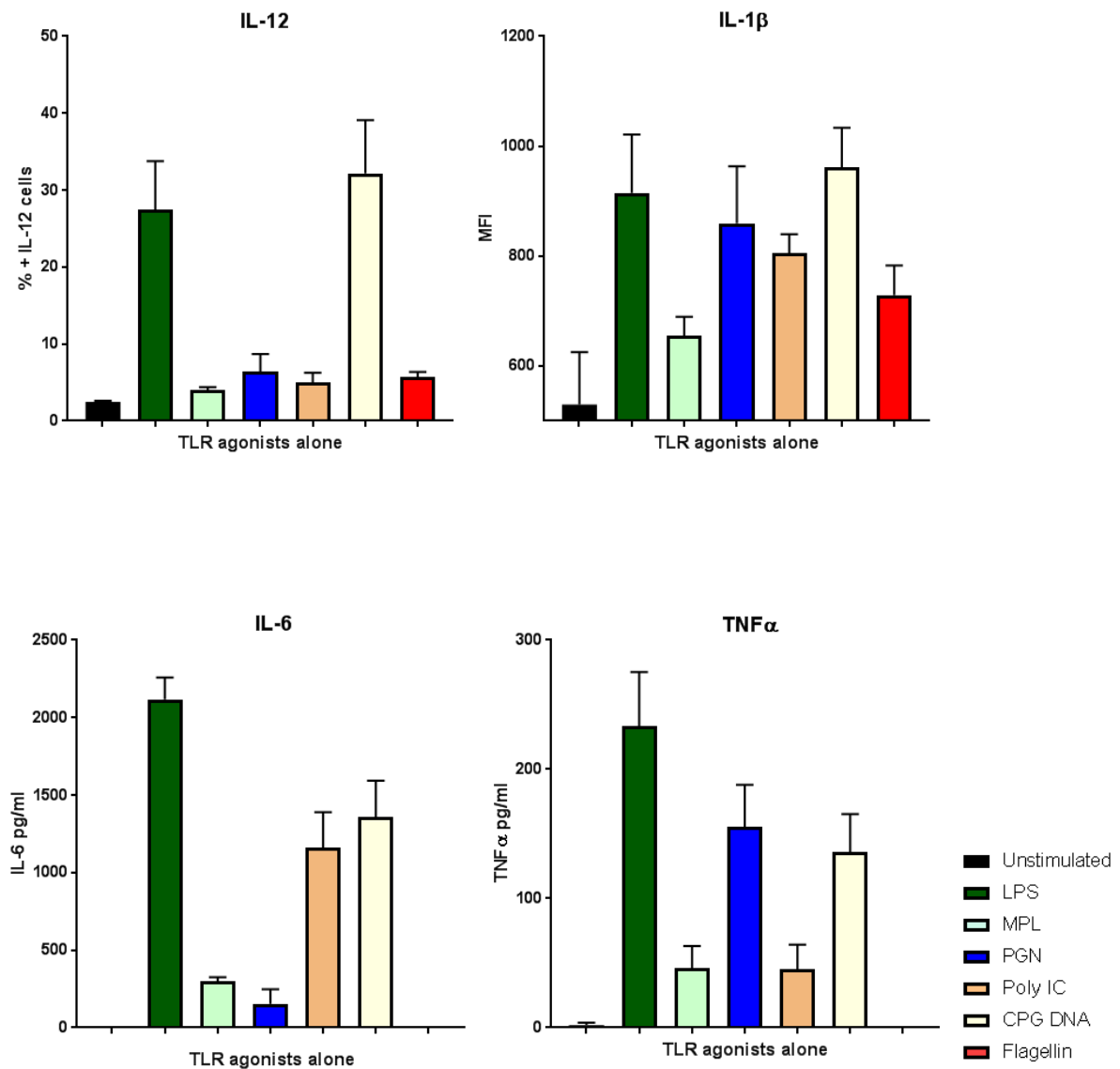


Figure 3.6.2. Effect of TLR agonists on BMDC cytokine expression. Changes in cytokine expression induced by TLR agonists are depicted. Duplicate wells of 1×10^5 BMDCs were stimulated with LPS, MPL, Peptidoglycan, Poly I:C, CPG DNA or Flagellin for 48 hours. The levels of IL-1 β , IL-6 and TNF α were measured by ELISA and the percentage of IL-12+ BMDCs was determined by intracellular flow cytometry. Mean values and $\pm$ standard deviations are shown (n=3).

3.7 Effects of TLR adjuvants on the immunogenicity of spores.

This study has found that the spore delivery system enhanced DC expression of TNF α , IL-12, IL-6 and IL-1 β by DCs, but not activation markers and MHC molecules. This finding was shown in figure 3.2.1. It was hypothesised that the spore vaccine delivery system could be combined with an adjuvant to increase the activation of dendritic cells. Six TLR agonists were tested as adjuvants for a spore delivery system; the TLR 4 agonists LPS and MPL, the TLR 2 agonist Peptidoglycan, the TLR 3 agonist Poly I:C, the TLR 9 agonist CPG DNA and Flagellin a TLR 5 agonist. Co-stimulation with TLR agonists and spores led to significant changes in the expression levels of activation markers and cytokines.

Spores and LPS

Co-stimulation with spores and LPS led to significantly enhanced expression of MHCI and MHCII. This is shown in Figure 3.7.1. The level of MHC I increased around twofold from 4,024 in unstimulated cells to 8,645 with Spores and LPS ($p<0.0001$). MHC II levels on BMDCs also increased in the presence of spores and LPS. LPS stimulation induced a MHC II expression level of 4,791. This rose to 12,336 when LPS was combined with spores ($p=0.0033$). CD80, CD86 and PD-L1 levels were also increased by combining Spores with LPS. This can be seen in figure 3.7.2. CD80 expression increased three fold from 1,493 in unstimulated cells to 4,866 in the presence of Spores and LPS ($p<0.0001$) CD86 levels also increased, more than eight fold, from 662 to 5,779 with spores and LPS ($p<0.0001$). The expression of the inhibitory molecule PD-L1 was raised, from 3,427 MFI in unstimulated cells to 6,572 in cells stimulated with spores and LPS and together ($p=0.0036$).

Figure 3.7.3 shows that spores alone elicited TNF α and IL-1 β responses. TNF α production was observed in BMDCs stimulated with spores and LPS. TNF α levels rose from 1.98 pg/ml to 296 pg/ml ($p<0.0001$). IL-1 β levels rose from 529 pg/ml to 943 pg/ml when BMDCs were stimulated with spores ($p=0.0195$). Combining spores with TLR agonists did not significantly increase IL-1 β levels further. Figure 3.7.4

depicts the effect of combining spores with LPS on IL-6 and IL-12 production. IL-6 levels rose from below detectable limits to 1,724 pg/ml ($p<0.0001$). 2.5% of unstimulated BMDCs expressed IL-12. With spore and LPS in combination, The percentage of IL-12 expressing BMDCs increased to 32% ($p<0.0001$).

Spores and MPL

Combining Spores with MPL resulted in increased levels of MHC I, MHC II, CD80, CD86 and PD-L1. In figure 3.7.1, the level of MHC I increased around twofold from 4,024 in unstimulated cells to 7,891 with spores and MPL ($p<0.0001$). MPL stimulation generated MHC II expression levels of 4,842. When MPL was combined with spores, this increased to 11,319 ($p=0.0166$). In figure 3.7.2, CD80 levels doubled from 1,493 to 3,540 ($p<0.0001$) and CD86 expression increased seven fold from 662 to 4,740 with spores and MPL ($p<0.0001$). PD-L1 levels simultaneously rose from 3,427 to 6,570 in the presence of spores and MPL ($p=0.065$). Figure 3.7.4 shows that this combination induced IL-6 as well. Unstimulated BMDCs generated no detectable IL-6. Stimulation with Spores and MPL raised the IL-6 concentration to 964pg/ml ($p=0.0017$).

Spores and peptidoglycan

Combining Spores with peptidoglycan led to increased levels of MHC I, CD80, CD86 and PD-L1. MHC I levels increased from 4,024 in unstimulated cells to 6,868 ($p=0.0004$). Figure 3.7.2 shows CD80 levels rose from 1,493 in unstimulated cells to 3,314 following stimulation with spores and peptidoglycan ($p=0.0002$). CD86 expression increased more than six fold from 662 to 4,332 ($p<0.0001$). PD-L1 levels rose from 3,426 in unstimulated cells to 6,117 following stimulation with PGN and spores together ($p=0.0332$). Combining spores with peptidoglycan resulted in increased production of IL-6, and TNFa. Figure 3.7.4 shows that IL-6 concentrations increased from below detectable levels to 795pg/ml

($p=0.0170$). TNFa levels are depicted in figure 3.7.3. These rose from 1.98pg/ml in unstimulated cells to 145pg/ml in the presence of spores and peptidoglycan.

Spores and Poly I:C

Co-stimulation with spores and Poly I:C resulted in higher levels of MHC I, MHC II, CD86 and PD-L1. Figure 3.7.1 depicts MHC I levels rising from 4,024 to 7,854 ($p<0.0001$). MHC II expression increased 2.6 fold from 3,738 with Poly I:C alone to 10,005 with spores and Poly I:C ($p=0.0225$). In Figure 3.7.2, CD86 levels rose fivefold from 662 to 3,563 ($p<0.0001$). Expression of the inhibitory molecule PD-L1 also increased on BMDCs stimulated with spores and Poly I:C. BV421 MFI was 3,426 on unstimulated cells, rising 1.8 fold to 6,437 in BMDCs stimulated with spores and Poly I:C ($p=0.0107$).

MyD88 and IRF-3 signalling is required for maximal dendritic cell activation and expression of MHC II, CD86 and CD80 (Shen, H. et al. 1849-1858). Spores act in concert with TLR 3 and TLR 4 agonists to increase expression of MHC II, CD86 and CD80. *B. subtilis* spores are known to induce MyD88 signalling through TLR2 (de Souza et al. e87454). Poly I:C induces IRF3 signalling. Combining spores with Poly I:C leads to both MyD88 and IRF3 signalling resulting in activation of DCs.

Spores and CPG DNA

CPG DNA in combination with spores led to BMDCs expressing higher levels of MHC I and MHC II. This is shown in figure 3.7.1. MHC I levels doubled from 4,024 to 8,165 ($p<0.0001$). MHC II expression levels were 5,026 with CPG DNA stimulation. When CPG DNA was combined with Spores, MHC II levels doubled to 10,793 ($p=0.0446$).

CD80, CD86 and PD-L1 levels were also raised in the presence of spores and CPG DNA. This is shown in figure 3.7.2. Stimulation with spores and CPG DNA raised the expression of CD80 from 1,493 MFI to

3,346 ($p=0.0001$). CD86 levels were similarly raised from 662 in unstimulated cells to 3,287 with CPG DNA and Spores ($p<0.0001$). PD-L1 levels increased from 3,426 MFI in unstimulated cells to 6,112 MFI in cells stimulated with CPG DNA and Spores ($p=0.0338$). Maximal activation of DCs requires IRF-3 signalling and MyD88 signalling (Shen, H. et al. 1849-1858) CPG DNA induces IRF3 signalling (Volpi et al. 1852). Co-stimulation with spores would lead to concomitant MyD88 and IRF-3 signalling, enhancing the MyD88 activatory signal generated by spores alone.

CPG DNA combined with spores to elicit IL-6, IL-12 and TNF α responses. Figure 3.7.3 shows TNF α concentrations increased 79 fold from 1.98 pg/ml to 158 pg/ml in the presence of spores and CPG DNA. Figure 3.7.4 shows that levels of IL-6 increased to 1,644 pg/ml ($p<0.0001$). The percentage of IL-12+ BMDCs increased from 2.4% to 32% with spores and CPG stimulation ($p=0.0007$). IL-12 plays an important role in the development of IFN γ responses (Belladonna et al. 5448-5454).

Spores and Flagellin

Figure 3.7.1 and 3.7.2 show that a combination of Spores and Flagellin activated BMDCs and increased their expression of MHC I and CD86. MHC I levels increased from 4,024 in unstimulated cells to 6,164 with spores and flagellin ($p=0.0161$). CD86 levels increased from 662 in unstimulated cells to 2,169 with a combination of spores and flagellin ($p=0.0090$). IL-6 levels also rose. This can be seen in figure 3.7.4, where the concentration of IL-6 increased from below detectable levels in unstimulated cells to 784 pg/ml with the addition of spores and flagellin.

Based on this data, it was thought that CPG DNA, MPL, LPS or Poly I:C would all be suitable adjuvants for a spore based vaccine as each of these adjuvants combine with spores to increase expression of MHC I, MHC II and CD86. It was notable though, that each of these TLR agonists increased expression of the inhibitory molecule PD-L1 when combined with spores. Poly I:C was selected for further testing as an adjuvant for Spore FP1.

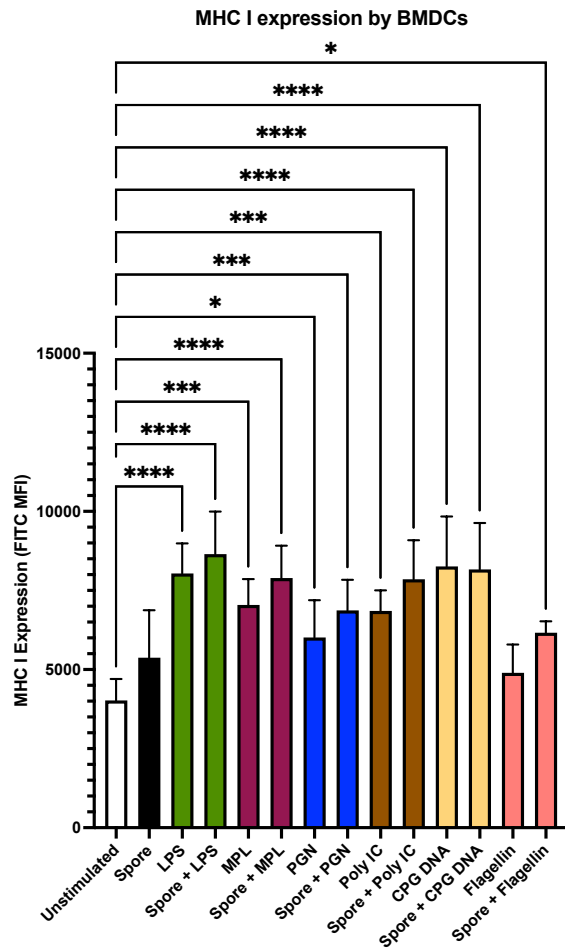
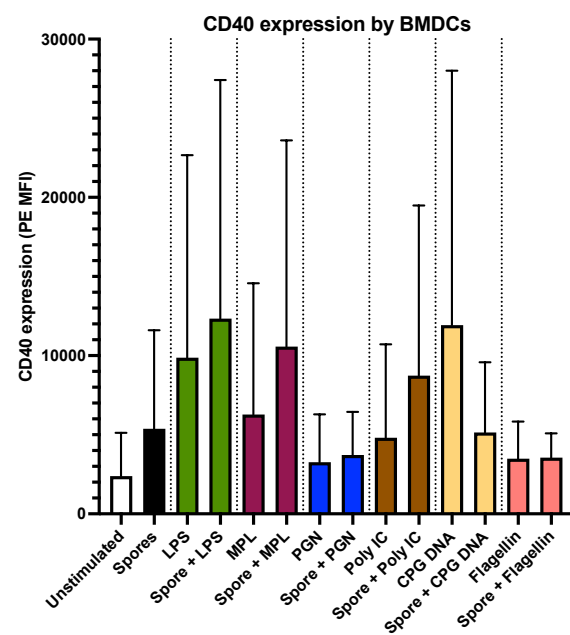
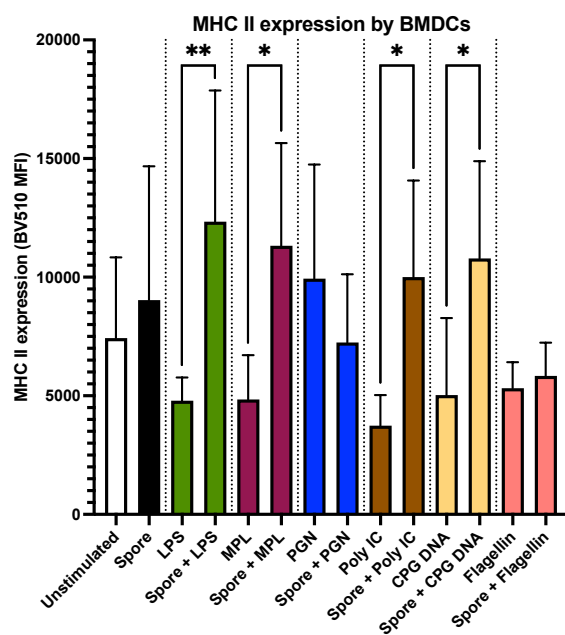


Figure 3.7.1. Effect of TLR agonists and spores on MHC molecules and CD40 expression. Duplicate wells of 1×10^5 BMDCs were stimulated with *B. subtilis* spores at an MOI 5:1 (cells to spores) and TLR adjuvants for 48 hours. Flow cytometry was used to measure the expression of MHC I, MHC II and CD40. Mean MFI values and standard deviations are shown. (n=3). Statistical analysis was performed using One Way ANOVA and Sidaks multiple comparisons test for (B). One way ANOVA and Dunnetts test were used for graphs A & C. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .



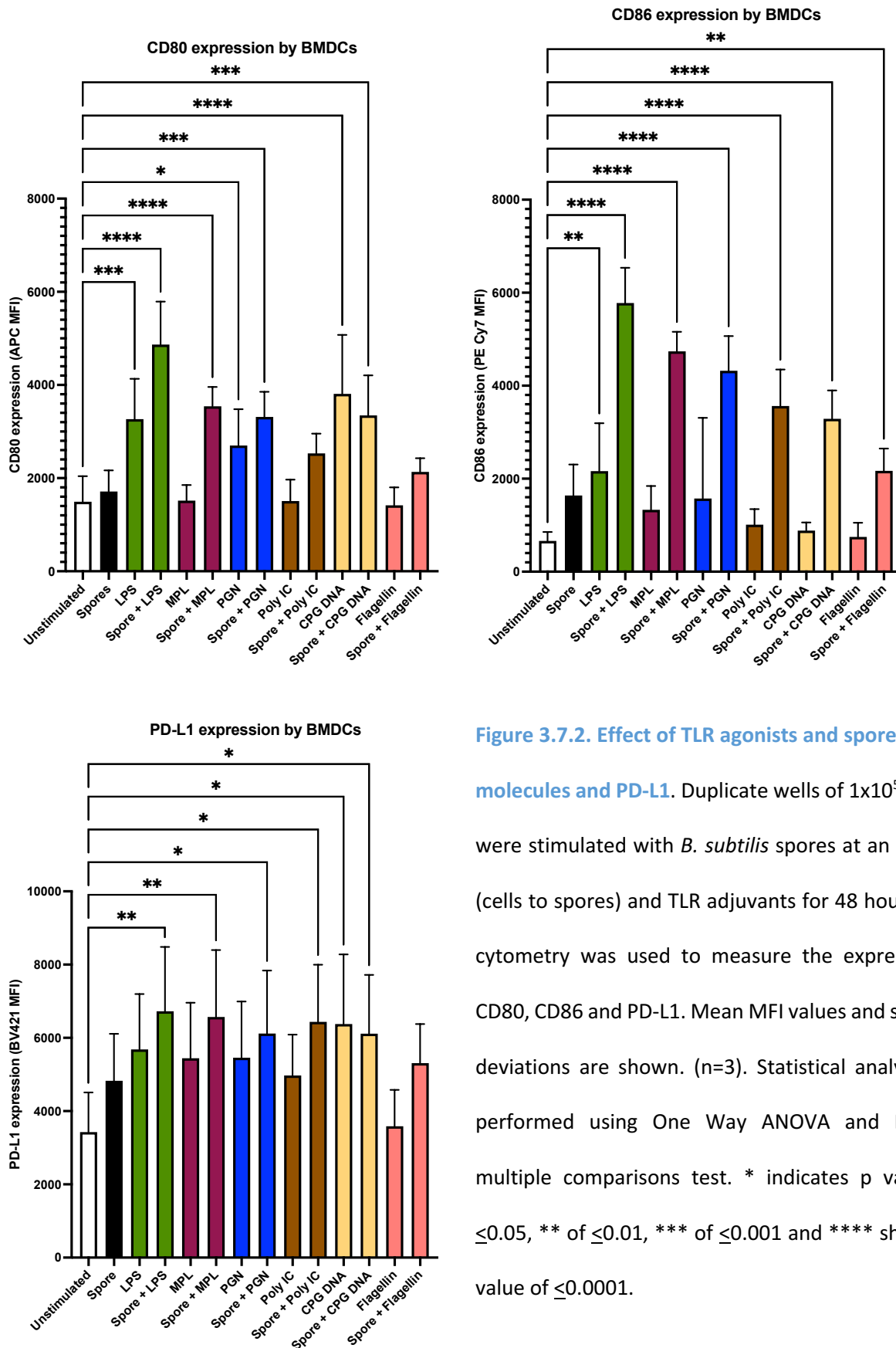


Figure 3.7.2. Effect of TLR agonists and spores on B-7 molecules and PD-L1. Duplicate wells of 1×10^5 BMDCs were stimulated with *B. subtilis* spores at an MOI 5:1 (cells to spores) and TLR adjuvants for 48 hours. Flow cytometry was used to measure the expression of CD80, CD86 and PD-L1. Mean MFI values and standard deviations are shown. (n=3). Statistical analysis was performed using One Way ANOVA and Dunnett's multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

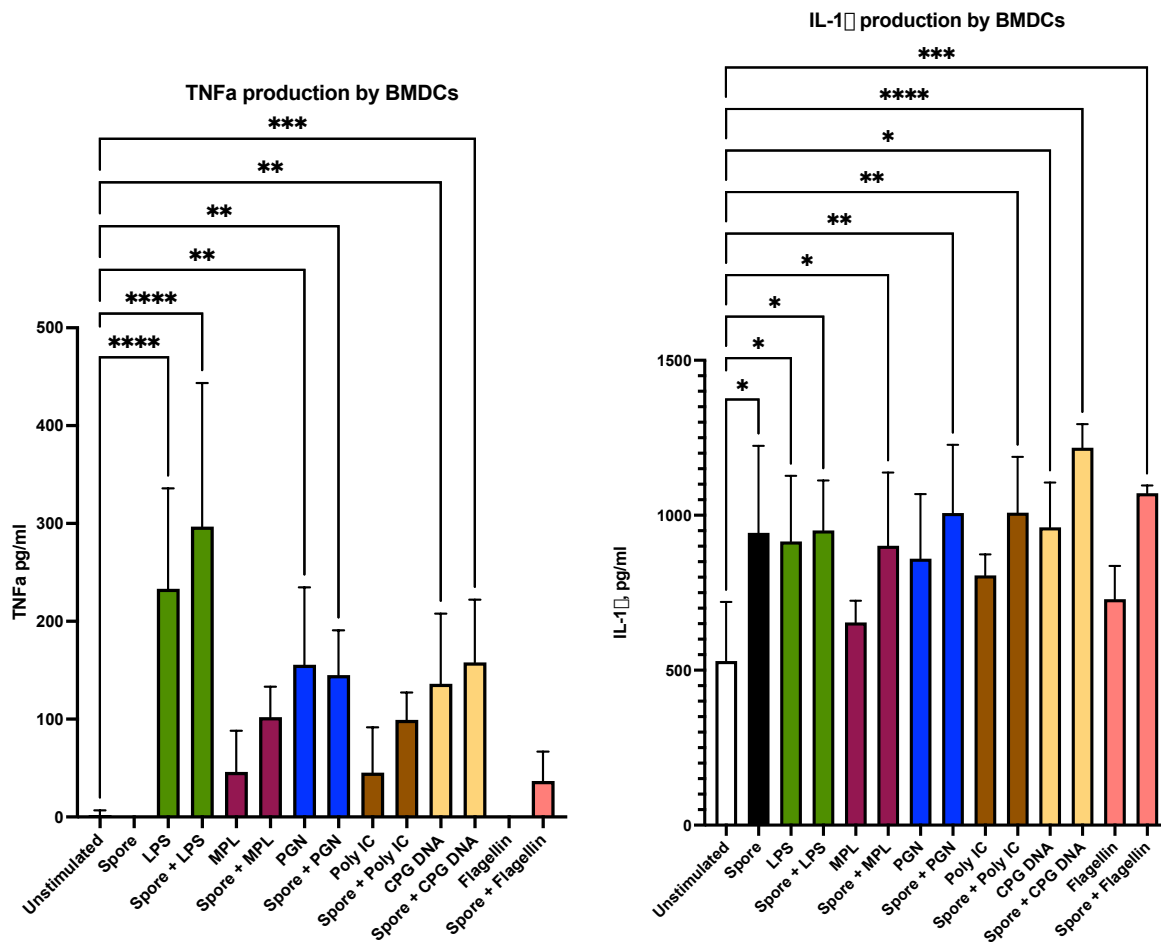


Figure 3.7.3. Effect of Spores and TLR adjuvants on TNFα and IL-1β production by BMDCs. 1×10^5 BMDCs in duplicate wells were stimulated with *B. subtilis* spores, MOI 5:1 (cells to spores) and TLR adjuvants for 48 hours. (n=3). The concentration of TNFα and IL-1β in the supernatant was measured by ELISA. Mean concentration values and standard deviations are shown for TNFα in graph A. The mean concentration of IL-1β and the standard deviations are shown in graph B. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

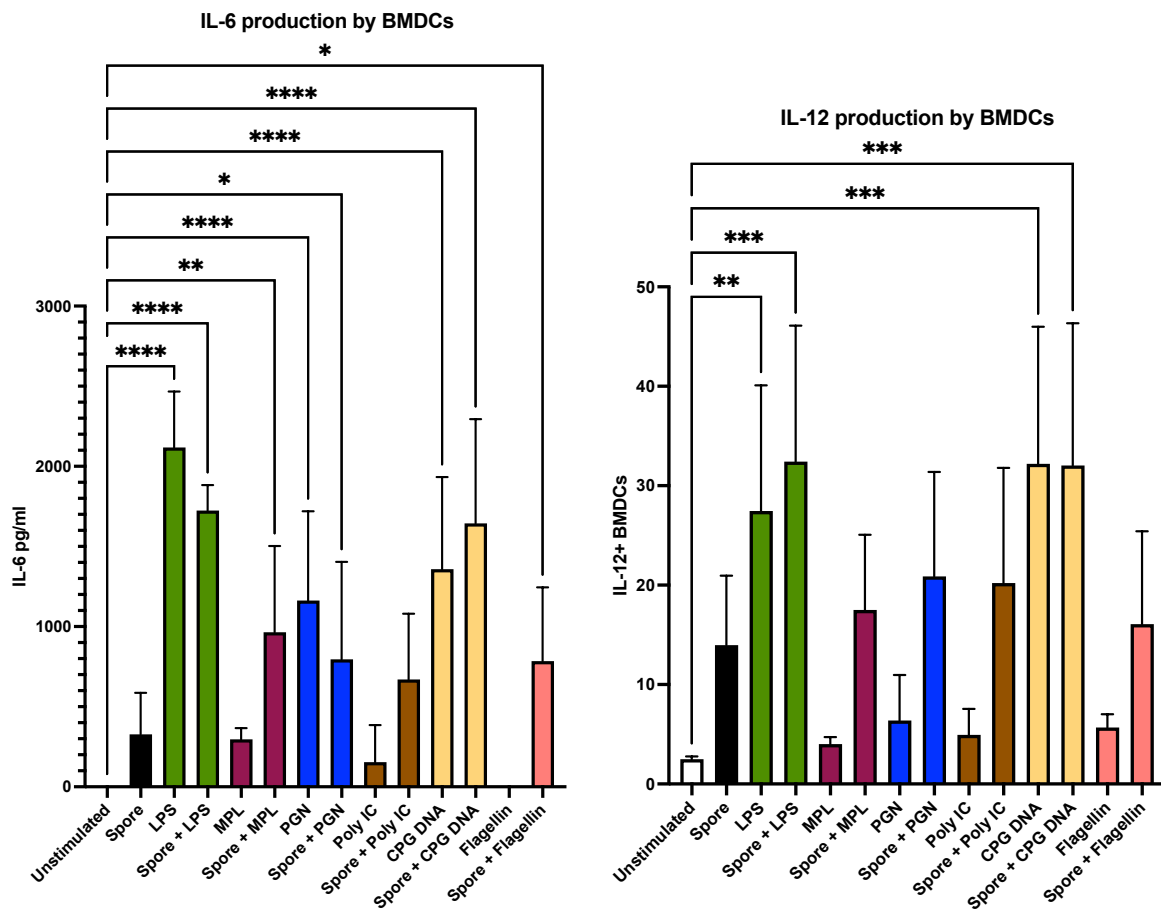


Figure 3.7.4 Effect of TLR agonists and Spores on IL-6 and IL-12 production by BMDCs. 1×10^5 BMDCs in duplicate wells were stimulated with Spores at an MOI of 5:1 (cells to spores) and TLR adjuvants for 48 hours ($n=3$). IL-6 levels in supernatants were measured by ELISA. Mean concentration values and standard deviations are shown for IL-6 in graph A. Flow cytometry was used to measure intracellular IL-12 within BMDCs. The mean percentage of IL-12+ cells and the standard deviations are shown in graph B. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

3.8 Combined effect of TLR adjuvants and YC NaMa on dendritic cells.

The YC NaMa delivery system alone had no detectable immunogenic effect on DCs. It was hypothesised that combining a TLR agonist adjuvant with YC NaMa could provide the activatory stimulus needed to activate DCs. Six TLR agonists were tested as adjuvants to YC NaMa; LPS, MPL, Poly I:C, Peptidoglycan, CPG DNA and Flagellin. The subsequent effect on DC activation marker expression and cytokine release was measured by flow cytometry and ELISA.

YC NaMa and LPS

YC NaMa and LPS together were highly immunogenic and activated DCs to increase their expression of MHC I, MHC II, CD80, CD86 and PD-L1. Figure 3.81 shows MHC I expression increasing from 4,024 to 7,846 ($p < 0.0001$). MHC II expression was also raised, from 4,791 with LPS alone to 11,441 when YC NaMa and LPS were used in combination ($p = 0.0189$). The results in figure 3.8.2 show that CD86 expression increased more than 7 fold from 662 to 5,063 with YC NaMa and LPS stimulation ($p < 0.0001$). The combination of LPS with YC NaMa also had the effect of increasing PD-L1 expression on BMDCs. PD-L1 expression doubled from 3,426 in unstimulated cells to 6,947 ($p = 0.0021$). Figure 3.8.3 shows that YC NaMa combined with LPS to generate TNF α responses. The concentration of TNF α rose from 1.9pg/ml to 249pg/ml ($p < 0.0001$). This combination also induced IL-6 and IL-12 responses, as can be seen in figure 3.8.4. IL-6 concentrations increased from below detectable levels in unstimulated cells to 1,800 in cells stimulated with YC NaMa and LPS ($p < 0.0001$). The percentage of IL-12 positive BMDCs increased from 2.4% to 23% following stimulation with YC NaMa and LPS.

YC NaMa and MPL

YC NaMa combined with MPL induced higher expression levels of MHC I, MHC II, CD86, and PD-L1. Figure 3.8.1 shows that YC NaMa and MPL increased MHC I expression from 4,024 to 6,891 ($p=0.0004$). MHC II expression also increased from 4,842 on MPL stimulated cells to 11,691 in the presence of both MPL and YC NaMa ($p=0.0143$). Figure 3.8.2 shows that CD86 expression increased fourfold from 662 on unstimulated cells to 2,675 with YC NaMa and MPL ($p<0.0001$). There was a corresponding increase in PD-L1 expression, from 3,426 on unstimulated cells to 6,691 in the presence of YC NaMa and MPL combined ($p=0.0054$).

YC NaMa and Poly I:C

YC NaMa in combination with Poly I:C increased expression of MHC I and MHC II. This can be seen in figure 3.8.1. MHC I levels increased from 4,024 on unstimulated cells to 6,639 ($p=0.0016$). MHC II levels amplified threefold from 3,738 on Poly I:C stimulated cells to 11,791 when the cells were stimulated with YC NaMa and Poly I:C together ($p=0.0024$). Figure 3.8.4 shows IL-6 levels were increased from below detectable levels in unstimulated cells to 1,185pg/ml in BMDCs stimulated with YC NaMa and Poly I:C ($p<0.0001$).

YC NaMa and CPG DNA

Stimulation with YC NaMa and CPG DNA led to increased expression of MHC I, CD80 and CD86. Figure 3.8.1 shows that MHC I levels increasing on DCs stimulated with YC NaMa and CPG DNA. Levels rose from 4,024 to 7,495 ($p<0.0001$). Figure 3.8.2 shows the effect of YC NaMa and CPG DNA on the expression of B-7 molecules. CD80 expression increased from 1,493 on unstimulated cells to 2,901 MFI ($p=0.0294$). Stimulation with YC NaMa and CPG DNA increased CD86 expression from 662 MFI on unstimulated cells to 2,045 MFI ($p=0.0111$).

YC NaMa in conjunction with CPG DNA generated strong cytokine responses. IL-1 β , IL-6 and TNFa concentrations were all elevated. Figure 3.8.3 shows IL-1 β levels rose from 529pg/ml to 925pg/ml ($p=0.0114$). TNFa levels also increased, from 1.9 pg/ml to 142pg/ml in the presence of YC NaMa and CPG DNA ($p=0.0053$). Figure 3.8.4 shows that the percentage of IL-12+ BMDCs concomitantly rose from 2.4% IL-12+ in the unstimulated cells to 28% IL-12+ in the cells stimulated with YC NaMa and CPG DNA. Figure 3.8.4 shows IL-6 levels also rose from below detectable levels to 746 pg/ml ($p=0.0019$).

YC NaMa and peptidoglycan

YC NaMa paired with peptidoglycan increased the expression of MHC I, CD86 and PD-L1 on DCs. The results in Figure 3.8.1 depict MHC I levels rising from 4,024 on unstimulated cells to 6,111 on BMDCs stimulated with YC NaMa and peptidoglycan together ($p=0.0234$). In figure 3.8.2, CD86 expression increased fivefold from 662 on unstimulated cells to 3,491 with YC NaMa and peptidoglycan ($p<0.0001$). PD-L1 expression also increased in cells stimulated with this combination. Unstimulated cells had a BV421 MFI of 3,426, which nearly doubled to 6,474 with the application of YC NaMa and peptidoglycan ($p=0.0121$). Combining YC NaMa with peptidoglycan also generated cytokine responses. Figure 3.8.3 depicts an increase in the TNFa concentration in BMDCs treated with YC NaMa and peptidoglycan. After 48 hours, TNFa levels rose 77 fold from 1.9pg/ml to 148pg/ml ($p=0.0031$).

Using TLR 3 and TLR 4 agonists in combination with YC NaMa lead to increased expression of MHC II and CD86 by DCs. This should improve the immunogenicity of YC NaMa vaccine formulations. TLR4 agonists, Poly I:C, CPG DNA, and peptidoglycan all combined with YC NaMa to enhance MHC I expression in DCs. This may be useful for vaccines designed to elicit antiviral responses. YC NaMa generated strong cytokine responses when paired with LPS or CPG DNA.

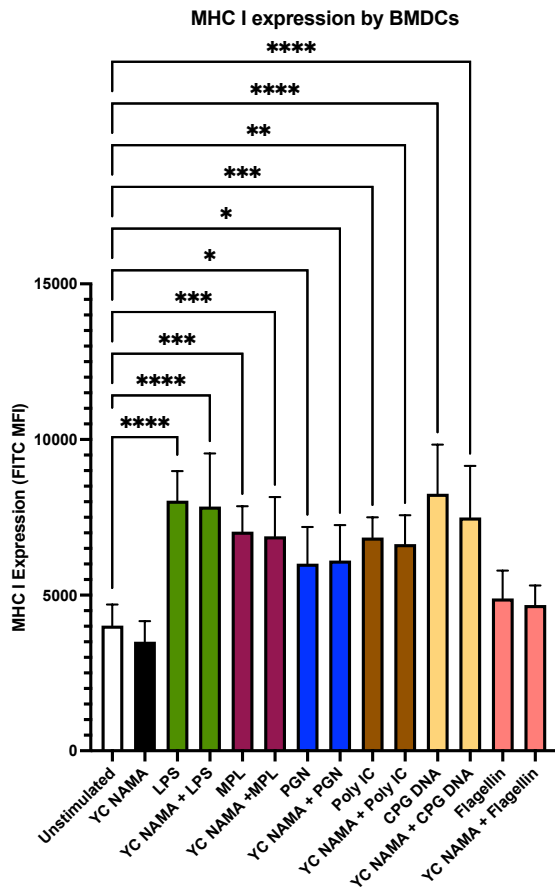
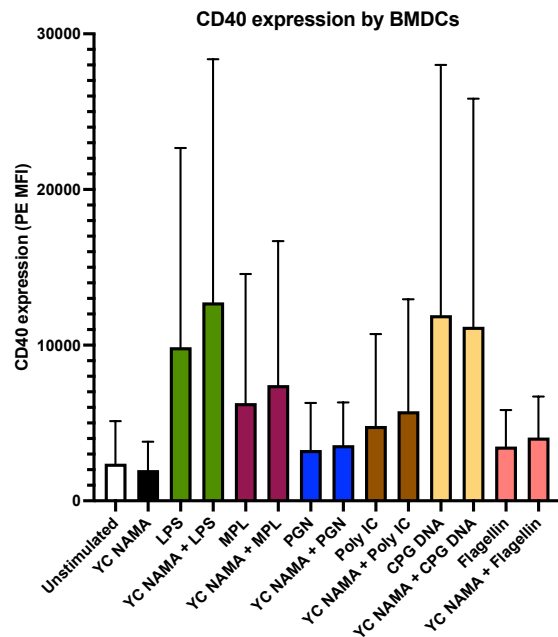
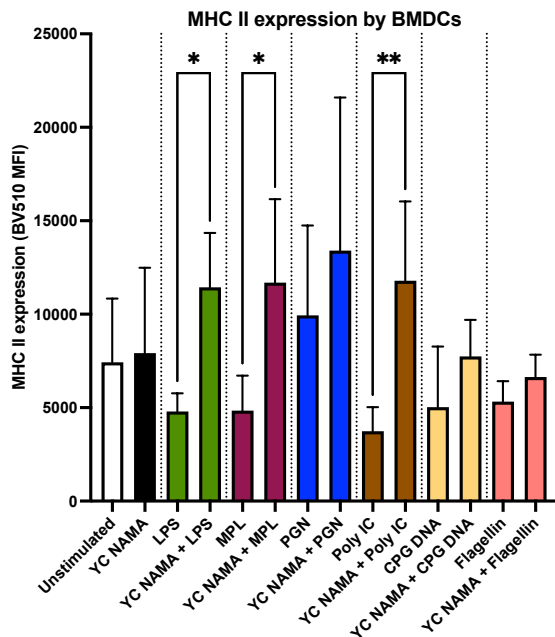


Figure 3.8.1. Effect of YC NaMa and TLR adjuvants on the expression of MHC molecules and CD40.

Duplicate wells of 1×10^5 BMDCs were stimulated with YC NaMa at $10 \mu\text{g/ml}$ and TLR adjuvants for 48 hours. ($n=3$). Flow cytometry was used to measure the expression of MHC I, MHC II and CD40. Mean MFI values and standard deviations are shown. Statistical analysis was performed using One Way ANOVA and Dunnett's multiple comparisons test for graphs A & C. One way ANOVA and Sidak's multiple comparisons test were used for graph B. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .



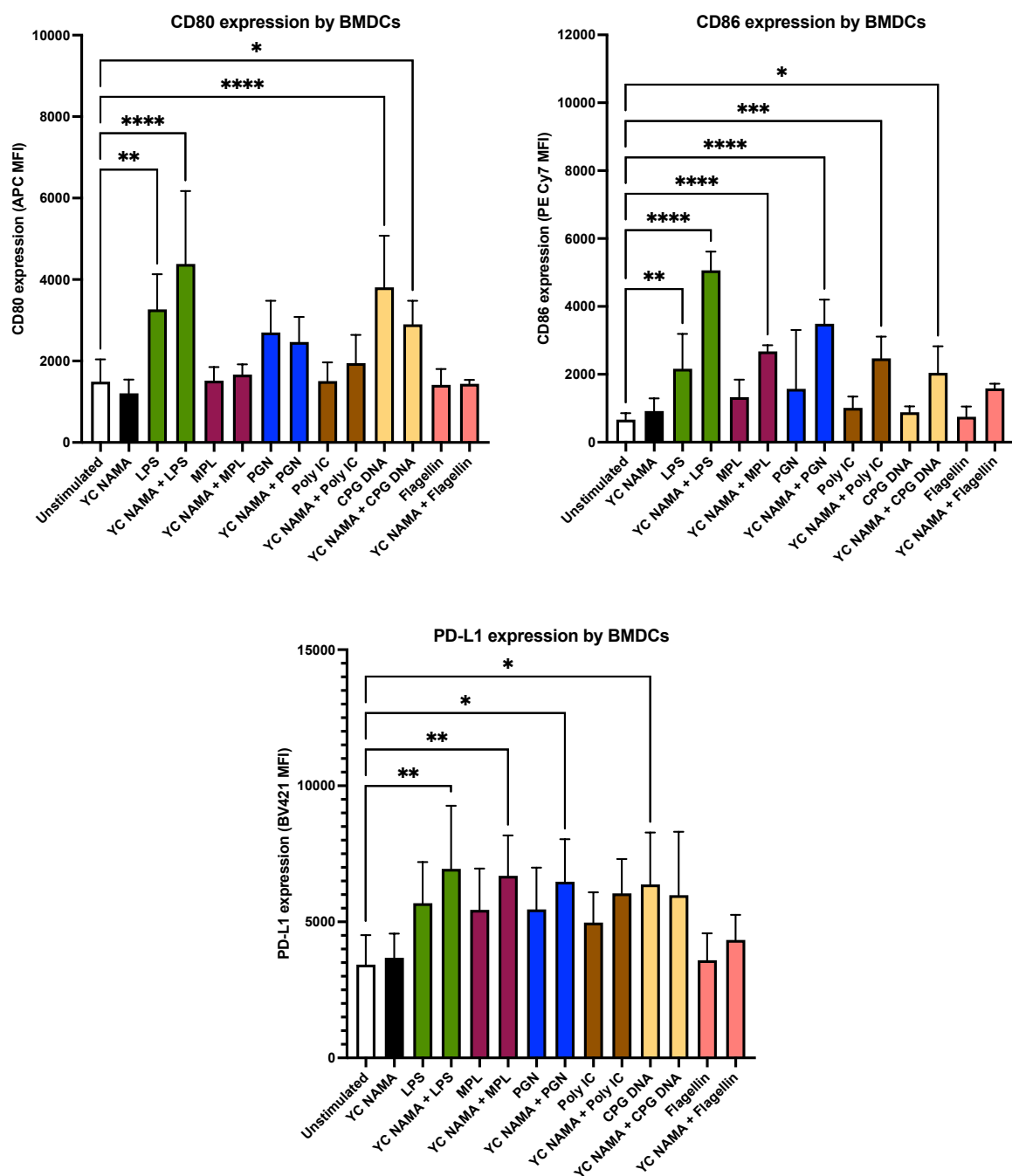


Figure 3.8.2. Effect of YC NaMa and TLR adjuvants on the expression of B-7 molecules and PD-L1.

Duplicate wells of 1×10^5 BMDCs were stimulated with YC NaMa at $10 \mu\text{g/ml}$ and TLR adjuvants for 48 hours. ($n=3$). Flow cytometry was used to measure the expression of CD80, CD86 and PD-L1. Mean MFI values and standard deviations are shown. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

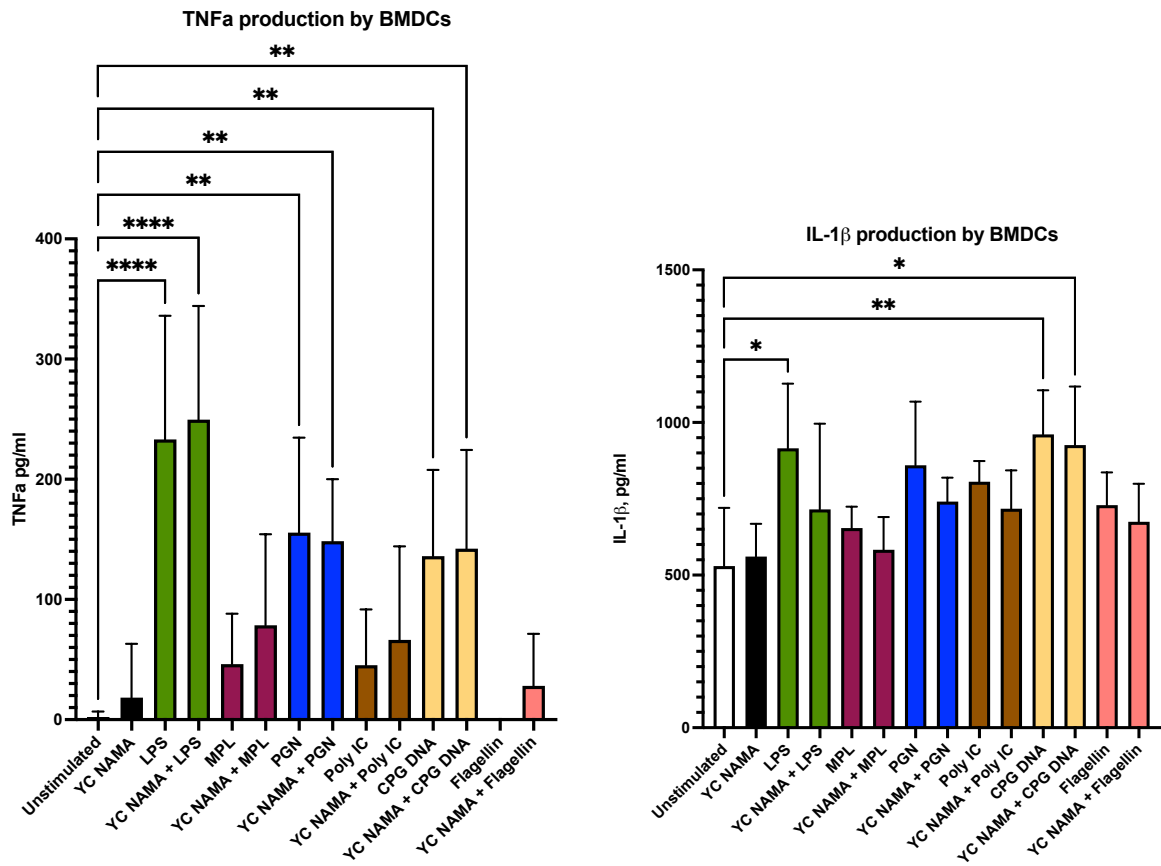


Figure 3.8.3. Effect of YC NaMa and TLR adjuvants on TNFα and IL-1β production by BMDCs.

Duplicate wells of 1×10^5 BMDCs were stimulated with YC NaMa at $10 \mu\text{g/ml}$ and TLR adjuvants for 48 hours. ($n=3$). The concentration of TNFα and IL-1β in the supernatant was measured by ELISA. Mean concentration values and standard deviations are shown for TNFα in graph A. The mean concentration of IL-1β and the standard deviations are shown in graph B. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

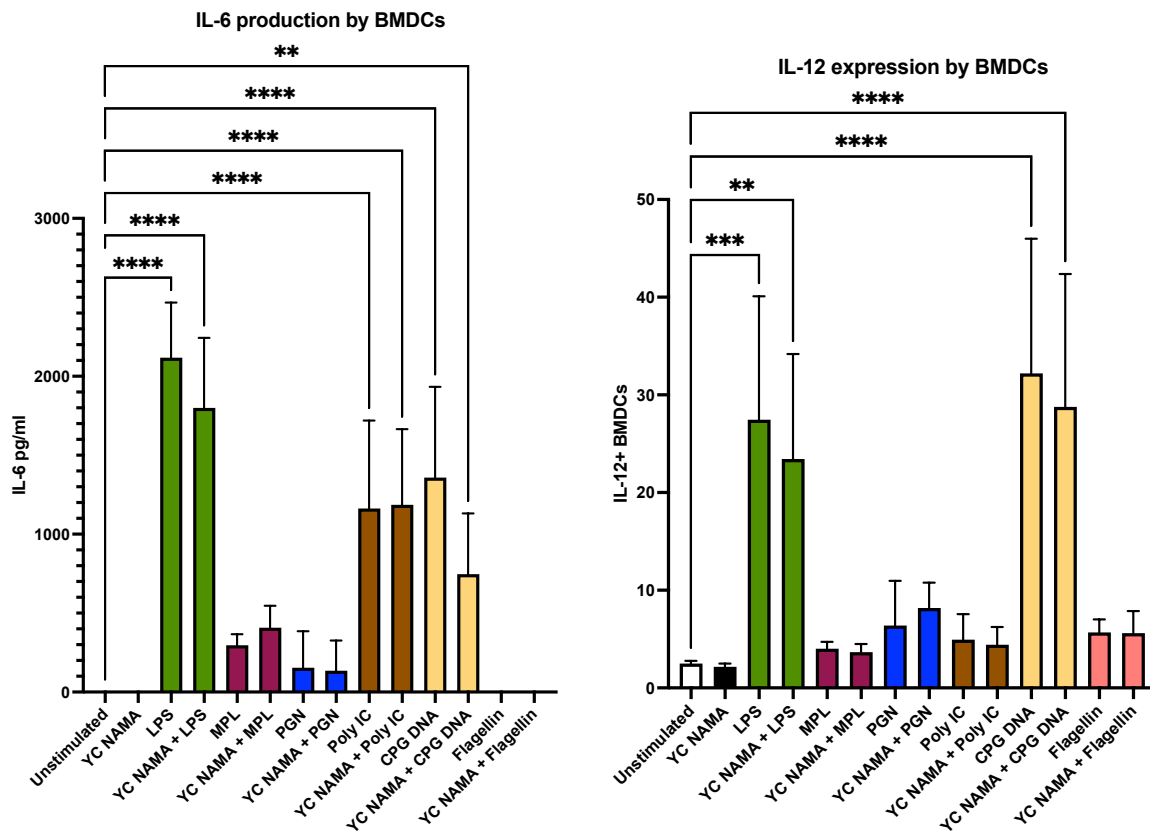


Figure 3.8.4 Effect of TLR agonists and YC NaMa on IL-6 and IL-12 production by BMDCs. Duplicate wells of 1×10^5 BMDCs were stimulated with YC NaMa at $10 \mu\text{g/ml}$ and TLR adjuvants for 48 hours ($n=3$). IL-6 levels in supernatants were measured by ELISA. Mean concentration values and standard deviations are shown for IL-6 in graph A. Flow cytometry was used to measure intracellular IL-12 within BMDCs. The mean percentage of IL-12+ cells and the standard deviations are shown in graph B. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

3.9 Combined effect of TLR adjuvants and Liposome P/S on dendritic cells

The liposome P/S delivery system did not exhibit any immunogenic effects on DCs. TLR agonists were examined as vaccine adjuvants which could enhance the immunogenicity of liposome P/S. Figure 3.9.1 shows the effect of combined stimulation with liposome P/S and TLR agonists on expression of MHC molecules and CD40 by dendritic cells. Figure 3.9.2 shows the effect of combining liposomes with TLR agonists on CD80, CD86 and PD-L1. Figures 3.9.3 and 3.9.4 show the effect of liposome P/S and TLR agonists on the production of proinflammatory cytokines.

Liposomes and LPS

Figure 3.9.1 shows MHC I levels doubling from 3,417 on unstimulated cells to 8,758 on cells stimulated with Liposomes combined with LPS ($p=0.0004$). CD86 expression also increased from 662 to 3,631 ($p=0.0008$), which can be seen in figure 3.9.2. The combination of Liposomes and LPS generated strong cytokine responses from BMDCs. Figure 3.9.3 shows TNF α levels increasing from a concentration below detectable levels in unstimulated cells to 140pg/ml after stimulation with Liposomes and LPS ($p=0.0010$). Figure 3.9.4 shows that IL-6 levels increased from 109 pg/ml to 1,658 pg/ml ($p=0.0023$). The percentage of IL-12+ BMDCs also increased from 4.4% to 24.5% when the cells were stimulated with Liposomes and LPS together ($p=0.0222$).

Liposomes and MPL

Stimulation with Liposomes and MPL increased MHC I levels from 3,417 to 8,308 in figure 3.9.1 ($p=0.0017$). The results in Figure 3.9.2 reveal that CD86 and PD-L1 expression was also elevated. CD86 levels rose from 662 to 2,883 ($p=0.0265$) and PD-L1 expression was increased on DCs treated with Liposomes and MPL. It rose from 2,498 to 8,906 ($p=0.0445$). The combination of liposomes with MPL did not influence the expression of MHC II, CD40 or CD80 by DCs. It also had no impact on the expression of proinflammatory cytokines.

Liposomes and Poly I:C

Figure 3.9.1A shows that Poly I:C and Liposomes induced MHC I expression, with levels rising from 3,417 to 7,862 ($p=0.0058$). Expression of MHC II, CD40, CD80 and CD86 was unaffected by liposomes and Poly I:C. Figure 3.9.2.C reveals that PD-L1 expression was raised 3.5 fold from 2,498 to 8,842 ($p=0.0485$).

Liposomes and CPG DNA

Figure 3.9.1A demonstrates that dendritic cells increase their expression of MHC I in the presence of liposomes and CPG DNA. MHC I levels rose from 3,417 on unstimulated cells to 7,951 on cells stimulated with liposomes and CPG DNA ($p=0.0046$). This was accompanied by an increase in PD-L1 expression, from 2,498 MFI on unstimulated cells to 9,383 ($p=0.0230$). This is shown in figure 3.9.2C. This combination of liposomes with CPG DNA also generated IL-12 responses, as depicted in figure 3.9.4B. The percentage of IL-12+ BMDCs increased more than six fold from 4.4% to 29% IL-12+ in the presence of Liposome and CPG DNA.

These results showed that Liposomes could be combined with LPS, MPL, Poly I:C and CPG DNA to enhance MHC I expression on DCs. These increases were all accompanied by increases in PD-L1 expression. These combinations may be useful for further vaccine development, particularly for vaccines aiming to elicit CD8+ T cell responses. These experiments did not show that liposome P/S inhibits TLR signalling in DCs. The “inhibitory” effect of phosphatidylserine which has been noted in previous papers (Birge et al., 2016), may be due in part to upregulation of PD-L1 on DCs stimulated with TLR agonists and phosphatidylserine.

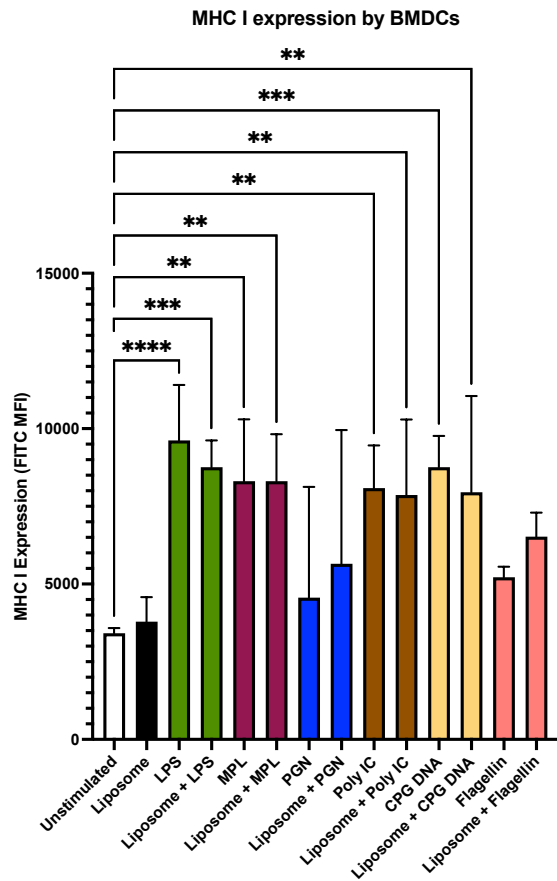
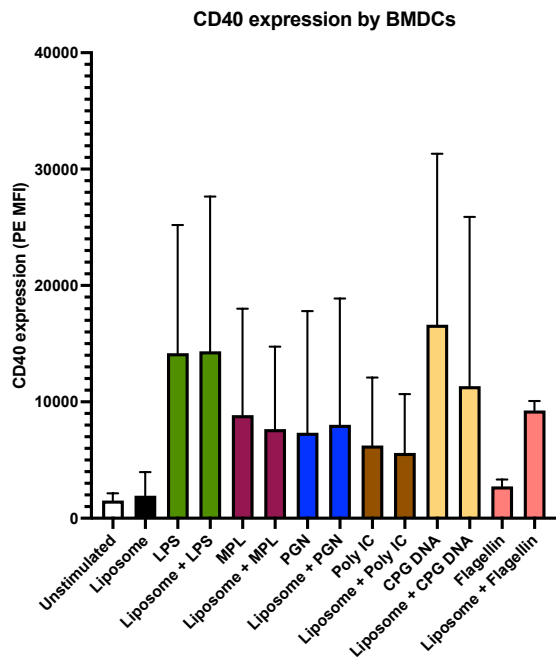
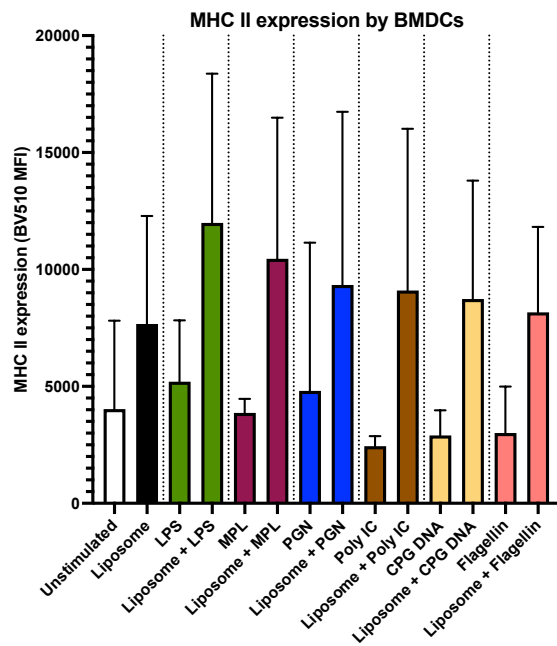


Figure 3.9.1. Effect of Liposomes and TLR adjuvants on the expression of MHC molecules and CD40.

Duplicate wells of 1×10^5 BMDCs were stimulated with Liposome P/S at a ratio of 5:1 and TLR adjuvants for 48 hours. (n=3). Flow cytometry was used to measure the expression of MHC I, MHC II and CD40. Mean MFI values and standard deviations are shown. Statistical analysis was performed using One Way ANOVA and Dunnett's multiple comparisons test for graphs A & C. One way ANOVA and Sidak's multiple comparisons test were used for graph B. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .



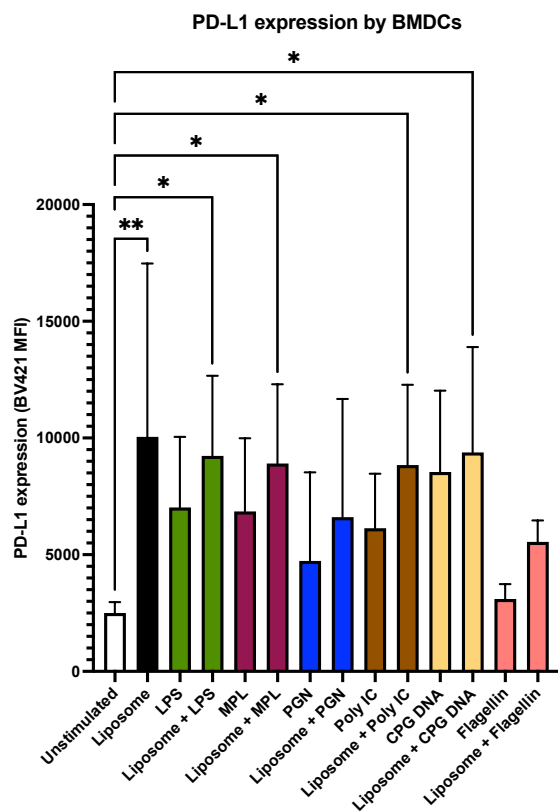
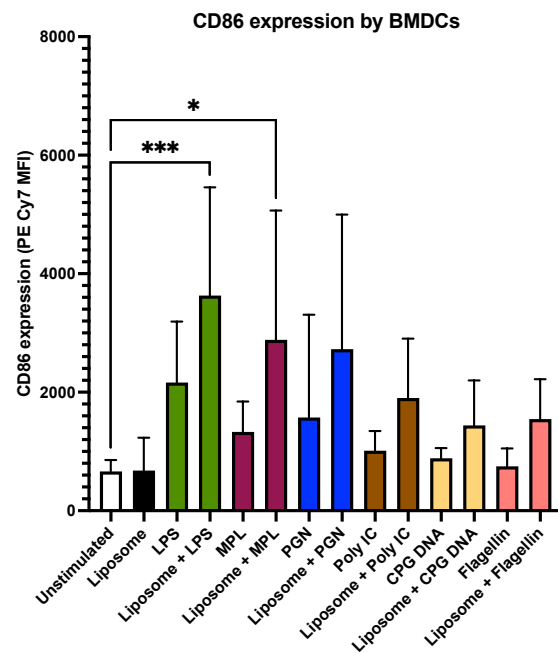
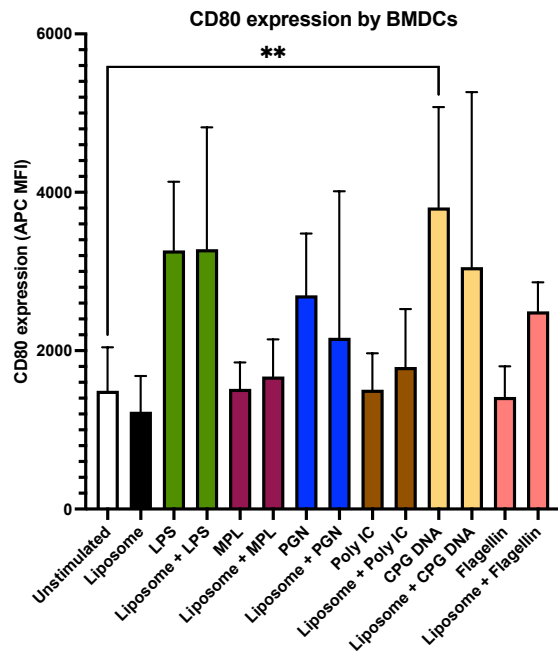


Figure 3.9.2. Effect of Liposomes and TLR adjuvants on the expression of B-7 molecules and PD-L1.

Duplicate wells of 1×10^5 BMDCs were stimulated with Liposome P/S at a ratio of 5:1 and TLR adjuvants for 48 hours. (n=3). Flow cytometry was used to measure the expression of CD80, CD86 and PD-L1. MFI values and standard deviations are shown. Statistical analysis was performed using One Way ANOVA and Dunnett's multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

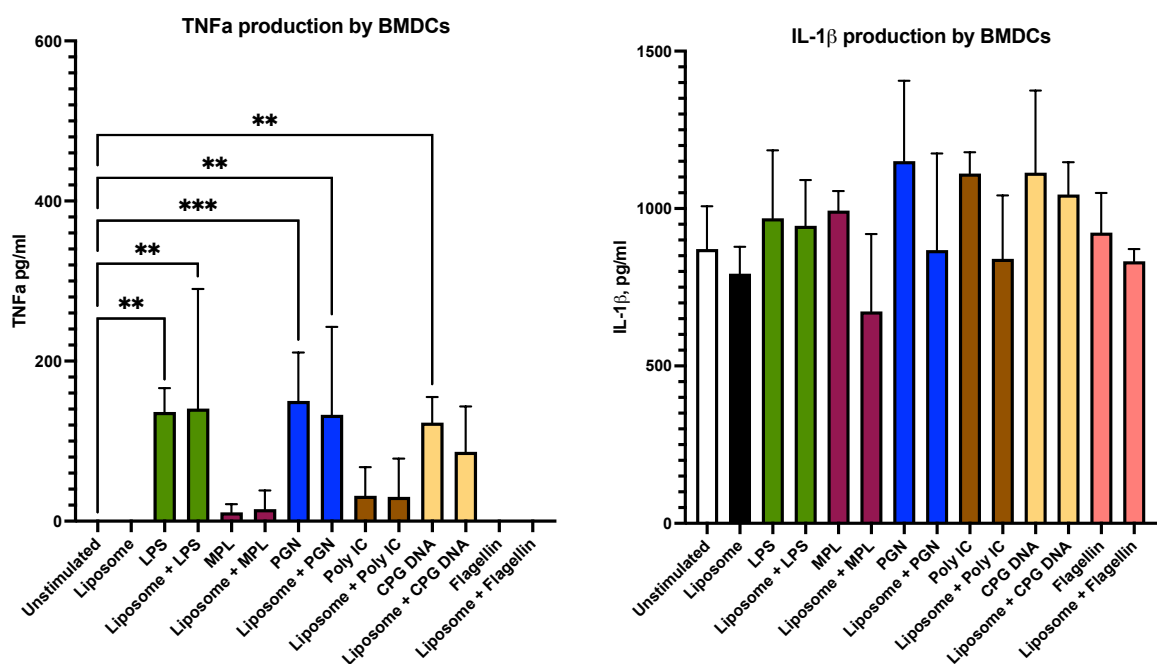


Figure 3.9.3. Effect of TLR agonists and Liposome P/S on TNFα and IL-1β production by BMDCs.

Duplicate wells of 1×10^5 BMDCs were stimulated with Liposome P/S at a ratio of 5:1 and TLR adjuvants for 48 hours ($n=3$). The concentration of TNFα and IL-1β in the supernatant was measured by ELISA. Mean concentration values and standard deviations are shown for TNFα in graph A. The mean concentration of IL-1β and the standard deviations are shown in graph B. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

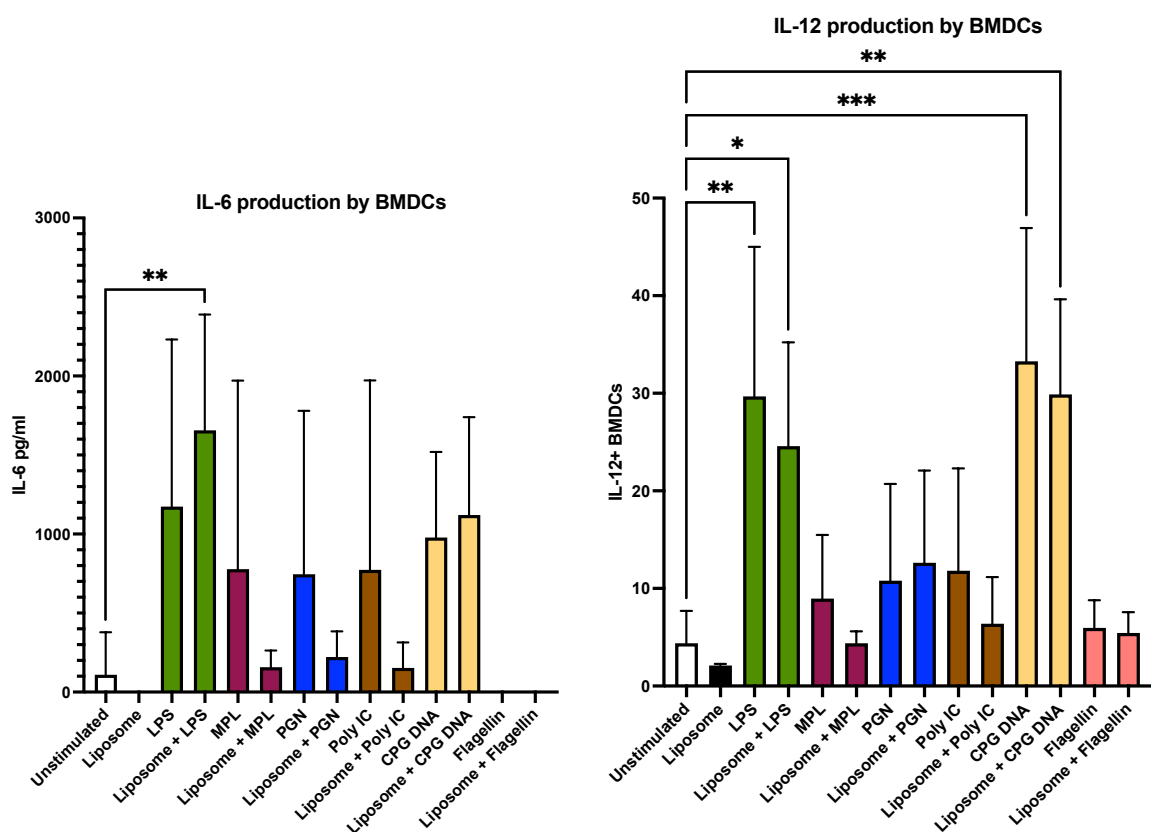


Figure 3.9.4. Effect of TLR agonists and Liposome P/S on IL-6 and IL-12 production by BMDCs.

Duplicate wells of 1×10^5 BMDCs were stimulated with Liposome P/S at a ratio of 5:1 and TLR adjuvants for 48 hours ($n=3$). IL-6 levels in supernatants were measured by ELISA. Mean concentration values and standard deviations are shown for IL-6 in graph A. Flow cytometry was used to measure intracellular IL-12 within BMDCs. The mean percentage of IL-12+ cells and the standard deviations are shown in graph B. Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

Liposomes and Flagellin

The graphs in figure 3.9.5 examine the effect of combining phosphatidylserine liposomes with flagellin. BMDCs stimulated with liposome and flagellin together showed significantly higher expression of CD40, CD80, CD86, MHC I, MHC II, and PD-L1 than cells stimulated with flagellin alone. Expression of CD86 was doubled, whilst MHC II and CD40 expression rose more than twofold. Increased expression of these markers is significant as it can be linked to the capacity of DCs to present antigen. This data reveals that liposome is capable of enhancing TLR5 signalling in DCs leading to strong activation of these cells. Flagellin may therefore be an appropriate adjuvant for liposome based vaccines. Interestingly, PD-L1 mean MFI values also rose, from 3,100 in flagellin stimulated cells to 5,552 MFI in cells stimulated with flagellin and liposomes. PD-L1 is an inhibitory marker which is upregulated as part of a negative feedback loop when cells are overstimulated. When flagellin was applied to DCs alone it had no impact on the expression of activation markers or cytokine production. Addition of liposomal phosphatidylserine allowed flagellin to activate these cells through TLR5 signalling. TLR5 signalling is regulated by the TIR domain containing adaptor inducing interferon β (TRIF). TRIF induces proteolytic degradation of TLR5 (Choi et al., 2010). It is plausible that phosphatidylserine binds to TRIF and alters its activity to permit TLR5 signalling.

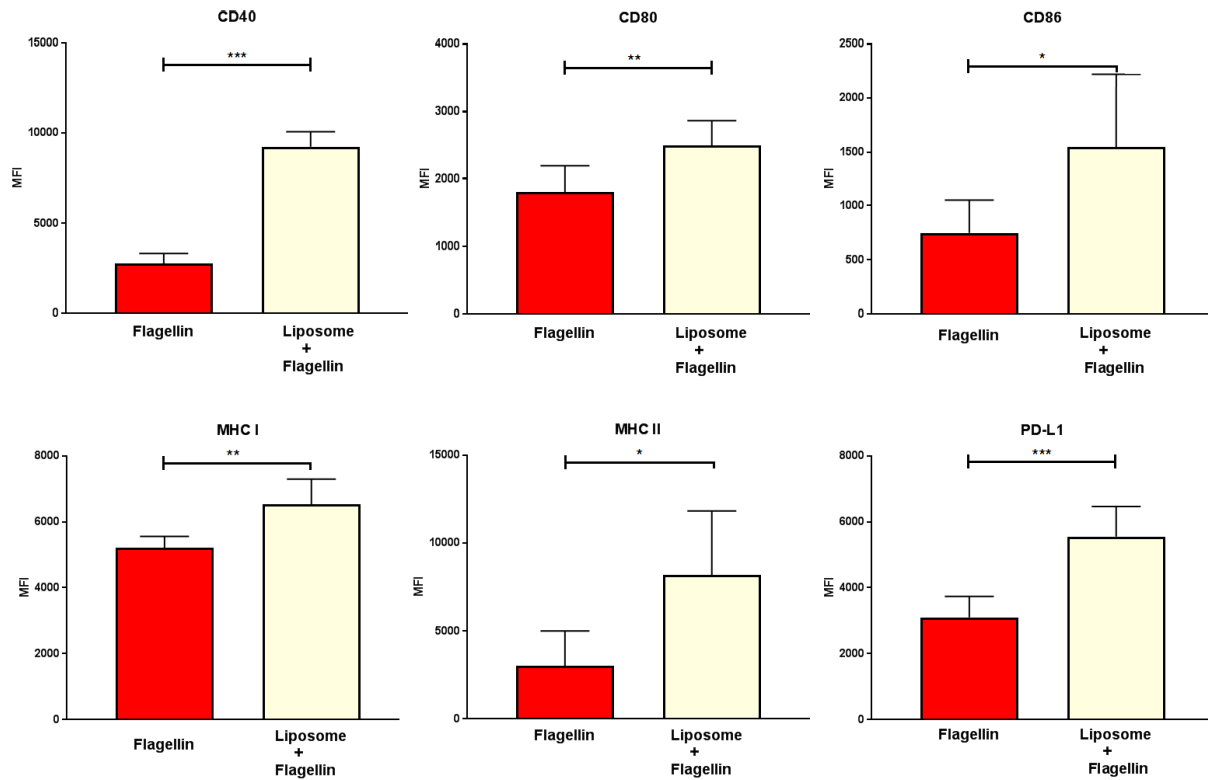


Figure 3.9.5. Immunogenicity of Liposome P/S and Flagellin. There were significant increases in cell surface marker expression in cells stimulated with both liposome and flagellin compared to flagellin alone. Expression of CD40, CD80, CD86, MHC I, MHC II and PD-L1 were all significantly increased following co-stimulation with Liposome P/S and flagellin. BMDCs were seeded at a density of 1×10^5 cells per well. Duplicate wells were stimulated with Liposome P/S at a ratio of 5:1 for 48 hours. Flow cytometry was used to determine the expression (MFI) of cell surface markers ($n=3$). Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using an unpaired T test.

Key Findings

- *B subtilis* spores combined with LPS or MPL upregulate expression of MHC I, MHC II, CD80, CD86 and PD-L1 on DCs as well as inducing production of IL-1 β , IL-6, IL-12 and TNFa.
- *B subtilis* spores combined with peptidoglycan upregulate expression of MHC I, CD80, CD86, PD-L1, IL-1 β , IL-6 and TNFa by DCs
- *B subtilis* spores combined with Poly I:C upregulate expression of MHC I, MHC II, CD86 and PD-L1 on DCs.
- *B subtilis* spores and CPG DNA in combination upregulate DC expression of MHC I, MHC II, CD80, CD86 and PD-L1. IL-6, IL-12 and TNFa production is also enhanced.
- *B subtilis* spores combined with flagellin upregulate DC expression of MHC I, CD86 and IL-6.
- YC NaMa can be combined with LPS and MPL to activate DCs, this results in upregulation of MHC I, MHC II, CD80, CD86 and PD-L1. IL-6, IL-12 and TNFa are also upregulated.
- YC NaMa combined with peptidoglycan upregulates MHC I, CD86, PD-L1 and TNFa expression by DCs
- YC NaMa combined with Poly I:C upregulates MHC I and MHC II expression on DCs. It also upregulates IL-6 production.
- YC NaMa combined with CPG DNA to activate DCs, upregulating MHC I, CD80 and CD86 expression. IL-1 β , IL-6, IL-12 and TNFa responses were also observed.
- Phosphatidylserine liposomes combined with LPS or MPL upregulated expression of MHC I, CD86 and PD-L1 on DCs. Production of IL-6, IL-12 and TNFa was also elevated.
- Liposomes combined with Poly I:C or CPG DNA upregulated expression of MHC I and PD-L1 on DCs, but had no effect on the production of proinflammatory cytokines.

3.10 Effect of Spore FP1 Poly I:C on DC induced T cell proliferation. (*in vitro*)

For further experimentation, the spore delivery system was combined with Poly I:C. This combination induced the expression of activation markers on DCs. It was important to determine whether this combination had other impacts on dendritic cells. A key function of dendritic cells is antigen presentation and the generation of T cell responses. This *in vitro* study examined the ability of DCs stimulated with spore FP1 Poly I:C to induce T cell proliferation. BMDCs were used in conjunction with naïve splenocytes to construct this *in vitro* model. DCs were pulsed with Spore FP1 Poly I:C, washed and then naïve splenocytes were co-cultured with the DCs. Proliferation of T cells was examined using flow cytometry to measure Ki67 in CD4+ and CD8+ T cells. The gating plan for this experiment is shown in supplemental figure 10.2. Statistical analysis was performed using one way ANOVA coupled with Dunnett's multiple comparisons test. The data is shown in figure 3.10.1.

Stimulation of DCs with Spore FP1 Poly I:C led to reductions in CD4+ and CD8+ T cell proliferation. The percentage of proliferating CD8+ T cells fell twofold in the cells co-cultured with DCs stimulated with Spore FP1 Poly I:C ($p=0.0332$). The percentage of proliferating CD4+ T cells also fell by around twofold proliferation ($p=0.0131$).

In contrast, DCs pulsed with FP1 combined with LPS did not inhibit proliferation of CD8+ T cells and indeed, they enhanced proliferation of CD4+ T cells. Stimulation with LPS and FP1 led to an increase in the percentage of Ki67+ CD4+ T cells from 45 % in unstimulated cells up to 61% ($p=0.0359$).

This indicated that the inhibition of T cell proliferation is not caused by the FP1 fusion protein, but instead by either spores, Poly I:C or the combination of both. This may have implications for the *in vivo* efficacy of spore FP1 Poly I:C as a candidate against tuberculosis. Further work was needed to establish whether Spore FP1 Poly I:C was a suitable vaccine formulation.

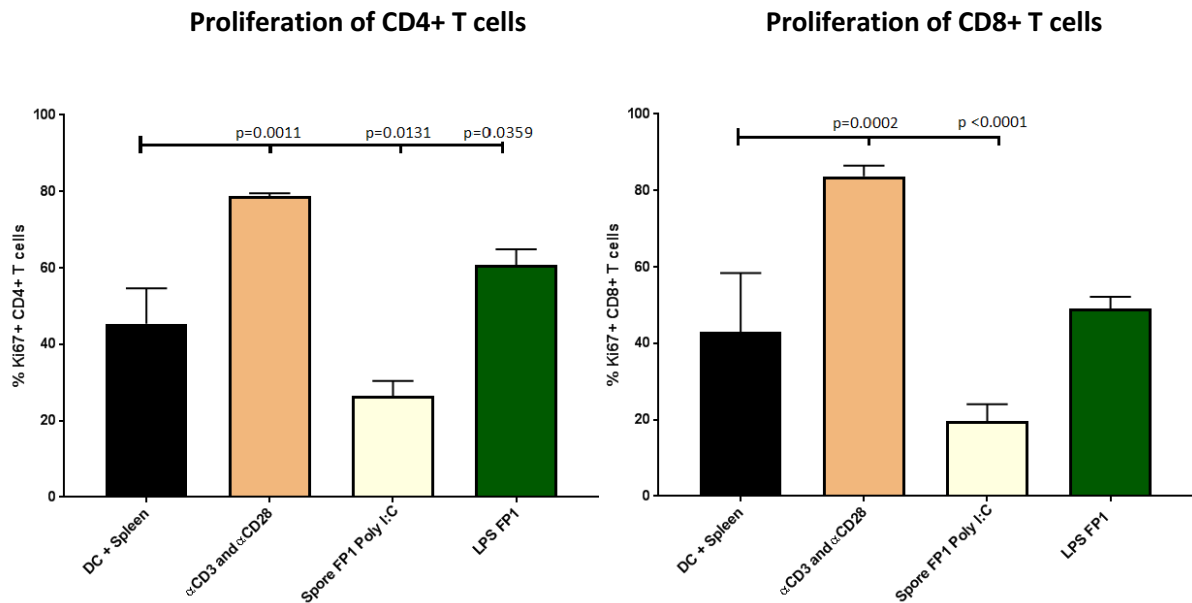


Figure 3.10.1. Effect of Spore FP1 Poly I:C on DC induced T cell proliferation. The percentage of proliferating T cells following stimulation with Spore FP1 Poly I:C is shown. DCs pulsed with Spore Poly I:C FP1 inhibited the proliferation of CD4+ and CD8+ T cells. LPS FP1 induced proliferation of CD4+ T cells but not CD8+ T cell proliferation. BMDCs were seeded at a density of 1×10^5 cells per well in a 96 well plate and stimulated for 24 hours with Spore FP1 Poly I:C. Naïve splenocytes were added to wells at a density of 1×10^6 cells per well, (n=3). After 5 days, cells were analysed using a FACS Canto flow cytometer. CD90+ T cells were gated for CD4 and CD8 and then the percentage of Ki67+ cells measured. Mean values and $\pm$ standard deviations are shown. Data was analysed using one way ANOVA and Dunnett's multiple comparison test.

3.11 *In Vivo* immunogenicity of Spore FP1 Poly I:C

Spore FP1 Poly I:C has been tested as a boost to BCG and shown to confer protection against MTB challenge in mice (Copland et al. 346). This study sought to determine whether spore FP1 Poly I:C was immunogenic without a prior BCG boost. In addition, this experiment tested an alternative delivery mechanism for Spore FP1 Poly I:C, syngeneic transfer of pulsed DCs. Syngeneic transfer of vaccine pulsed DCs has previously been used to enhance T cell responses against TB (Griffiths et al. 13894). In this experiment, DCs stimulated with Spore FP1 Poly I:C were administered to C57/BL6 mice along with Spore FP1 Poly I:C. DCs are known to represent a bottleneck in the development of vaccine responses against TB, combining spore FP1 Poly I:C with pulsed syngeneic DCs was intended to enhance the immunogenic effects of the formulation.

The *in vivo* effect of Spore FP1 Poly I:C on T cell proliferation and expression of IFN γ and IL-17 were measured. This study also attempted to determine whether these responses were antigen specific, using FP1 as a recall antigen. The effect of Spore FP1 Poly I:C on T cell proliferation is shown in Figure 3.11.1 A and B, IFN γ responses are shown in figure 3.11.1C whilst IL-17 responses are presented in figure 3.11.1D.

Animals in this study were divided into three groups. Group 1 received PBS alone, Group 2 received *B. subtilis* spores combined with the antigen FP1 and a Poly I:C adjuvant. Group 3 received Spore FP1 Poly I:C along with syngeneic transfer of DCs stimulated with Spore FP1 Poly I:C. Splenocytes were taken from the animals in each group and cultured for 24 hours in the presence of PBS or PBS. Proliferation of CD4 $^{+}$ and CD8 $^{+}$ T cells was measured by flow cytometry using Ki67 as a proliferation marker. Supernatants were collected from cells and levels of IFN γ and IL-17 were measured. The data was then analysed using one way ANOVA and Sidak's multiple comparisons test.

Spore FP1 with Poly I:C may have induced antigen specific IFN γ production by splenocytes. The concentration of IFN γ doubled in the presence of FP1 recall antigen. Supernatants from Group 2 splenocytes had an IFN γ concentration of 1,163 pg/ml. With FP1 recall antigen, this doubled to 2,761

pg/ml. This increase was not statistically significant. Spore FP1 Poly I:C did not have any detectable effect on IL-17 responses or the proliferation of CD4⁺ T cell or CD8⁺ T cells. This was true both with and without the addition of the recall antigen FP1.

Vaccinating mice with syngeneic DCs pulsed with Spore FP1 Poly I:C led to increased CD4⁺ T cell proliferation ($p < 0.0001$). Because this effect was seen both with and without FP1 antigen recall, it is not possible to conclude whether this was an antigen specific response. A similar pattern was seen in CD8⁺ T cells, with increased Ki67 MFI observed in CD8⁺ T cells from mice vaccinated with syngeneic pulsed DCs. Proliferating CD8⁺ T cells were seen with and without FP1 antigen recall ($p < 0.0001$).

Administering pulsed syngeneic DCs to mice induced IFN γ responses. Splenocytes from Group 3 animals generated significant IFN γ responses. Group 3 splenocytes treated with antigen recall FP1 produced a mean IFN γ concentration of 36 ng/ml. This was more than 10 fold higher than the concentration of IFN γ generated by Spore FP1 Poly I:C alone ($p = 0.0022$). Supernatants from the splenocytes were also tested for IL-17, these results are shown in figure 3.11.1D. Spore FP1 Poly I:C alone did not induce IL-17 production. Combining spore FP1 poly I:C with syngeneic pulsed DCs led to higher levels of IL-17. The concentration of IL-17 in the supernatant increased from 46pg/ml to 63pg/ml ($p = 0.0030$).

This experiment revealed that the spore FP1 Poly I:C vaccine was not suitably immunogenic in mice. It did not generate significant IFN γ responses, IL-17 responses or induce proliferation of T cells. When Spore FP1 Poly I:C was administered in combination with pulsed syngeneic DCs, it led to CD4⁺/CD8⁺ T cell proliferation and dramatic increases in the expression of IFN γ and a small but significant increase in IL-17 production by splenocytes. This indicates that the immune response to Spore FP1 Poly I:C can be improved with the addition of syngeneic pulsed DCs. Combining spore FP1 Poly I:C with syngeneic pulsed DCs is not a feasible vaccination strategy for humans. However, this study does demonstrate that it may be possible to improve the immunogenicity of this vaccine candidate by altering the formulation to enhance DC responses.

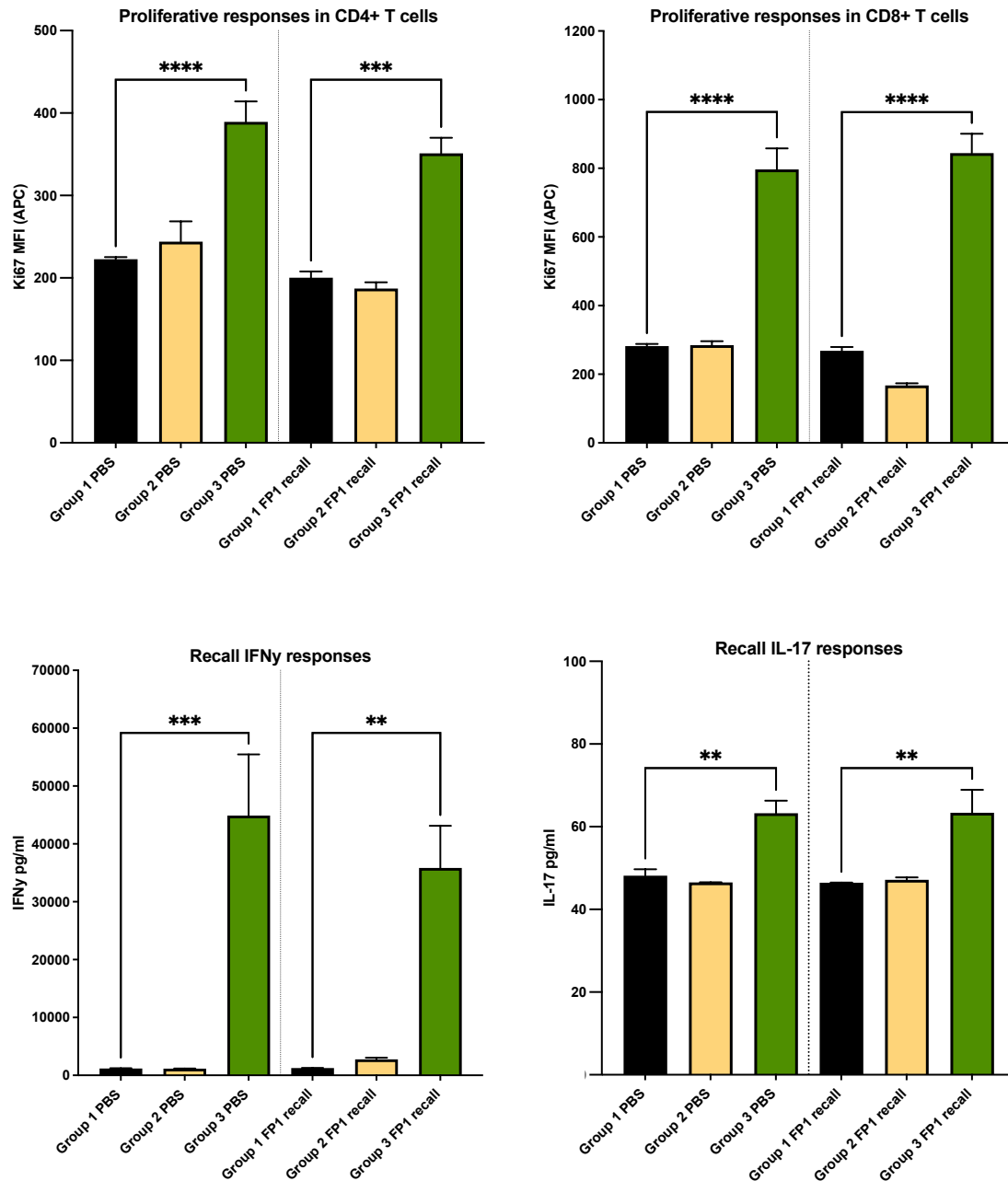


Figure 3.11.1. Recall responses to Spore Poly I:C (with syngeneic transfer). Mice in Group 1 received s.c. PBS. Animals in Group 2 were administered Spore FP1 Poly I:C s.c. in three doses. Mice in Group 3 received three s.c. doses of Spore FP1 Poly I:C and 1×10^5 syngeneic BMDCs which had been pulsed for 24 hrs with Spore FP1 Poly I:C ($n=3$). The doses were administered at two week intervals. Spleens were collected and splenocytes were treated with either $5 \mu\text{g/ml}$ FP1 antigen recall or PBS for 24 hours. Proliferation of CD4+ T cells (A) and CD8+ T cells was measured by flow cytometry. IFN γ (C) and IL-17 (D) concentrations were measured by ELISA. Mean values and $\pm$ standard deviations are shown. This result was analysed using One way ANOVA and Sidaks multiple comparisons test.

3.12 Effect of Spores and Poly I:C on PD-L1 expression by murine BMDCS

This study attempted to rationally design a TB vaccine which would activate DCs and induce strong immunogenic responses. The delivery system of *Bacillus subtilis* spores and the TLR 3 agonist Poly I:C, in combination, upregulated expression of activation markers on DCs and enhanced proinflammatory cytokine production. It was expected therefore that Spore FP1 Poly I:C would be a highly immunogenic formulation. Instead, it was found that this combination was poorly immunogenic in T cells. It is important to investigate why this vaccine candidate failed to activate T cell responses in our culture system.

Programmed Death Ligand 1 (PD-L1) is a transmembrane protein expressed by a wide range of cells. PD-L1 expression on DCs has a tolerogenic effect and suppresses the development of adaptive immune responses. It inhibits TCR mediated activation and proliferation of T cells. This study has shown that DCs stimulated with Spore FP1 Poly I:C were unable to induce T cell proliferation *in vitro* (figure 3.10.1). Figure 3.11.1A shows the same effect in mice; Spore FP1 Poly I:C did not induce T cell proliferation. Figure 3.11.1D showed that the vaccine did not induce IL-17 either. The experiment shown in figure 3.11.1 established that the *in vivo* immunogenicity of spore FP1 Poly I:C was enhanced by the addition of pulsed syngeneic DCs. This raised the possibility that the Spore FP1 Poly I:C by itself was not optimally activating DCs. To explore this possibility further, the results from figure 3.7.2 were re-examined.

Spore FP1 Poly I:C activates DCs, as previously discussed. However, it also induces the upregulation of PD-L1 on the surface of DCs. This is shown in Figure 3.12.1. Stimulation with spores alone or Poly I:C increased the expression level of PD-L1. Spores increased PD-L1 expression from 3,427 to 4,826 ($p=0.0038$). Poly I:C increased PD-L1 expression to 4,971 ($p=0.0011$).

Combined stimulation with spores and Poly I:C increased PD-L1 MFI from 3,427 to 6,438 ($p=0.0016$). This was significantly higher than the PD-L1 expression level seen with either spores or Poly I:C alone ($p=0.0062$) and ($p=0.0215$).

Combining spores with Poly I:C overstimulated the DCs, causing a negative feedback loop. Increased PD-L1 expression on DCs led to abrogated T cell responses, as PD-L1 blocks the activation of T cells. This explains why the DCs showed upregulation of activation markers following stimulation with Spore Poly I:C but were unable to activate T cells.

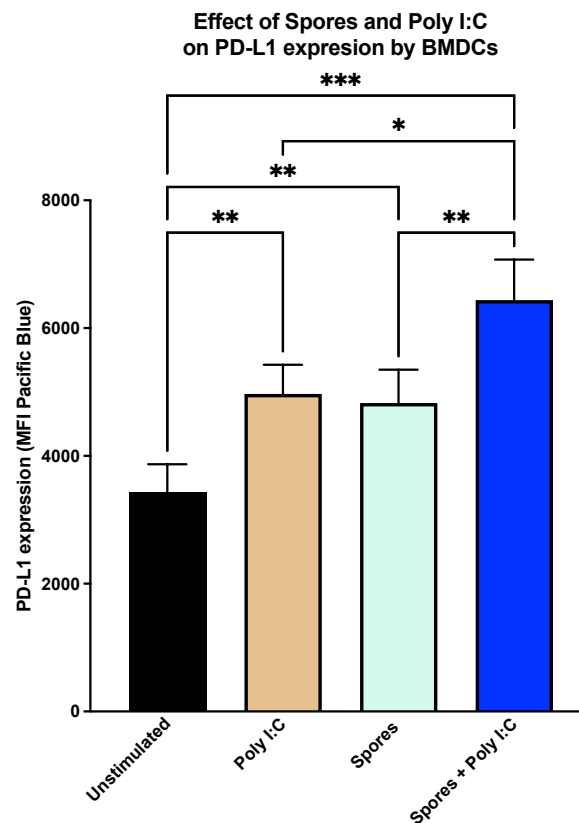


Figure 3.12.1. Effect of Spores and Poly I:C on PD-L1 expression by BMDCs. PD-L1 expression increased following stimulation with both Spores and Poly I:C compared to unstimulated cells. Triplicate wells with 1×10^5 BMDCs were stimulated with spores at an MOI of 5:1. Poly I:C was added at a concentration of $10 \mu\text{g/ml}$. Cells were incubated for 48 hours and then cells were processed and MFI values analysed by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Graphs representative of three repeated experiments ($n=3$). Data was analysed using one way ANOVA and Tukeys multiple comparisons test.

3.13 Effect of BCG infection on expression of PD-L1.

BCG vaccination is known to have variable efficacy against tuberculosis. BCG has previously been shown to upregulate PD-L1 expression on bladder cancer cells (Wang et al. 2891). The effect of BCG on the expression of PD-L1 by antigen presenting cells (APCs) was unknown. PD-L1 is a tolerogenic cell surface molecule, capable of abrogating T cell responses and limiting T cell proliferation. This experiment used flow cytometry to determine the effect of BCG infection on PD-L1 expression by J774 macrophages and murine BMDCs. Cells were infected with BCG at an MOI of 1 or stimulated with 100ng/ml LPS. Cells were also stimulated with filtered supernatant from J774 cells infected with BCG at an MOI of 1. This filtered supernatant did not contain BCG, but did contain soluble factors released during BCG infection. As a control, filtered supernatant from uninfected cells was added to separate wells.

The effect of BCG on the expression of PD-L1 by macrophages is shown in figure 3.13.1. PD-L1 expression in J774 cells, was significantly increased (nearly twofold) following stimulation with LPS, BCG at an MOI of 1 and the filtered supernatant from BCG infected cells. PD-L1 expression (measured by Pacific Blue MFI) increased from 11,251 to 20,278 following stimulation with LPS ($p=0.0017$). BCG infection increased PD-L1 expression from 11,251 to 18,567 ($p=0.0044$). Filtered supernatant from BCG infected cells increased PD-L1 expression from 11,251 to 20,961 ($p=0.0012$). Filtered supernatant from uninfected cells did not induce PD-L1 expression ($p=0.8812$).

The same effect was seen in BMDCs. LPS was able to raise the expression of PD-L1 on BMDCs. The Pacific Blue MFI rose from 1,974 to 4,998 ($p<0.0001$). BCG infection at an MOI of 1 increased PD-L1 expression. The MFI for Pacific Blue, rose from 1,974 in the unstimulated cells to 3,937 on the BCG infected cells ($p=0.0002$). Filtered supernatant from BCG infected cells was also able to induce raised PD-L1 expression. Pacific Blue MFI rose from 1,974 in unstimulated cells to 7,861 in cells treated with filtered supernatant from BCG infection ($p<0.0001$). Filtered supernatant from uninfected cells did not alter the expression of PD-L1, revealing that the upregulation of PD-L1 was a BCG induced effect.

These results show that BCG infection induces PD-L1 expression on macrophages and DCs by means of soluble factors, possibly cytokines. PD-1 is a tolerogenic receptor responsible for downregulating Induction of T cells. Upregulation of PD-L1 by BCG may limit the ability of APCs to generate effective T cell responses.

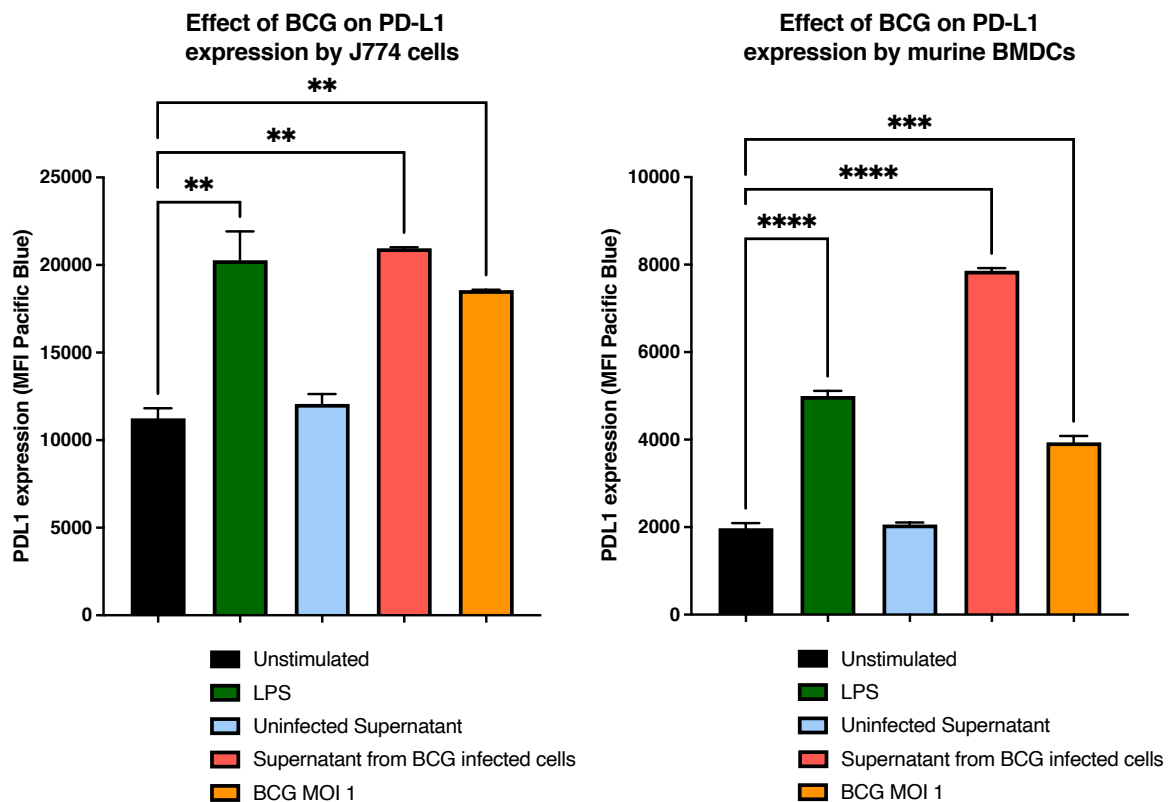


Figure 3.13.1. Effect of BCG infection on expression of PD-L1. J774 cells or BMDCs were seeded at a density of 1×10^6 cells in duplicate well. Cells infected with BCG at an MOI of 1 or stimulated with 100ng/ml LPS or stimulated with filtered supernatant from J774 cells infected or not infected with BCG at an MOI of 1. (n=3). Cells were stained with PDL1-Pacific Blue antibodies and flow cytometry was performed. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Results were analysed using one way ANOVA and Dunnett's multiple comparisons test.

3.14 Effect of BCG infection on expression of B7 family cell surface markers.

The previous experiment showed that PD-L1 is upregulated in macrophages following BCG infection. This experiment examined the ability of BCG to upregulate CD80, CD86, PD-L1 and PD-L2 on APCs. CD80 and CD86 are costimulatory markers and PD-L1 and PD-L2 are important tolerogenic molecules (Zhang, Y., Chung, Bishop, Daugherty, Chute, Holst, Kurahara, Lott, Sun, Welcher, and Dong 11695-11700).. Expression of these B-7 proteins is disparately controlled between macrophages and DCs. J774 cells were used as a model for macrophages, whilst murine BMDCs were used as a model for DCs. Figure 3.14.1 shows the effect of BCG on the expression of CD80, CD86, PD-L1 and PD-L2.

Macrophages and DCs were stimulated with LPS as a positive control. They demonstrated different patterns of upregulation of B-7 receptors following LPS treatment. Macrophages stimulated with LPS showed upregulation of CD86 and PD-L1, with 2.5 fold upregulation of both compared to 1.5 fold upregulation of CD80 and PD-L2. DCs stimulated with LPS showed a 3.5 fold increase in CD86 and PD-L2 expression and a 2.5 and 2 fold increase in CD80 and PD-L1.

Stimulation with BCG led to increased expression of PD-L1, PDL-2, CD80 and CD86 in both macrophages and DCs. At a MOI of 2, the expression of PD-L2 and CD80 increased 2 fold, whilst PD-L1 and CD86 increased by 3 and 2.5 fold. In macrophages, there was a clear dose dependent effect of BCG infection, with higher doses inducing higher expression of the four B-7 proteins. For example, CD86 increased 1.5 fold following infection with BCG at a MOI of 0.1. At an MOI of 1, CD86 increased 2 fold. Infection with BCG at an MOI of 2 increased CD86 by 2.5 fold compared to the unstimulated cells.

Infection of DCs with BCG at an MOI of 1 led to a 2.5 fold increase in CD86 expression and more than 2 fold increase in PD-L1 and PD-L2 expression. CD80 expression was increased 1.5 fold following BCG infection at a MOI of 1. PD-L1 and PD-L2 are both immunomodulatory receptors which limit antigen presentation and dendritic cell activation of T cells. This experiment has established that BCG upregulates expression of both of these tolerogenic markers on APCs.

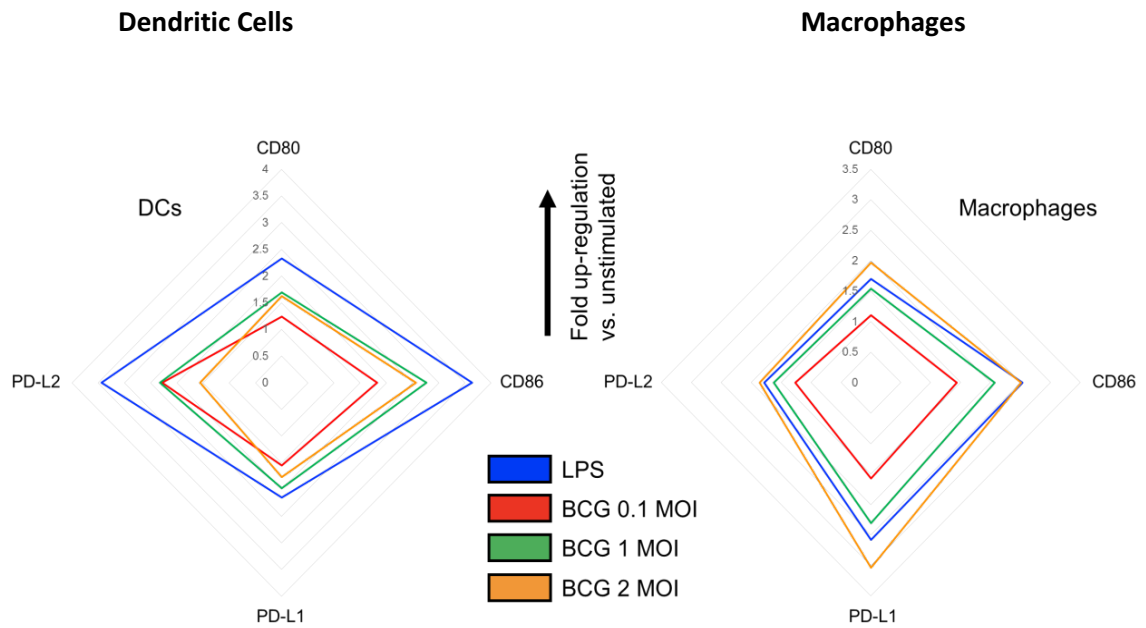


Figure 3.14.1. Effect of BCG infection on expression of B7 family proteins. Expression of CD80, CD86, PD-L1 and PD-L2 in macrophages and DCs following BCG infection is depicted as fold increases in MFI relative to the unstimulated control. Infection with BCG or stimulation with LPS led to increased expression of PD-L1, PDL-2, CD80 and CD86 in both macrophages and DCs. DCs or J774 macrophages were seeded at a density of 1×10^6 cells per well. Cells were infected with BCG at a MOI of 0.1, 1 or 2. LPS was used as a positive control at 100 ng/ml. Cells were cultured for 24 hours. Flow cytometry was used to determine expression levels of CD80, CD86, PD-L1 and PD-L2 expression (n=3). Data generated by A. Sparrow, Graph made by A. Copland.

Key Findings

- *Bacillus subtilis* spores elicit proinflammatory cytokine responses in DCs.
 - YC NaMa and Liposome delivery systems do not induce activation of DCs.
 - Spores can be combined with CPG DNA, MPL, LPS, flagellin or Poly I:C to enhance the activation of DCs.
 - YC NaMa can be combined with LPS, MPL, CPG DNA, peptidoglycan or Poly I:C to activate DCs.
 - Liposomes in combination with LPS, MPL, Poly I:C and CPG DNA induce the activation of DCs.
 - DCs pulsed with Spore FP1 Poly I:C could not induce proliferation in naïve T cells *in vitro*.
-
- Vaccinating mice with Spore FP1 Poly I:C did not generate T cell proliferative responses.
 - Vaccinating mice with Spore FP1 Poly I:C did not generate IL-17 responses.
 - Mice vaccinated with Spore FP1 Poly I:C did not generate significant antigen specific IFN γ responses in T cells.
 - Mice vaccinated with Spore FP1 Poly I:C combined with syngeneic pulsed DCs, generated antigen specific IFN γ responses and IL-17 in T cells. Proliferation of T cells was also observed.
-
- Spore FP1 Poly I:C overstimulates DCs leading to upregulation of PD-L1 expression.
 - BCG upregulates the expression of PD-L1, PD-L2, CD80 and CD86 on macrophages and DCs.

Chapter 4 - Characterisation of tonsil mononuclear cell cultures

Abstract

The BMDC model can be used to generate useful insights into vaccine immunogenicity. Further vaccine development still requires the use of *in vivo* animal models. This study sought to develop *in vitro* models using human tonsils to replace the need for animal experimentation. There are several advantages to using tonsillar mononuclear cells (TMCs). They are a readily available source of primary human tissue. TMCs are composed of a heterogeneous mixture of key immune cell populations, principally, B cells, CD4+ T cells, CD8+ T cells and monocytes. The TMCs used in this study contained 60 % B cells, 15 % T cells and 2 % monocytes. Of the T lymphocytes, 60% were CD4+ whilst 30% were CD8+ cells. Around 20% of CD4+ T cells had a naïve phenotype, expressing CD45ra. The percentage of CD45ra+ naïve CD4+ T cells increased as the cells were cultured *in vitro*. It was found that T lymphocyte cells developed a CD4 and CD8 double positive phenotype over the span of 12 days. This is a novel phenomenon which bears further investigation.

The TMC cultures were revealed to have a high viability. They can be grown for at least 12 days with no reduction in viability. The TMCs were able to respond to cytokine stimulation and proliferate. Work was carried out to identify suitable stimulants capable of activating T cells and B cells and inducing cytokine production. PMA / Ionomycin is capable of polyclonally activating tonsillar T cells and B cells and was identified as a positive control for TB immunogenicity experiments. In anticipation of working on the development of TB vaccines, the TMCs were screened for PPD reactivity. 11% of patients showed PPD reactivity – broadly in line with the known incidence of latent TB.

Preliminary work was also undertaken to establish a COVID 19 infection model using TMCs. It was found, however, that TMCs are unsuitable for this purpose as they do not express the ACE2 receptor which mediates cell entry. This characterisation of TMCs provided a basis upon which vaccine immunogenicity models and infection models could then be established.

Aims and objectives

Phenotype of tonsil mononuclear cells

Measure the percentage of Myeloid, Monocyte, CD4+ and CD8+ T cells and B cells within TMCs.

Long term viability of TMC cultures

Determine the viability of TMCs over 12 days of culture and measure the percentage of Myeloid, Monocyte, CD4+ and CD8+ T cells and B cells over 12 days of culture.

Effect of cytokines on long term viability of TMCs.

Measure the effect of various cytokines on the long term viability of tonsil cells.

Grow TMCs with THP1 conditioned media, IL-2, IL-21 and BAFF.

Ability of TMCs to respond to immunogenic stimulation

Stimulate cells with PMA Ionomycin, PHA, Resiquimod and Con A to determine whether tonsillar B and T cells are able to respond to known immunogens.

Identification of memory and naïve T cells.

Measure CD45ra expression on CD4+ T cells and CD8+ T cells to determine the proportions of naïve cells and effector cells

Mycobacterial antigen recall

Stimulate TMCs with PPD to identify patients with prior TB exposure or LTBI

Suitability of the tonsil model for a COVID 19 infection model

Measure ACE 2 expression on T cells, B cells, Monocytes and Myeloid cells to establish whether TMCs can be used to develop an in vitro infection model for SARS COV 2.

4.1 Developing *in vitro* models using tonsil mononuclear cells (TMCs)

This project aims to test vaccine immunogenicity using an *in vitro* model based on tonsil tissue. Tonsils are formed of mucosal and lymphoid tissue, containing a high proportion of lymphocytes. This study seeks to establish whether these TMCs can be used as the basis for a range of novel *in vitro* models. In this study, Dengue Fever and Tuberculosis have been selected as model pathogens because protection against these diseases requires different immune responses. The relevance and robustness of the TMC *in vitro* model can be established by developing immunogenicity models for both of these diseases.

4.2 Phenotype of TMCs

The viability of the TMCs was examined. It was not known whether the tonsil tissue would be healthy enough to be useful for the development of immunogenicity models. Figure 4.2.1 shows that the TMCs had a high viability of around 80%. This was adequate for further cell culture and experimentation. The subsets of immune cells within the TMCs were then examined. The percentage of cells which were CD4+ T cells, CD8+ Cells, CD19 B cells was determined. The results are shown in figure 4.2.2. Figure 4.2.3 shows the percentage of CD11b+ Myeloid cells and CD14+ monocytes present in the TMC cultures. TMCs from 16 different patients were used. The phenotype of the TMCs was determined by flow cytometry. TMCs contain approximately 15% T lymphocytes, 60% B lymphocytes, 2% myeloid cells and 1% monocytes. The Myeloid cells express CD11b and contain a mixture of macrophages, dendritic cells, NK cells and granulocytes. Figure 4.2.4C shows that CD14+ monocytes constitute 0.2% of cells in adult TMCs and 1.2% of the TMCs from children. This data suggests that tonsillar monocytes are around 6 fold more abundant in children than in adults. Monocytes are important antigen presenting cells. The greater abundance of monocytes in childrens' TMCs may make them more suitable for vaccine immunogenicity studies.

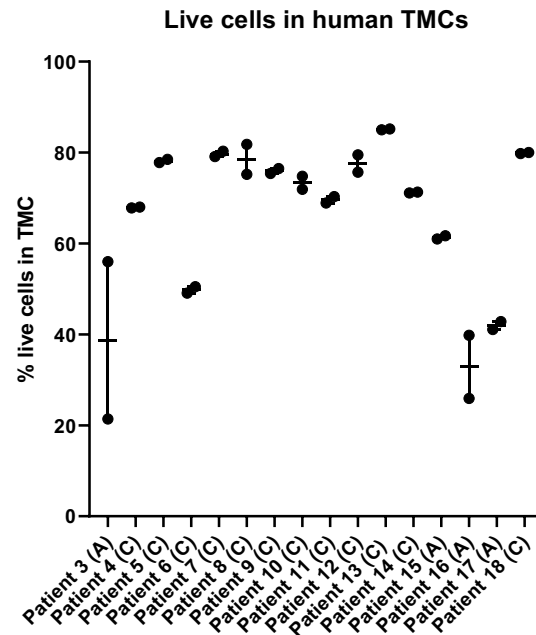


Figure 4.2.1. Phenotype of tonsillar mononuclear cells (TMCs) - Viability. Cells in TMC cultures were around 80% viable at the start of culture. TMCs from 16 patients' tonsils were stained using viability dye and analysed by flow cytometry. FMOs were used to gate positive cells and determine the percentage of each sub population (n=2). Mean values and $\pm$ standard deviations are shown. Tonsils from adults are labelled (A), tonsils from children are marked (C).

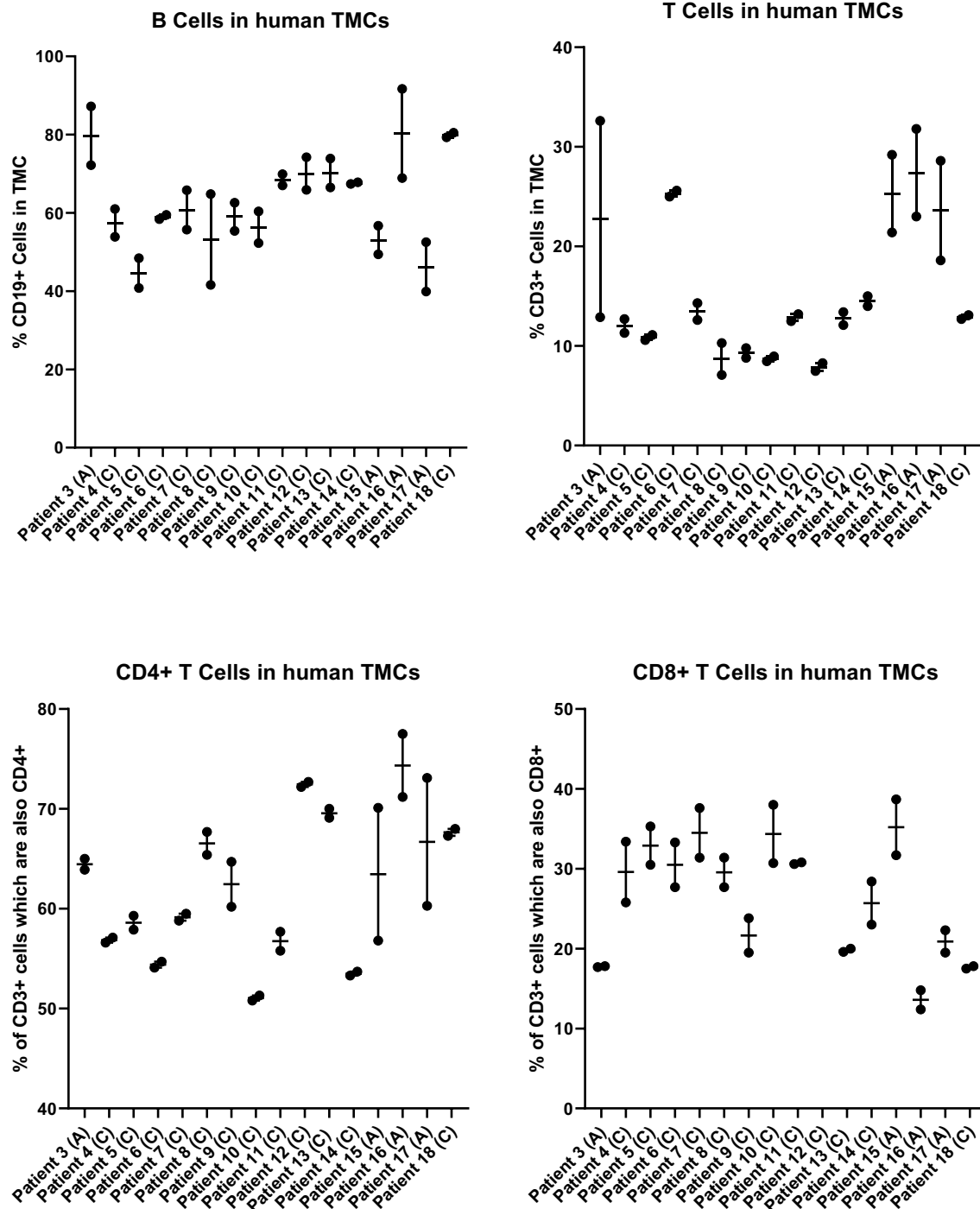


Figure 4.2.2. Phenotype of tonsillar mononuclear cells (TMCs) – Lymphocytes. The majority of cells in TMC cultures, around 60%, were CD19+ B cells (B). Approximately 20% of cells were CD3+ T cells (C). Of these CD3+ T cells, 60% were CD4+ T helper cells and 30% were CD8+ cytotoxic T cells (D). TMCs from 16 patients were stained for CD3, CD4, CD8 and CD19 and analysed by flow cytometry. FMOs were used to gate positive cells and determine the percentage of each sub population.

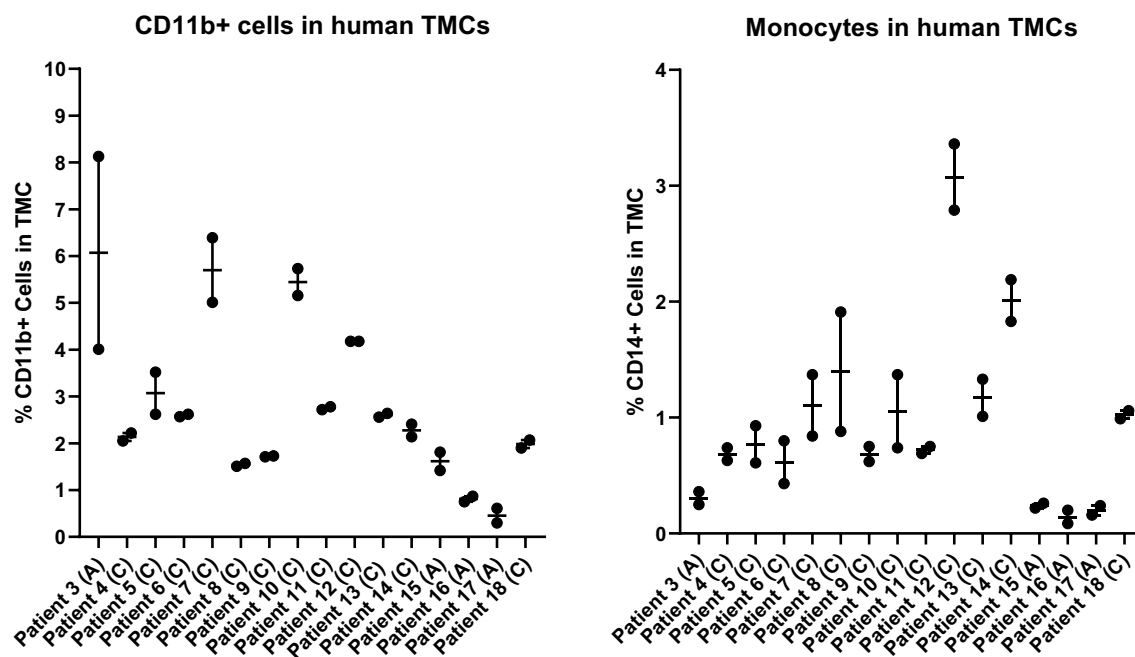


Figure 4.2.3. Phenotype of tonsillar mononuclear cells (TMCs) – Myeloid cells and Monocytes. 2% of TMCs were myeloid cells expressing CD11b and 1% of TMCs were monocytes expressing CD14. TMCs from 16 patients' tonsils were stained for CD11b and CD14 and then analysed by flow cytometry. FMOs were used to gate positive cells and determine the percentage of each sub population. Mean values and $\pm$ standard deviations are shown (n=2).

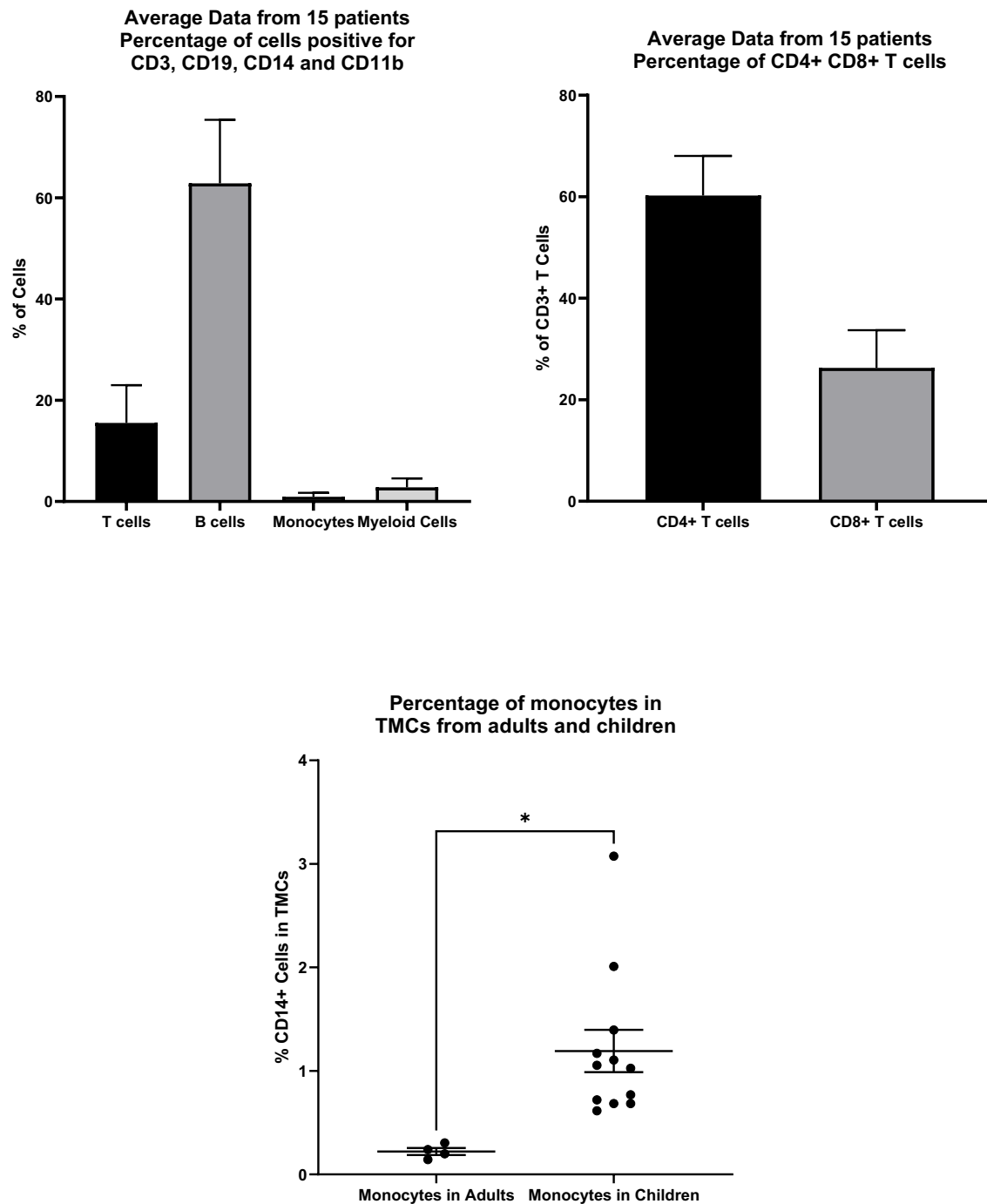


Figure 4.2.4. Average TMC phenotype and the percentage of Monocytes in TMCS from adults and children. Graphs A and B show flow cytometry phenotype data from 15 patients. Mean values are shown. Graph C shows average data from 12 children's tonsils and 4 adult's tonsils (n=2). Mean values and $\pm$ standard deviations are shown. Statistical analysis was performed using an unpaired T test.

4.3 Viability of long term cultures

To investigate immune responses in TMCs, it is necessary to maintain long term cultures. This study intends to model antigen specific responses to novel vaccine candidates. Antigen specific responses take weeks to develop and the cells must stay alive during that time. The effect of long term culture on TMCs is shown here. Populations of B cells, T cells, myeloid cells and monocytes within TMCs were examined over a 12 day period.

TMCs from six patients were used for this study. The cells were cultured in 96 well plates. Wells were seeded with 2×10^5 cells in 200ul of RPMI media. The media was replenished, 100ul removed and replaced, on Day 5 and Day 10. Samples were taken at the start of the experiment and on days 6, 9 and 12. The cells were stained for CD3, CD4, CD8, CD11b, CD14, CD19 and CD45ra. The percentage of immune cell populations and their viability was measured by flow cytometry.

This study demonstrated that using this protocol tonsillar mononuclear cells can be cultured for 12 days with no loss of viability. This is presented in figure 4.3.1. The TMCs were 55% viable at baseline. Their viability increased to 72% after 12 days ($p < 0.0001$). This was due to proliferation of the tonsil cells, leading to more viable cells being present. A 12 day time point was selected for this study as previous studies have detected the generation of antibody responses within this timeframe (Wagar et al., 2021b). The fact that this study showed high viability of TMC cultures after 12 days indicates that longer incubation periods may be possible with this culture technique. Follicular dendritic cells from tonsil cultures have been maintained for 150 days (Tsunoda et al., 1990).

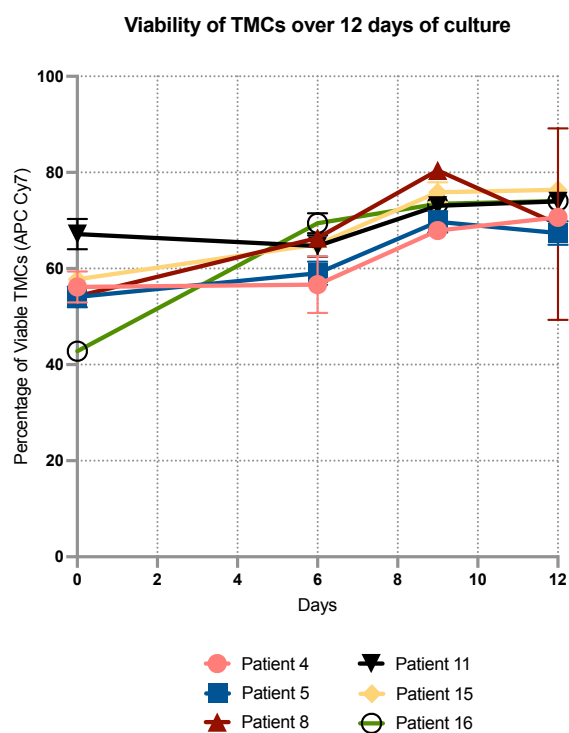


Figure 4.3.1. Viability of TMCs. The effect of long term culture on TMC viability is shown. Cell viability did not diminish over 12 days. TMCs from 6 randomly selected patients were cultured over a period of 12 days with regular replenishment of media. Samples taken at baseline and day 6, day 9 and day 12 were stained with Efluor 780 viability dye. The percentage of viable cells was determined by flow cytometry. Mean values and $\pm$ standard deviations are shown (n=3).

The phenotype of CD4+ T cells altered during the 12 days of *in vitro* culture. The CD4+ population expressed CD8+ and developed a CD4+ CD8+ double positive phenotype. Figure 4.3.4 shows the percentage of CD4+ T cells diminishing. The population of CD4+ T cells were not dying, instead they were expressing both CD4 and CD8 at the same time. Figure 4.3.5 shows that the percentage of CD4+ CD8+ double positive T cells increased. It is possible that CD4+ T cells were reverting to a naïve phenotype in the absence of stimulation. Figure 4.3.6 shows the expression of CD45ra on CD4+ T cells. On CD4+ T cells, CD45Ra and CD45R0 are immunophenotype markers for naïve and memory cells respectively. 20% of CD4+ T cells were naïve cells expressing CD45Ra. After 12 days of culture, this rose, with 29% of CD4+ T cells expressing CD45ra ($p=0.0105$). This confirmed that the number of CD4+ T cells exhibiting a naïve phenotype had increased.

It was notable that the CD8+ T cells in the culture changed their phenotype over the course of the 12 days. Figure 4.3.3 shows the percentage of CD8+ T cells decreasing over 12 days. The CD8+ T cells co-expressed both CD4 and CD8. After 12 days these double positive cells represented around 80.2% of all T cells. This can be seen in figure 4.3.5. CD4+ CD8+ double positive cells are rare and poorly understood (Overgaard et al., 2015). These tonsil cultures present a good model to examine these rare cells in more detail. The development of double positive CD4+ CD8+ T cells was consistent in all six of the TMC samples shown in figure 4.3.5. The same phenomenon was regularly observed in further work with the TMCs. Figure 4.3.6 shows that at baseline, 66% of CD8+ T cells were CD45ra. This is consistent with an effector CD8+ phenotype. After 12 days of culture, this fell to 42% ($p<0.0001$). This demonstrated that the percentage of effector CD8+ T cells was falling.

Differentiated CD4+ T cells have previously been observed to express the CD8 alpha chain (Mucida, D. et al 2013). The same effect is seen in mature CD8+ T cells, which express CD4 during *in vitro* activation (Kitchen, S.G. et al 2002). Peripheral double positive T cells have been described in numerous disease states (Overgaard N.H. et al 2014). Further work will be needed to understand the significance of this phenomenon in tonsil *in vitro* cultures.

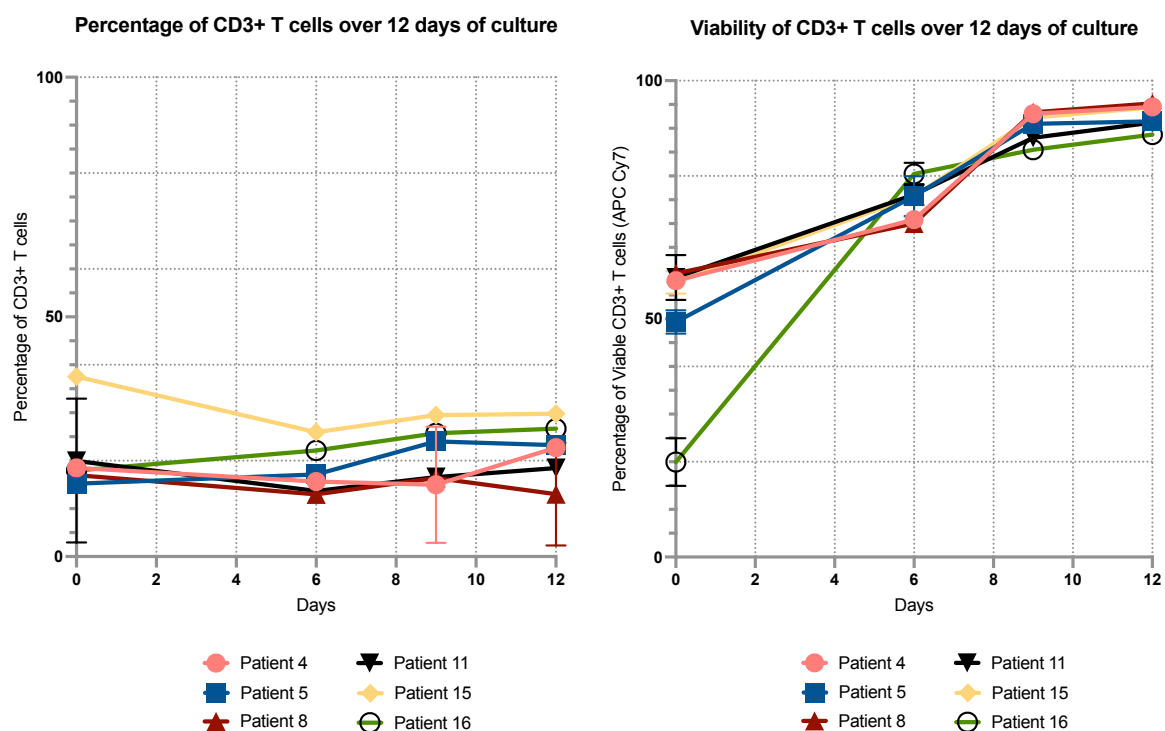


Figure 4.3.2. Percentage and viability of CD3+ T cells. The percentage of CD3+ T cells did not significantly alter of the course of 12 days (A). The viability of the CD3+ T cells increased from around 50% at the baseline to 90% viable after 12 days of culture. TMCs from 6 patients were cultured over a period of 12 days with regular replenishment of media. Samples taken at baseline and day 6, day 9 and day 12 were stained and measured by flow cytometry. Mean values and $\pm$ standard deviations are shown (n=3).

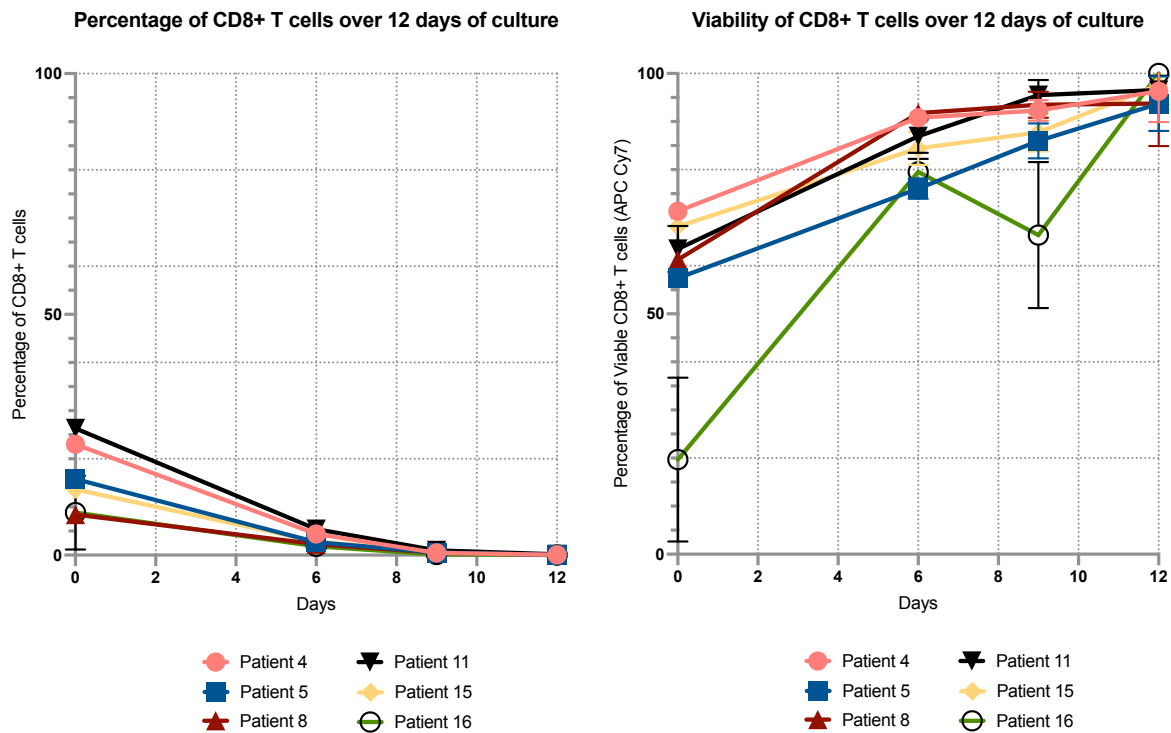


Figure 4.3.3. Percentage and viability of CD8+ T cells. The CD8+ T cell populations changed dramatically over 12 days. Approximately 20% of CD3+ T cells were CD8+ cytotoxic T cells at day 0. After 12 days of culture the population of CD8+ T cells had effectively disappeared. TMCs from 6 patients were cultured over a period of 12 days with regular replenishment of media. Samples taken at baseline and day 6, day 9 and day 12 were stained and measured by flow cytometry. Mean values and $\pm$ standard deviations are shown (n=3).

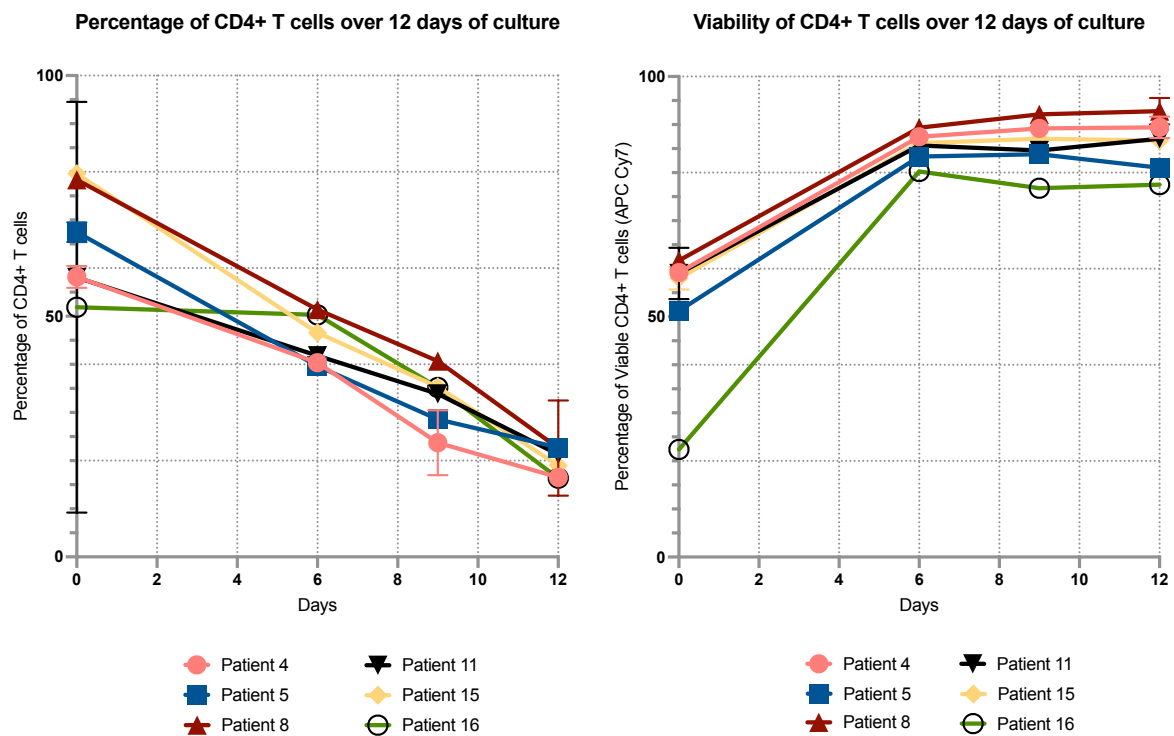


Figure 4.3.4. Percentage and viability of CD4+ T cells. TMCs from 6 patients were cultured for 12 days. Samples taken at day 0, day 6, day 9 and day 12 were stained and measured by flow cytometry. The percentage of CD3+ T cells which are CD4+ T cells is shown along with the viability of those cells. Mean values and $\pm$ standard deviations are shown ($n=3$). CD4+ T cells represent around 70% of CD3+ T cells at baseline. This falls to 20% after 12 days of culture. The viability of these cells increased from 60% to 90% viable following in vitro culture.

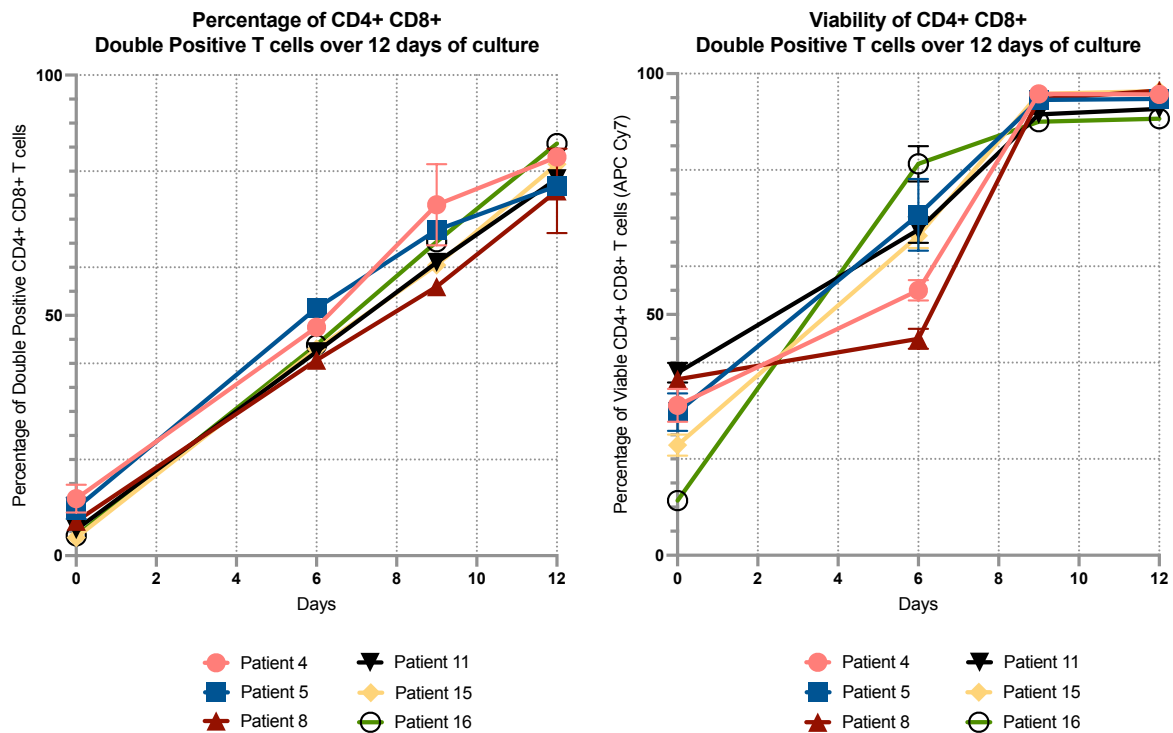


Figure 4.3.5 Percentage and viability of CD4+ CD8+ Double Positive cells. Tonsillar T lymphocytes were identified using flow cytometry. Cells were stained with anti CD3, CD4 and CD8 antibodies. Mean values and $\pm$ standard deviations are shown (n=3). At baseline, CD3+ T cells were either CD4+ or CD8+, in distinct populations. After *in vitro* culture for 12 days, the lymphocytes developed a CD4+ CD8+ double positive phenotype. These cells were approximately 90% viable and formed 80% of the T cell population.

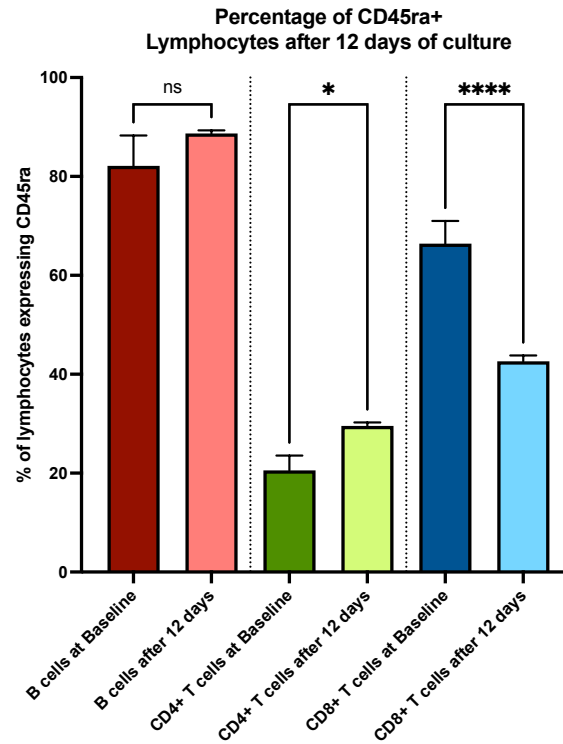


Figure 4.3.6. The expression of CD45ra by tonsillar lymphocytes. The expression of CD45ra by tonsillar lymphocytes was measured at baseline and after 12 days in vitro culture. TMCs were stained with anti CD45ra antibody. CD4+ T cells, CD8+ T cells and CD19+ B cells were identified and the percentage of CD45ra+ cells was measured by flow cytometry. The percentage of CD45ra+ CD4+ T cells is shown in A. The percentage of CD45ra+ CD8+ T cells is shown in B. The percentage of CD45ra+ CD19+ B cells is shown in C. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . (n=3). Statistical analysis was performed using One way Anova and Sidaks multiple comparison test.

The percentage of CD19+ B cells was 46% at baseline in the TMC cultures. After 12 days, this rose to 60.7% ($p<0.001$). B cell viability also rose over this timeframe, from 80% to 91% ($p=0.0005$). These results are shown in figure 4.3.7. These culture conditions were suitable for the long term maintenance of B cell populations. It has previously been documented that a high percentage of tonsillar B cells express CD45Ra (Morikawa et al., 1991). This matched our finding that more than 80% of tonsillar B cells were CD45Ra positive (figure 4.3.6).

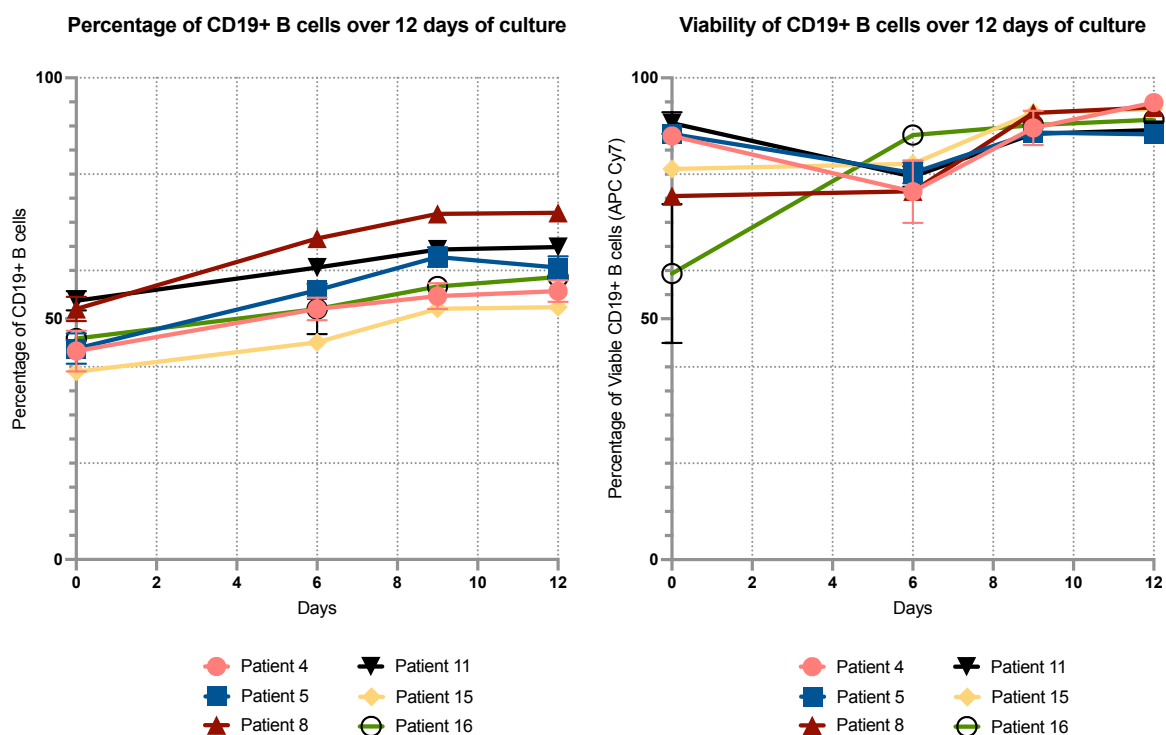


Figure 4.3.7. Percentage and viability of B cells. TMCs from 6 patients were cultured for 12 days. Flow cytometry was used to measure viability and CD19 expression. Samples were assessed on Day 0, Day 6, Day 9 and Day 12. The percentage of TMCs which expressed CD19 over 12 days of culture is shown in graph A. The viability of these cells is shown in graph B. Mean values and $\pm$ standard deviations are shown ($n=3$). The percentage of CD19+ B cells was around 60% at baseline and did not alter significantly over the course of the culture. The viability of these cells remained high, with 90% viable after 12 days.

CD11b+ Myeloid cells and CD14+ Monocytes are present in low numbers in tonsil cultures. The percentage of monocytes within the culture remained broadly unchanged over 12 days of culture. Figure 4.3.8 shows that over 12 days of culture, the viability of the monocytes increased from 28.8% to 80.67% viable ($p < 0.0001$). In figure 4.3.9, The percentage of CD11b+ Myeloid cells increased from 2.995% to 7.068% ($p < 0.0001$). Over the same timespan the viability of the myeloid cells also increased, from 55% to 86.98% ($p < 0.0001$). These results reveal that there is a healthy population of APCs within the TMC culture.

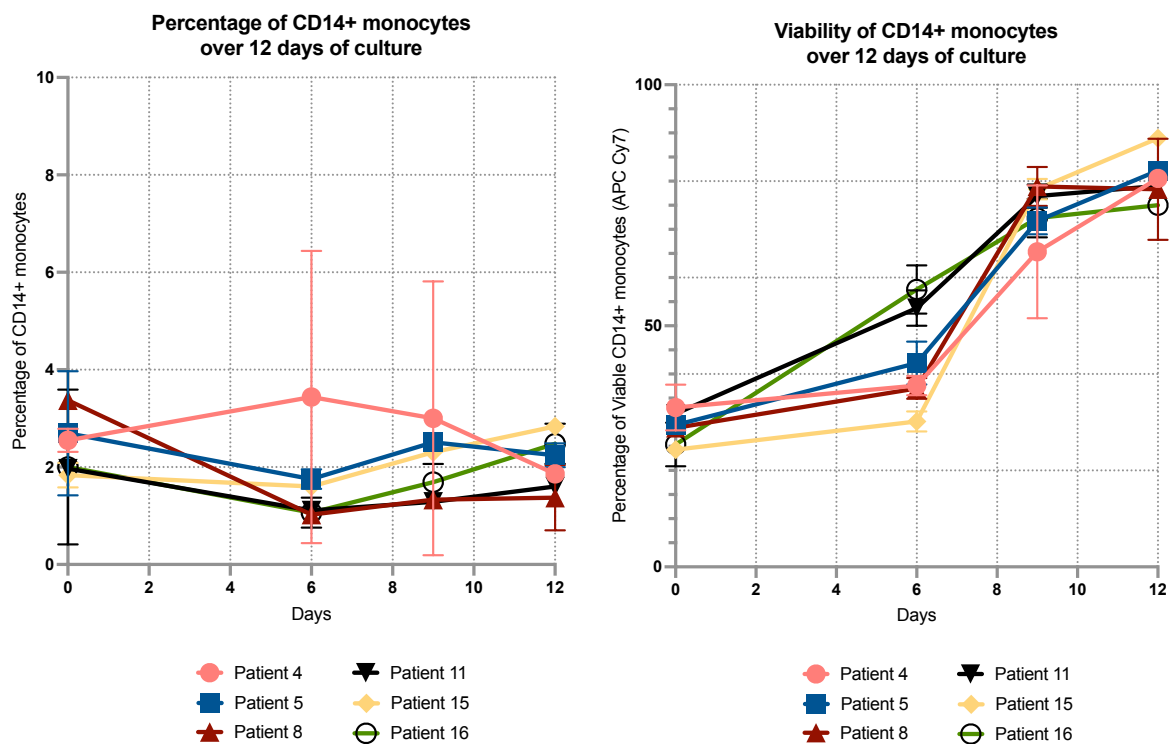


Figure 4.3.8. Percentage and viability of monocytes. TMCs from 6 patients were cultured for 12 days. Flow cytometry was used to measure cell viability and expression of CD14. The percentage of CD14+ monocytes in the TMC culture is shown in graph A. Mean values and $\pm$ standard deviations are shown ($n=3$). This remained constant at 2% of total cells. The viability of these cells was measured and is shown in graph B. Monocyte viability increased over the 12 days from 30% viable to 80%.

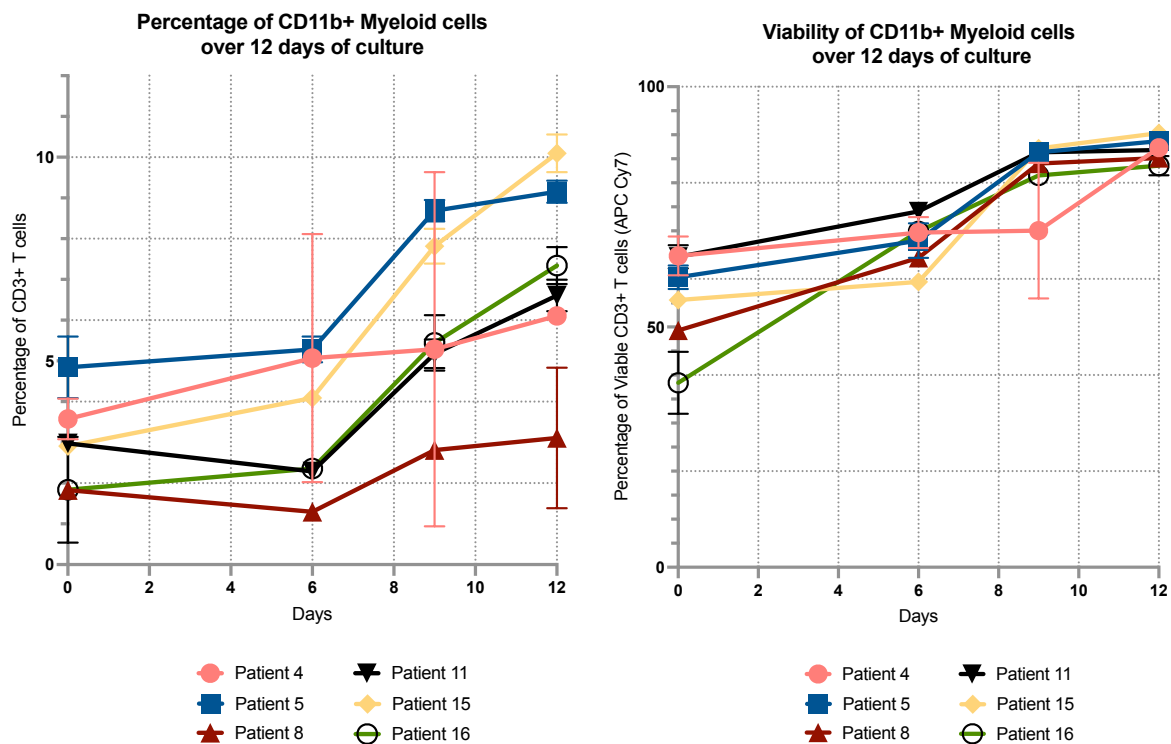


Figure 4.3.9. Percentage and viability of CD11b myeloid cells. TMCs from 6 randomly selected patients were cultured for 12 days. Flow cytometry was used to measure cell viability and identify myeloid cells expressing CD11b. Mean values and $\pm$ standard deviations are shown (n=3). The percentage of CD11b+ myeloid cells increased from 3% of the total cells to 6% over 12 days of in vitro culture (A). The percentage of viable myeloid cells increased from 60% on Day 0 to 80% on Day 12 (B).

These results show that TMCs can be cultured in vitro for long periods without any loss of viability. The TMCs contain a heterogenous mixture of T cells, B cells and APCs. These characteristics make TMCS suitable for the development of immunogenicity models for novel vaccines. The next step is to demonstrate that the cells respond appropriately to known stimuli. This is particularly important as the TMCs have been excised from inflamed lymph nodes, so it is unclear whether they will react to further stimulation.

4.4 Effect of cytokines on long term culture of tonsillar cells

The development of humoral immunity requires appropriate responses to cytokines and proliferation of daughter B cells. This experiment was performed to demonstrate that TMCs are capable of responding to cytokine stimulation. An additional aim is to find *in vitro* culture media which supports the proliferation of B cells and the development of B cell responses.

This study examined the effect of 3 cytokines, IL-2, IL-21 and B cell activating follicular factor (BAFF), which are known to be important for the proliferation of B cells (Ettinger et al., 2007) (Karnell & Ettinger, 2012). These cytokines were added to TMC cultures to examine their effect on the long term survival of the tonsil cells.

IL-2 is a proliferative cytokine, required for the proliferation of B cells (Mingari et al., 1984). It induces the differentiation of activated B cells into plasma cells (Le Gallou et al., 2012) (Hipp et al., 2017). IL-2 also combines with BAFF to enhance the viability and survival of B cells (Gui et al., 2016).

IL-21 is a type I cytokine produced by activated T cells. IL-21 elicits STAT3 signalling in resting human B cells (Habib et al., 2002)(Diehl et al., 2008) It is required for B cell activation following B cell/ T cell interaction (Kuchen et al., 2007) (Diehl et al., 2008). IL-21 plays an important role in generating B cell activation, expansion and the development of plasma cells (Avery et al., 2010). IL-21 has different effects on B cells depending on whether CD40L signalling is present. BCR signalling and IL-21, In the absence of CD40L, IL-21 delivers a death signal to B cells. It induces production of granzyme B leading to B cell death (Hagn et al., 2009) (Jahrsdörfer et al., 2006). Conversely, BCR signalling in the presence of both IL-21 and CD40L drives B cell activation, proliferation and plasmacyte differentiation.

B cell activating follicular factor (BAFF) is produced by a range of cell types including myeloid cells, dendritic cells, monocytes, activated T cells and B cells (Craxton et al., 2003a) (Scapini et al., 2008) (Nardelli et al., 2001). BAFF binds to B cell maturation protein A (BCMA) on tonsillar B cells. It promotes the survival of B cells (Avery et al., 2008) and is required for the macrophage / DC driven expansion

of B cells (Craxton et al., 2003b). BAFF and IL-21 act in combination to maintain memory B cell populations (Ettinger et al., 2007). In spite of this, BAFF does not save B cells from the death signal generated by IL-21 signalling in the absence of CD40L.

In addition, supernatant was collected from cultures of THP1 cells. This supernatant was filter sterilised and added at 10% concentration to cultures of TMCs. THP1 cells are a monocytic cell line. In this experiment, THP1 conditioned media was used as a source of soluble molecules which might be lacking from the culture media. In particular, the THP1 conditioned media was used as a source of soluble CD40L to help maintain B cell cultures. Macrophages and monocytes are known to express soluble CD40L (Mach et al., 1997) (Katsiari et al., 2002). THP1 conditioned media contains CD40L, but it does not contain all of the nutrients required for cell growth as these will have been exhausted by the THP1 cells. Because of this, THP1 media is only used at a concentration of 10% in the culture media.

To establish the optimal growth conditions, the cell number and viability were measured. Cells were treated with IL-2, THP1 conditioned media, IL-2, IL-21 and BAFF or THP1 conditioned media with IL-2, IL-21 and BAFF.

Figure 4.4.1 shows that culturing TMCs with 10% THP1 conditioned media led to decreased viability after 12 days. 63% of the unstimulated cells were viable compared to only 34% of the cells cultured with THP1 conditioned media ($p=0.0007$). The number of TMCs in the culture also fell from 88,200 per ml in the unstimulated cells down to 45,000 per ml in the culture supplemented with THP1 conditioned media. Supplementation with THP1 conditioned media alone is unsuitable for long term culture of TMCs.

TMCs cultured with IL-2 had a similar level of viability compared to unstimulated cells after 12 days. Figure 4.4.1 also shows that the number of TMCs increased in the presence of IL-2, from 88,200/ml to 358,333/ml ($p<0.0001$). This demonstrated that it is possible to induce proliferation of TMCs *in vitro* using IL-2.

The culture media containing a combination of IL-2, IL-21 and BAFF had a similar effect on TMCs. TMC viability was unchanged compared to the unstimulated cells. The number of TMCs increased from 88,200/ml to 186,667/ml after 12 days ($p=0.0160$). This was not significantly different compared to IL-2 stimulation alone.

A combination of THP1 conditioned media, IL-2, IL-21 and BAFF together maintained the viability of the TMCs and significantly increased the number of TMCs in the culture. After 12 days, the number of TMCs had increased more than six fold, from 88,200 cells/ml to 604,607 cells/ml and these were around 60% viable.

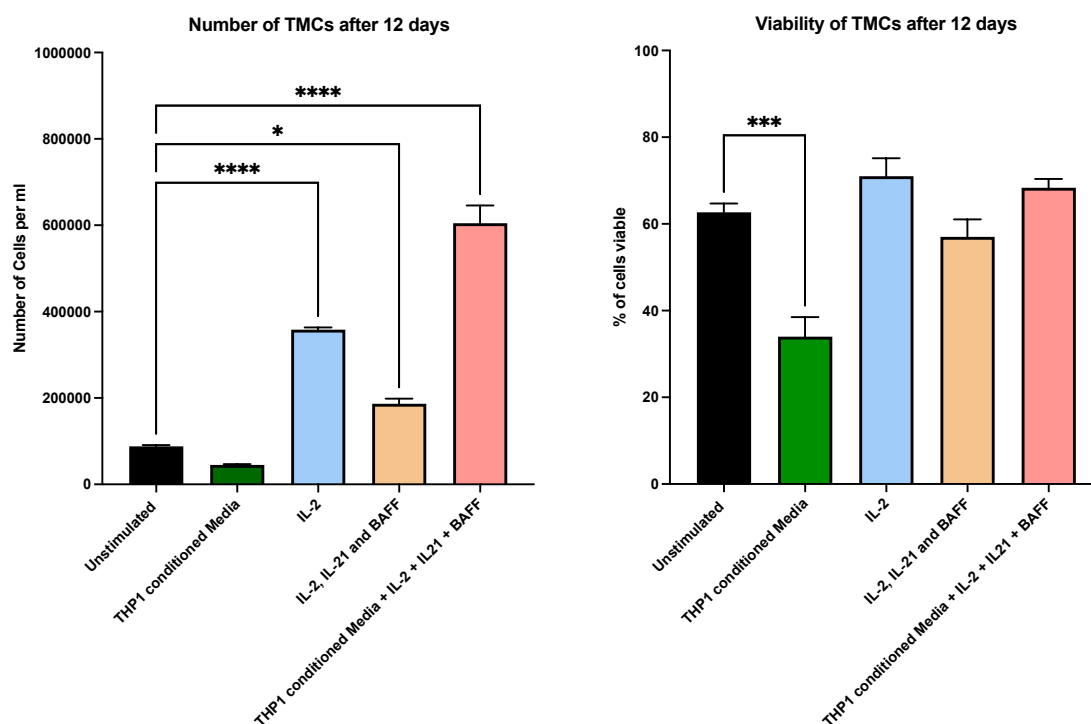


Figure 4.4.1. Effect of cytokines on long term culture of tonsillar cells. TMCs were seeded at a density of 1×10^5 cells/well. The cells were grown in complete RPMI media supplemented with (1) 10% THP1 conditioned media, (2) IL-2 alone, (3) IL-2, IL-21 and BAFF or (4) 10% THP1 conditioned media with IL-2, IL-21 and BAFF. After 12 days, the number of cells (A) and the percentage of viable cells (B) was measured using trypan blue staining ($n=3$). Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 .

The proliferation of T cells and B cells was investigated by measuring the percentage of Ki67+ cells by flow cytometry. These results were inconclusive (figure 4.4.2). Ki67 expression is a transient phenomenon observable only as the cells are proliferating. In this experiment, Ki67 was measured after 12 days of culture. No significant differences in Ki67 expression were seen between the different groups. The optimal timepoint for measuring Ki67 was likely before day 12. Subsequent experiments measured Ki67 at an earlier timepoint.

The expression of CCR5 on T cells was also measured and is shown in figure 4.4.2C. CCR5 is a chemokine receptor which is only expressed on activated T cells (Contento et al., 2008). Growing TMCs in THP1 conditioned media did not impact the expression of CCR5 on T cells. Incubating cells with IL-2 led to a significant reduction in the percentage of T cells expressing CCR5. 20% of unstimulated T cells expressed CCR5. This fell 30 fold to 0.67% when IL-2 was added to the culture media ($p < 0.0001$). Similar declines in CCR5 expression were seen when cells were incubated with additional IL-2, IL-21 and BAFF together. Culturing TMCs with IL-2, IL-21, BAFF and THP1 also significantly reduced CCR5 expression on T cells. This is consistent with an increasing population of naïve T cells.

The significance of this experiment is that it shows that tonsillar cultures are capable of proliferating *in vitro*. With a combination of cytokines and THP1 conditioned media, the number of TMCs was increased more than 6 fold over 12 days. This is useful for any future work which seeks to develop B cells antibody responses or investigate the immunological responses elicited by novel vaccine candidates. Tonsillar B cells have previously been shown to form germinal centres *in vitro* and respond to recall antigens with antibody responses. It may therefore be possible to use TMC cultures to recapitulate the normal behaviour of lymphoid cells.

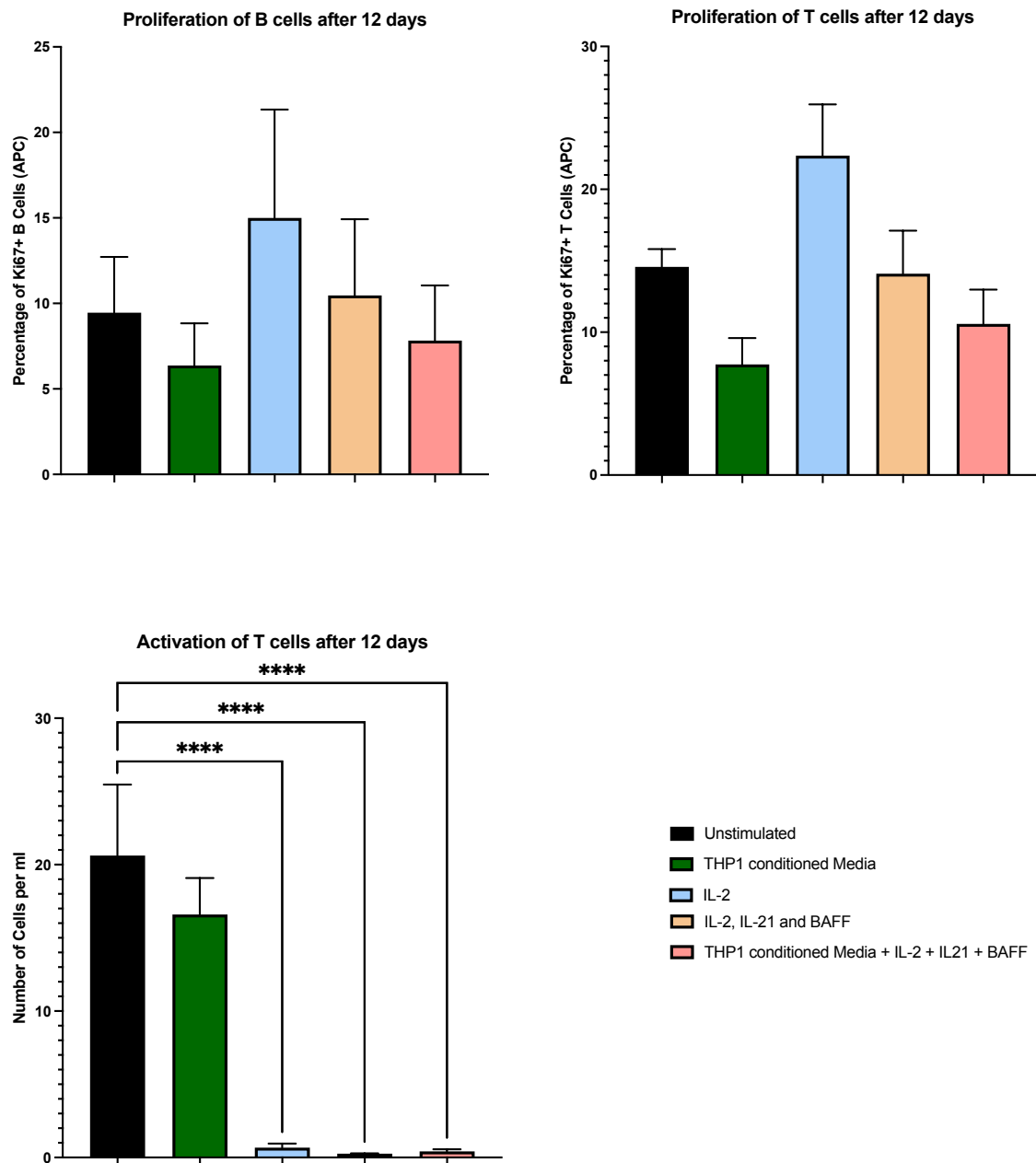


Figure 4.4.2. Effect of cytokines on long term culture of tonsillar cells. TMCs were seeded at a density of 1×10^6 cells/ml. The cells were grown in complete RPMI media supplemented with 10% THP1 conditioned media or IL-2 or IL-2, IL-21 and BAFF or 10% THP1 conditioned media, IL-2, IL-21, BAFF. After 12 days, the percentage of proliferating B cells (A) and T cells (B) were recorded. the percentage of T cells expressing CCR5 (C) was also measured using flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . (n=3).

4.5 Ability of TMCs to respond to immunogenic stimulation

This study explores the possibility of using *in vitro* tonsil models for a variety of novel purposes. Firstly, *in vitro* models were developed to test the immunogenicity of vaccine candidates for tuberculosis and Dengue Fever. Secondly, tonsil cells were used to model mycobacterial infection using BCG GFP. Finally, the interactions between BCG GFP and tonsillar B cells were examined. For these studies, the expression of activation markers and intracellular cytokine production by tonsillar B cells and T lymphocytes were measured. For each of these investigations, it was important to demonstrate that tonsil cells are capable of responding appropriately to compounds which upregulate relevant immunological markers on tonsillar lymphocytes. TMCs were cultured with four chemicals; PMA/Ionomycin, Resiquimod, PHA and Con A. These molecules are known to elicit proinflammatory responses and induce proliferation of T cells. TMCs were stimulated with these formulations for 5 days. The expression of the activation markers CD40, CD69, CD80 and CD86 by lymphocytes was determined by flow cytometry. Production of intracellular cytokines was also measured by flow cytometry. For T cells, IFN γ , IL-10, IL-4, IL-17 and TNF α were measured whilst for B cells IFN γ , IL-2, IL-4, IL-10, IL-12 and TNF α were detected. The proliferation of lymphocytes was also measured using Ki67 as a proliferation marker. Testing the TMCs in this way will demonstrate that the cells are healthy and their ability to respond to immunological stimuli is unimpaired. This study will also provide information for the development of immunogenicity assays.

4.6 Development of a TB immunogenicity T cell panel

An *in vitro* model could be used to screen novel TB vaccine formulations and determine their effect on T cells. T cell responses are pivotal for protection against TB infection. IFN γ and IL-17 responses in T helper cells are strongly associated with protective responses against TB (Torrado & Cooper, 2010). These responses were measured using flow cytometry. IL-2 production and Ki67 expression were used to determine whether induced T cell responses would be robust and sustained. Stimulation with anti CD3 and anti CD28 activates T cells through the T cell receptor (TCR). PMA/Ionomycin activates T cells downstream of the TCR receptor through protein kinase C. The TMCs will be stimulated with both of these compounds to test whether tonsillar T cells are capable of being activated through the TCR. This study will also establish whether tonsillar T cells are capable of expressing IL-17, IFN γ , IL-2 and Ki67 in response to TCR stimulation.

Stimulating cells with 10ng of PMA and 500ng/ml Ionomycin for 5 days generated suitable responses in both CD4 $^{+}$ T cells and CD8 $^{+}$ T cells. Significant IFN γ and IL-17 cytokine responses were observed, along with increased Ki67 expression. These results are shown in figures 4.6.1. and 4.6.2. This demonstrates that the TMCs are capable of responding appropriately to immunogenic stimulation. A PMA / Ionomycin combination would be a suitable positive control for this TB immunogenicity panel. In CD4 $^{+}$ T cells, PMA / Ionomycin stimulation increased the percentage of IFN γ^{+} cells tenfold from 3% to 37.8% ($p=0.0006$). The percentage of proliferating cells increased sevenfold from 6.63% to 47.57% ($p<0.0001$). These results are depicted in figure 4.6.1. The percentage of IL-17 $^{+}$ cells increased twenty fold from 0.57% to 11.73% ($p,0.0001$). This is seen in Figure 4.6.2

In CD8 $^{+}$ T cells, a similar pattern was seen. PMA / Ionomycin increased the percentage of IFN γ^{+} CD8 $^{+}$ T cells from 15.7% to 56% ($p=0.0035$). Figure 4.6.2 shows that the percentage of IL-17 $^{+}$ cells rose from 1.53% to 10.5% ($p<0.0001$). in Figure 4.6.1, proliferation was induced, with 79.7% Ki67 $^{+}$ compared to 2.5% in the unstimulated CD8 $^{+}$ T cells ($p<0.0001$). PMA/Ionomycin would be a suitable positive control for a TB immunogenicity study which investigates IFN γ , IL-17, Ki67 and IL-2 levels.

It is known that healthy T cells can be induced to proliferate by anti CD3 and anti CD28 antibodies. Stimulation of TMCs with anti CD3 and anti CD28 antibodies resulted in proliferation of T lymphocytes. In CD4+ T cells, the percentage of Ki67+ cells rose from 6.67% to 39.8% ($p < 0.0001$). This is shown in figure 4.6.1. In CD8+ T cells, the percentage of Ki67+ cells increased from 2.5% to 29.3% ($p = 0.0014$). Stimulation with anti CD3 and anti CD28 had no effect on the expression of IFN γ or IL-17 by T cells, as can be seen in figure 4.6.1 and 4.6.2.

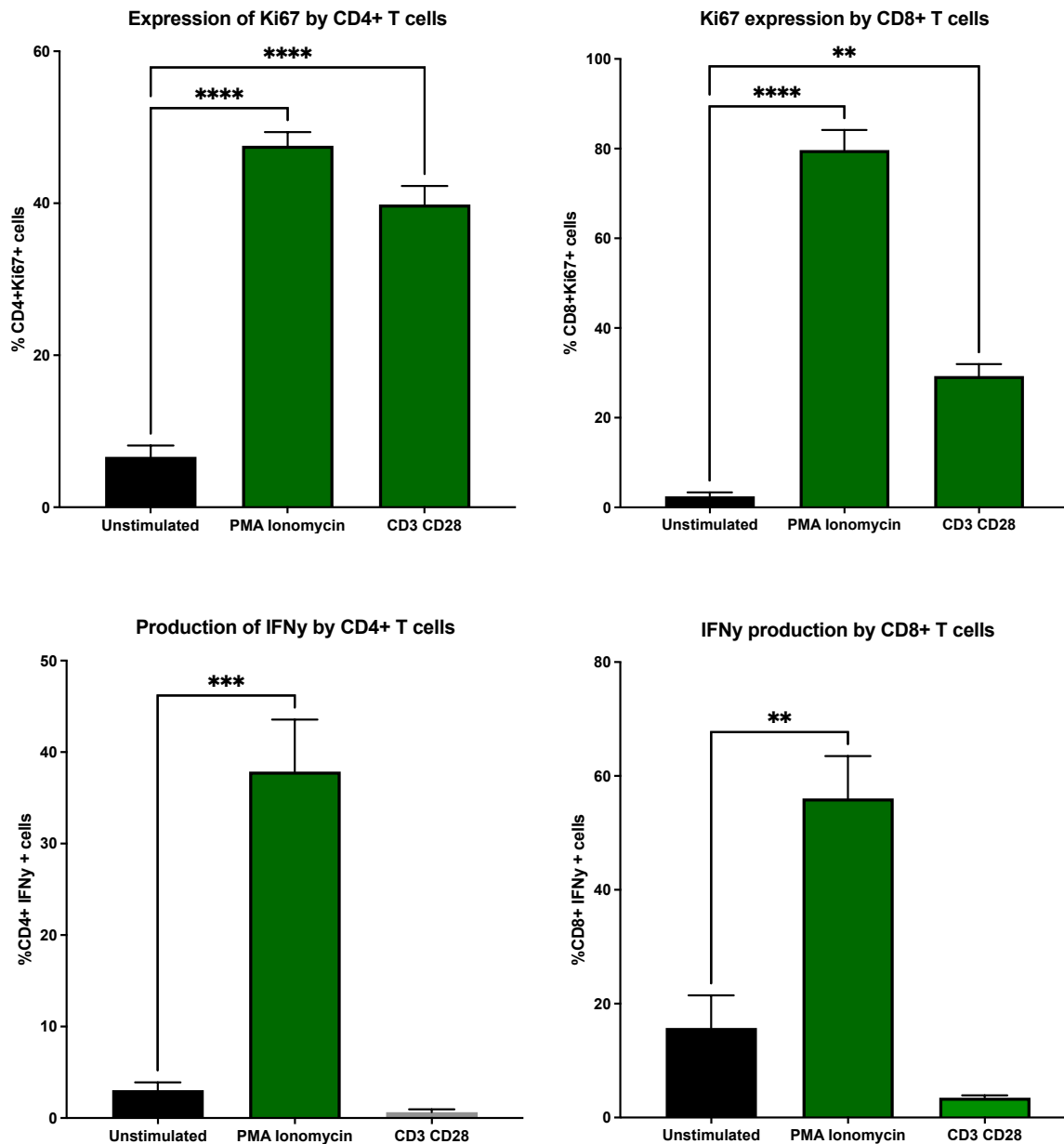


Figure 4.6.1. Development of a TB immunogenicity T cell panel. TMCs were seeded at 1×10^5 cells/well. Cells were stimulated with PMA/ Ionomycin or anti CD3 / anti CD28 for 5 days. Cells were treated with 10ng of PMA and 500ng/ml Ionomycin or $1 \mu\text{g/ml}$ of anti CD3 and $5 \mu\text{g/ml}$ of CD28. Cells were stained with CD3-FITC, CD4-PerCP Cy5.5 and CD8-BV510 antibodies. The percentage of CD4+ and CD8+ T cells expressing IL-17, IFN γ or Ki67 was recorded by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . (n=3). The results for IL-17 production are shown on the following page.

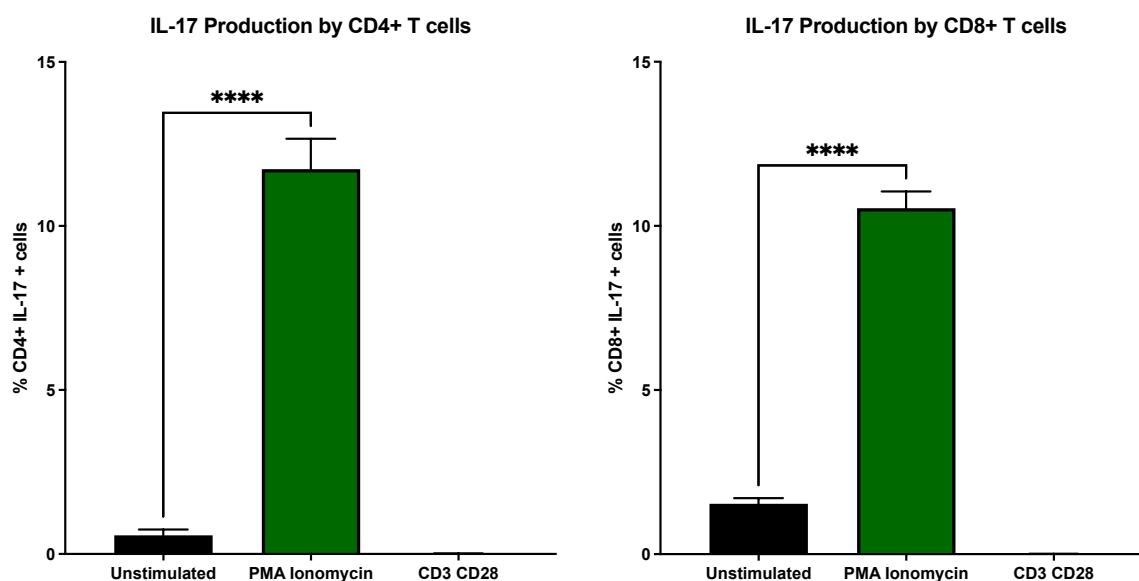


Figure 4.6.2. Development of a TB immunogenicity T cell panel. TMCs were seeded at 1×10^5 cells/well. Cells were stimulated with PMA/ Ionomycin or anti CD3 / anti CD28 for 5 days. Cells were treated with 10ng of PMA and 500ng/ml Ionomycin or $1 \mu\text{g/ml}$ of anti CD3 and $5 \mu\text{g/ml}$ of CD28. Cells were stained with CD3-FITC, CD4-PerCP Cy5.5 and CD8-BV510 antibodies. The percentage of CD4+ and CD8+ T cells expressing IL-17 was recorded by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . (n=3).

4.7 Production of IL-4, IL-10 and TNF α by tonsillar T cells.

During TB infection, IFN γ and IL-17 generated by T helper cells is important for protective immune responses. In contrast, IL-4 and IL-10 are associated with worse outcomes during TB infection (Pooran et al., 2019) (Abdalla et al., 2016).

When developing novel TB vaccines, it may be useful to examine their effect on the production of IL-4, TNF α and IL-10. This experiment has investigated three potential positive controls which can be used to induce cytokine production by T cells and B cells. PMA/ Ionomycin has been used along with PHA and Con A at concentrations of 500ng/ml, 5 μ g/ml and 50 μ g/ml.

Figure 4.7.1 shows stimulation with 50 μ g/ml of Con A upregulated the intracellular production of IL-4 by CD4 $^{+}$ T cells. Con A treatment increased the MFI of APC Cy7 in CD4 $^{+}$ T cells from 522 to 772 ($p=0.0101$). Con A also upregulated the production of IL-10 in CD4 $^{+}$ T cells. The MFI for PE increased from 694 to 1,802 reflecting an increase in intracellular IL-10 ($p=0.0068$). Stimulation with 50 μ g/ml Con A also induced IFN γ production by CD4 $^{+}$ T cells. BV421 MFI doubled from 1,316 to 3,523 ($p=0.0002$). Con A could be used as a suitable positive control to induce the production of IL-4, IFN γ and IL-10 by tonsillar T helper cells.

PHA did not induce production of IL-10, IL-4 or IFN γ by CD4 $^{+}$ T cells and as such would not be a suitable positive control for these parameters.

The levels of intracellular TNF α in the tonsillar CD4 $^{+}$ T cells was unaffected by stimulation with PMA / Ionomycin, Con A or PHA.

This work establishes that tonsillar T cells are capable of generating IL-4 and IL-10 responses *in vitro*. This is significant because these cytokines are associated with worse outcomes for TB patients. Novel TB vaccines can be tested using tonsillar immunogenicity models. TB vaccine candidates which induce excessive production of IL-4 or IL-10 can be identified and removed from development.

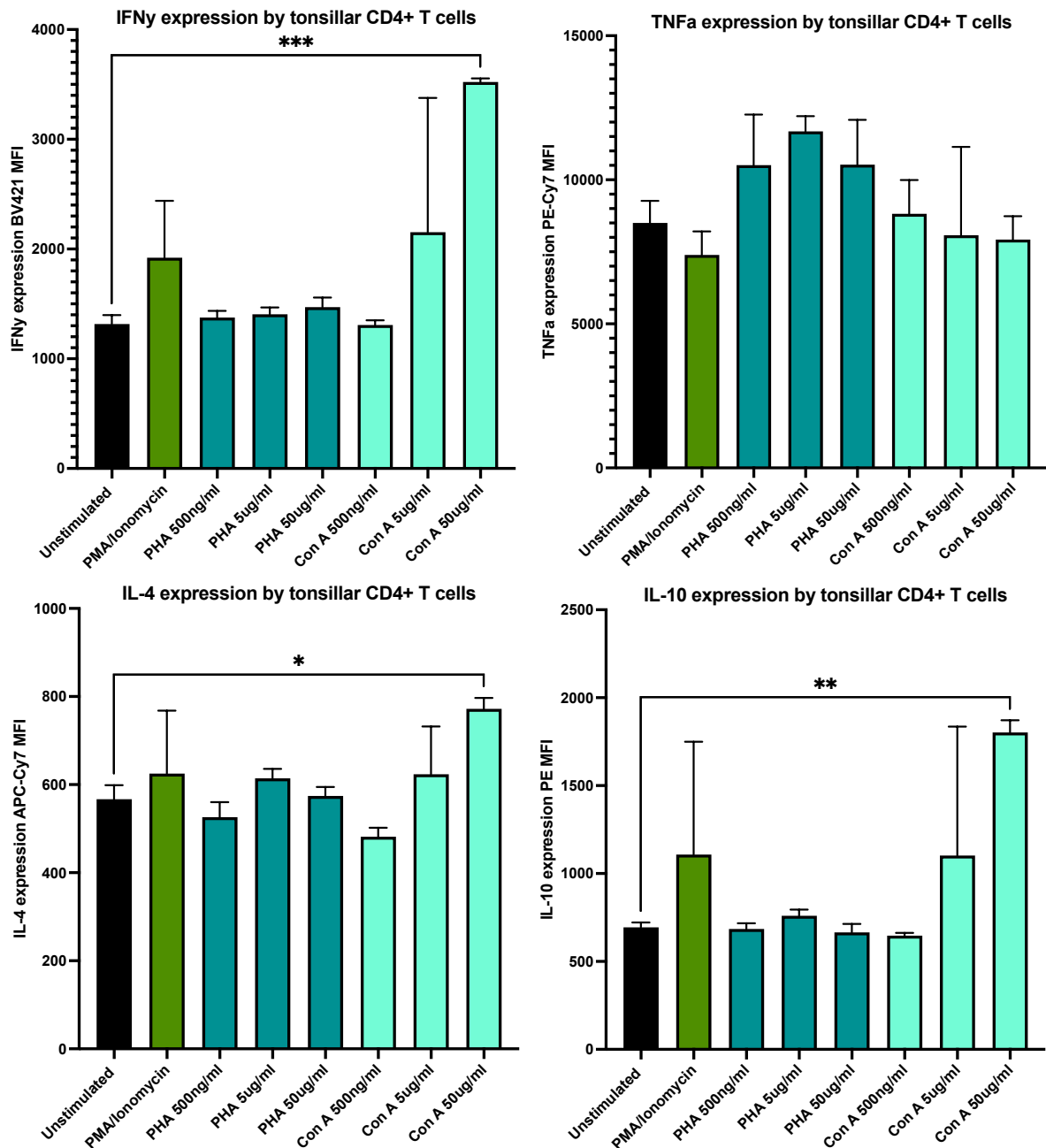


Figure 4.7.1 Production of IFN γ , TNF α , IL-4 and IL-10 by tonsillar T cells. Triplicate wells were seeded with 1×10^5 TMCs. Cells were stimulated with PHA or Con A at 50 μ g/ml, 5 μ g/ml or 500 ng/ml for 5 days. Cells were stained with CD3-FITC, CD4-PerCP Cy5.5 and CD8-BV510, IFN γ -BV421, TNF α -PE-Cy7, IL-4-APC-CY7 and IL-10-PE antibodies. MFI values were recorded and $\pm$ Standard deviations are shown. The statistical analysis was performed using One way ANOVA and Dunnetts multiple comparisons test. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 and *** of ≤ 0.001 .

4.8 Production of intracellular cytokines by tonsillar B cells

The production of cytokines is an important function of B cells which drives the development of lymphocytes. Tonsillar B cells were stimulated with PMA/ Ionomycin or Con A or PHA at concentrations of 50 µg/ml, 5 µg/ml or 50ng/ml. Flow cytometry was used to measure intracellular cytokines. The levels of IFN γ , IL-2, IL-4, IL-10, IL-12, TNF α were quantified after 5 days. The aim of this work was to demonstrate that tonsillar B cells are capable of generating cytokines in response to immune stimulation. Figure 4.8.1 reveals that Con A induced expression of IL-4, IL-2 and IL-10 in B cells. Figure 4.8.1 also shows that IL-2 was induced by PMA / Ionomycin, PHA and Con A.

3.9% of unstimulated B cells were positive for intracellular IFN γ . This was 12%, threefold higher, in the presence of Con A ($p < 0.0001$). Only 3.4% of tonsillar B cells were IL-4+ at baseline. This increased fourfold to 16.3% with 50 µg/ml of Con A ($p < 0.0001$). A similar pattern was seen with IL-10. Around 5.7% of B cells expressed IL-10 at baseline. This increased sixfold with 38.5% of B cells expressing IL-10 after stimulation with Con A ($p < 0.0001$).

The proliferative cytokine IL-2 was expressed by 4.5% of tonsillar B cells at baseline. With PMA/Ionomycin this rose to 12.4% of B cells expressing IL-2 ($p < 0.0278$). PHA stimulation caused 15.6% of B cells to produce IL-2 ($p = 0.0020$). Con A stimulation had the greatest effect on IL-2 production. 19% of B cells became IL-2+ in the presence of Con A ($p < 0.0001$).

A high percentage of tonsillar B cells express TNF α . 90% of B cells were TNF α +. This percentage was so high that it was unaltered by additional chemical stimulation (data not shown).

7% of unstimulated tonsillar B cells were IL-12+. This was unchanged by the addition of PMA/ Ionomycin, PHA or Con A (data not shown).

These results reveal that Con A is capable of eliciting production of IFN γ , IL-4, IL-10 and IL-2 by tonsillar B cells. Con A can be used as a positive control for further experiments which examine cytokine production by B cells.

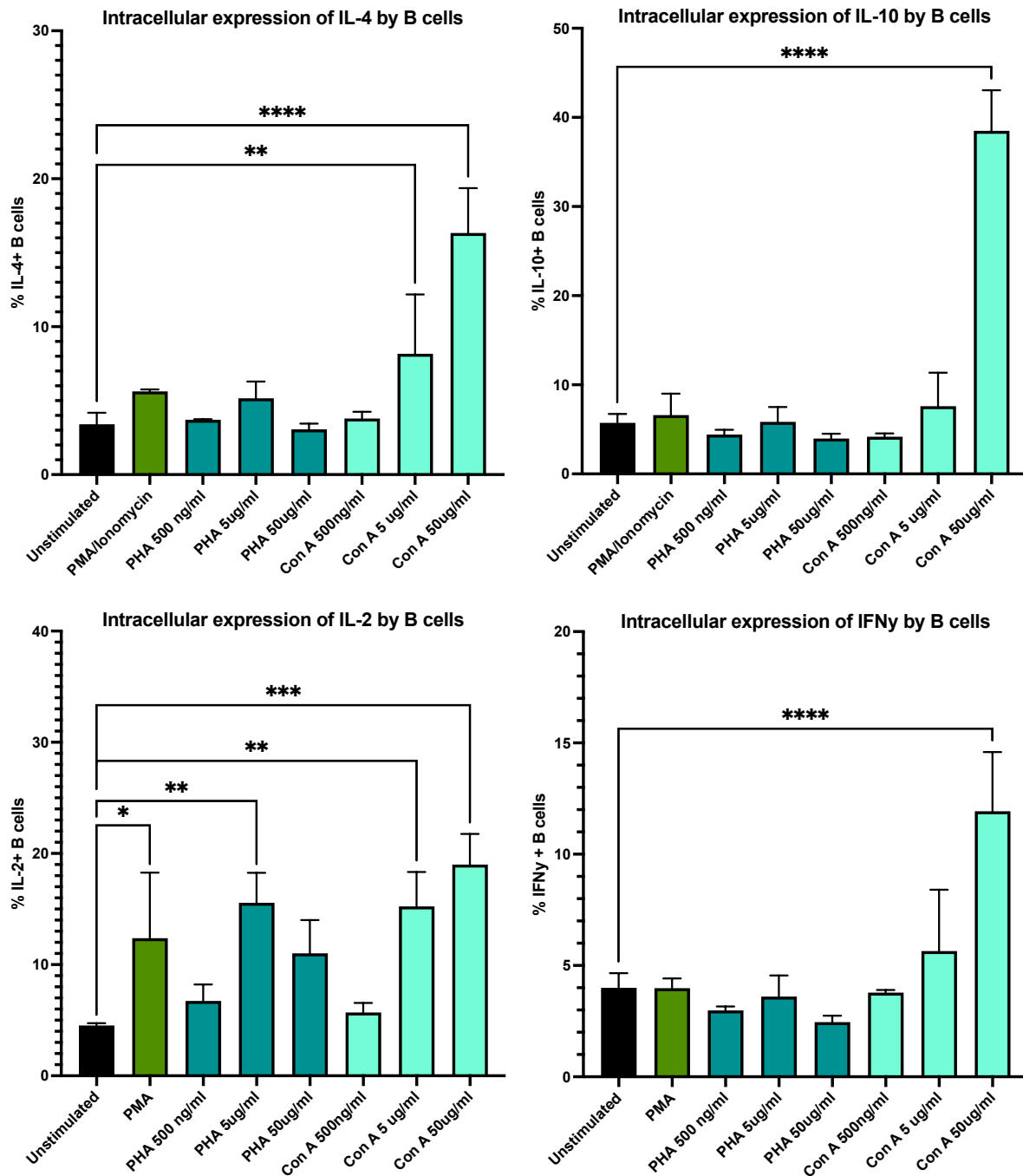


Figure 4.8.1. Production of intracellular cytokine by tonsillar B cells. Triplicate wells containing 1×10^5 TMCs were stimulated with PMA/Ionomycin, PHA or Con A for 5 days ($n=3$). The cells were prepared for intracellular staining and treated with antibodies against IL-4, TNF α , IL-12, IL-10, IL-2 and IFN γ . B cells were identified by flow cytometry and the percentage of cells containing each cytokine was recorded. Mean values and $\pm$ standard deviations are shown. Statistical analysis was performed using One way ANOVA and Dunnetts multiple comparison test.

4.9 Tonsillar T cell recall response to mycobacterial antigens

The tonsillar tissue collected in this study was used for the development of novel anti tuberculosis vaccines. Prior exposure to TB would be a confounding factor in these experiments. It is important to know that if a novel TB vaccine elicits strong antigen responses against TB that this is a new response and not merely a reactivation of a pre-existing anti TB response. Because of this, patients with known TB were excluded from this study. Infection with Tuberculosis is relatively common. Around 15% of people in the UK have latent TB. It was important to determine whether some participants had pre-existing reactivity to mycobacterial antigens. To test this, TMCs were treated with 0.1µg/ml (5 TU) of PPD for six days. CD4+ and CD8+ T cells were identified by flow cytometry. Proliferation was measured using the marker Ki67. Intracellular IFN γ was also measured along with the activation marker CD69. *In vitro* T cell responses to PPD have been used previously to detect LTBI and have a comparable efficacy to tuberculin skin tests (Arya et al., 2018).

Aims

To determine whether tonsillar T cells reacted to PPD *in vitro*.

4.10 Effect of PPD on tonsillar T cells

Tonsillar cells from patients 1 and 16 reacted to PPD. In patient 1, PPD recall responses were observed, with proliferation of CD4+ T cells and production of IFN γ by CD8+ T cells.

CD4+ T cells from patient 1 proliferated in response to PPD. The percentage of Ki67+ CD4+ T cells increased from 31% to 42% with PPD ($p=0.0017$). This is depicted in Figure 4.10.1. IFN γ was also generated by CD8+ T cells from patient 1. Figure 4.10.6 shows that IFN γ levels (BV421 MFI) rose from 156,995 in unstimulated CD8+ T cells to 436,398, representing a 2.7 fold increase in intracellular IFN γ ($p=0.0042$)

T cells from patient 16 also reacted to PPD. There was proliferation of CD4+ T cells and increased expression of CD69 by CD8+ T cells. The percentage of CD4+ T cells expressing Ki67 increased from 23% to 32% following PPD stimulation ($p=0.0242$). This is depicted in figure 4.10.1. PPD stimulation also led to increased CD69 expression on CD8+ T cells from patient 16. The PE MFI rose from 4,745 in the unstimulated cells to 7,234 in the PPD stimulated cells ($p=0.0250$). This is shown in figure 4.10.5.

PPD stimulation induced proliferation of CD4+ T cells and activation of CD8+ T cells from patients 1 and 16. As such these are unequivocal evidence of PPD reactivity in patients 1 and 16.

Ambiguous responses were seen in patients 3, 12, 13 and 14. CD8+ T cells from patients 3, 12 and 13 showed PPD responses, with upregulated expression of CD69. This is shown in figure 4.10.5. CD8+ T cells from patient 14 showed upregulated IFN γ production following PPD stimulation in figure 4.10.6. In TMCs from these patients, only one measured parameter was upregulated following PPD stimulation, whilst all other measured parameters were unchanged in the presence of PPD.

PPD stimulation raised expression of CD69 in CD8+ T cells from patients 3, 12, 13. These results can be seen in figure 4.10.5. PE MFI was used to measure CD69 expression. CD69 expression rose from 4,949 to 7,754 in CD8+ T cells from patient 3 with PPD stimulation ($p=0.0065$). In CD8+ T cells from patient 12, CD69 expression was increased from 5,029 to 8,444 by the addition of PPD ($p=0.0004$).

CD69 expression on patient 13's CD8+ T cells rose from 5,332 to 8,919 with PPD stimulation ($p=0.0002$).

Figure 4.10.6 shows that IFN γ was generated in response to PPD by CD8+ T cells from patient 14. The level of intracellular IFN γ (as measured by BV421 MFI) was 76,662 in unstimulated cells. This rose to 376,126 with PPD stimulation ($p=0.0016$).

18 patients were recruited into this study. Of these, 2 were found to have PPD reactivity. This represents 11% of our sample. The UK TB prevention and control LTBI programme found a LTBI positivity rate of 14.9% in 2018 (UK Health Security Agency,). It is therefore plausible that this PPD reactivity data represents 2 patients with latent TB. It is a limitation of this work that it is not possible to determine the patients latent TB status by TST or any other method. This precludes any definitive diagnosis of latent TB. Nonetheless, this study provides early evidence that if tonsil tissue was routinely collected from tonsillectomies it could be used to diagnose latent TB infections.

Another possibility is that these two PPD reactive patients have previously received a BCG vaccine. The BCG vaccination status of the participants of this study was unknown. This was a limitation when using these tonsils for TB research. BCG vaccination increases the likelihood that patients will respond to PPD. T cell responses to PPD have been detected *in vitro* following BCG vaccination. 12 months after BCG vaccination, PPD recall of CD4+ and CD8+ T cells led to IFN γ production (Smith, S. G. et al., 2010). In this experiment, it was not possible to link the observed PPD reactivity of tonsil tissue to the BCG vaccination status of the study participants.

For the purpose of TB vaccine immunogenicity studies, it is important to exclude any TMCs which are already PPD reactive. As a result, TMCs from patients 1 and 16 were not used for the development of novel TB vaccines.

Key Finding

Tonsils from Patients 1 and 16 showed reactivity to PPD.

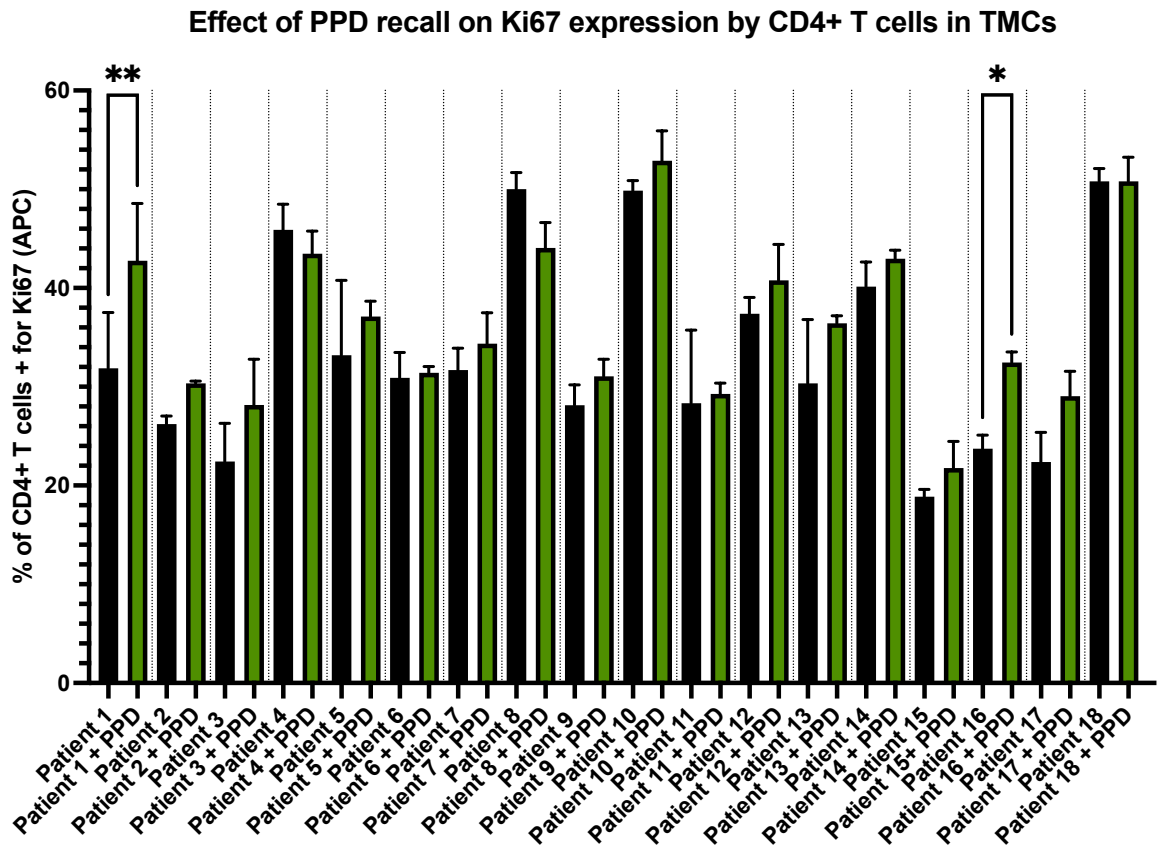


Figure 4.10.1. Effect of PPD recall on Ki67 expression by tonsillar CD4+ T cells. Proliferation of CD4+ T cells following PPD stimulation was measured by seeding triplicate wells with 1×10^5 TMCs on 96 well plates. These were incubated for 6 days with 5TU of PPD. The percentage of proliferating CD4+ T cells expressing Ki67 was measured by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using one way ANOVA and Sidaks multiple comparison test. There were significant increases in the percentage of Ki67+ CD4+ T cells from patients 1 and 16, following PPD stimulation.

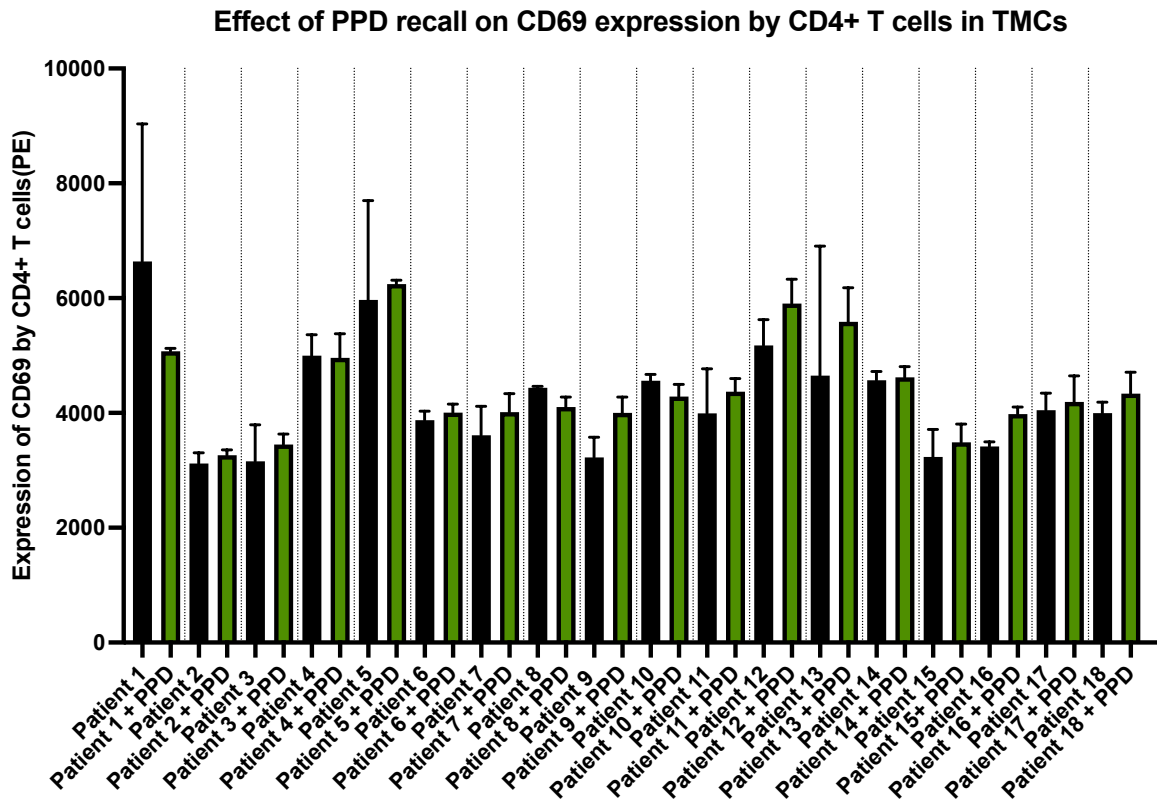


Figure 4.10.2. Effect of PPD recall on CD69 expression by tonsillar CD4+ T cells. 1×10^5 TMCs/well were seeded in triplicate into 96 well plates. These were incubated for 6 days with 5TU of PPD. Activated CD4+ T cells expressing CD69 were identified by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using one way ANOVA and Sidaks multiple comparison test. No significant increases in CD69 expression were detected.

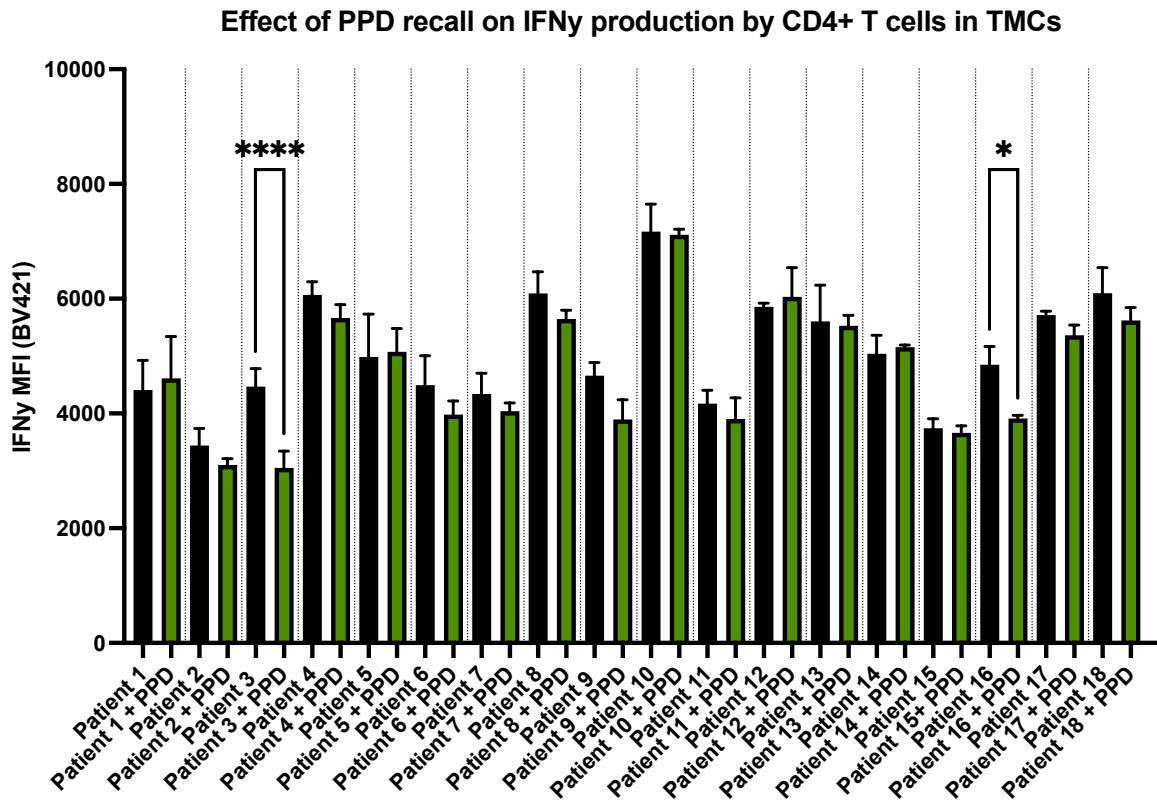


Figure 4.10.3. Effect of PPD recall on IFN γ production by tonsillar CD4 $^{+}$ T cells. 1×10^5 TMCs/well were seeded in triplicate into 96 well plates. These were incubated for 6 days with 5TU of PPD. Flow cytometry was used to identify CD4 $^{+}$ T cells. Intracellular production of IFN γ was measured. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using one way ANOVA and Sidaks multiple comparison test. PPD stimulation did not induce significant increases in IFN γ production in tonsillar CD4 $^{+}$ T cells.

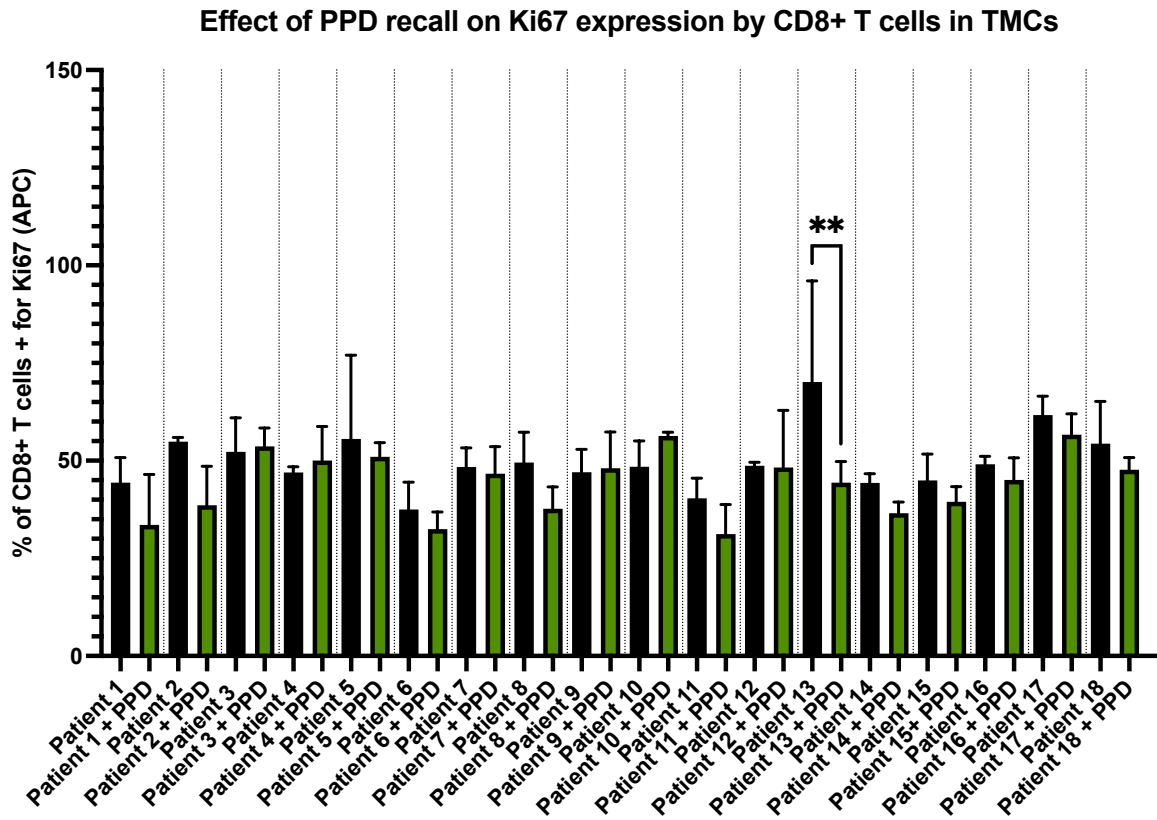


Figure 4.10.4. Effect of PPD recall on Ki67 expression by tonsillar CD8+ T cells. Proliferation of CD8+ T cells following PPD stimulation was measured by seeding triplicate wells with 1×10^5 TMCs. These were incubated for 6 days with 5TU of PPD. The percentage of proliferating CD8+ T cells expressing Ki67 was measured by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using one way ANOVA and Sidaks multiple comparison test. There were no significant increases in the percentage of Ki67+ CD8+ T cells following PPD stimulation.

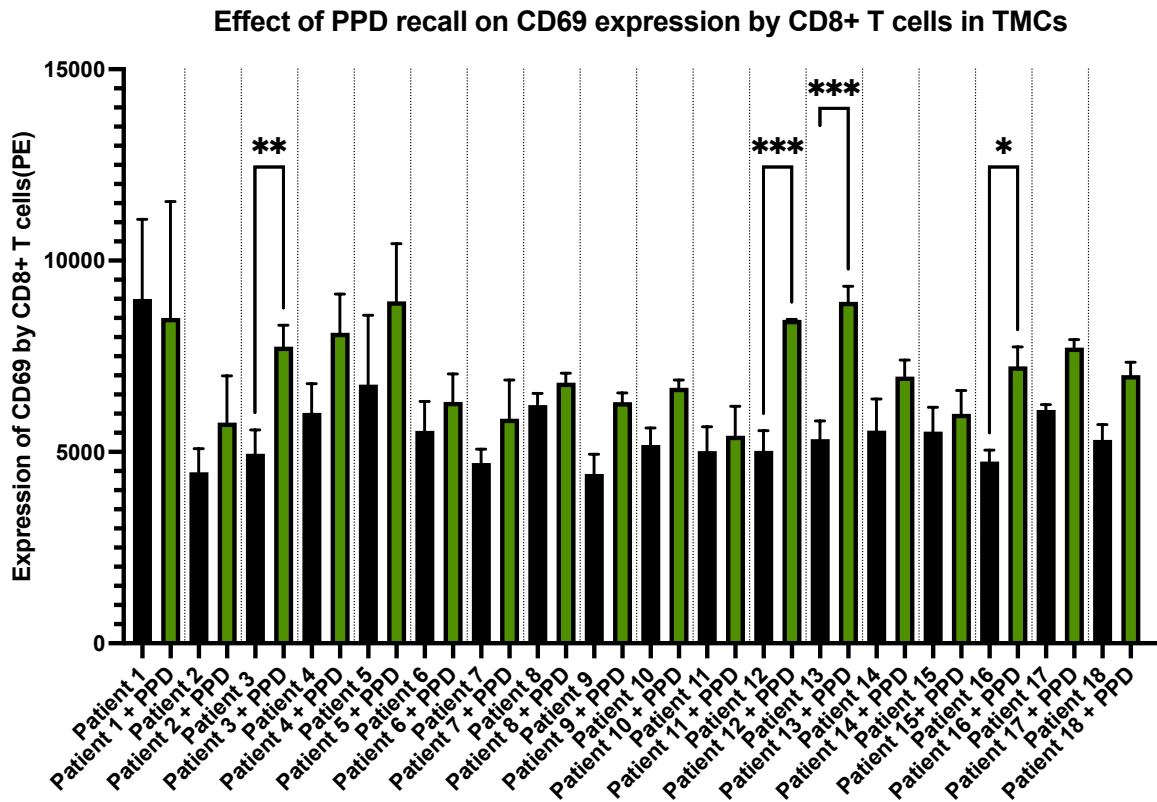


Figure 4.10.5. Effect of PPD recall on CD69 expression by tonsillar CD8+ T cells. 1×10^5 TMCs/well were seeded in triplicate into 96 well plates. These were incubated for 6 days with 5TU of PPD. Flow cytometry was used to identify CD8+ T cells. Activated CD8+ T cells expressing CD69 were identified. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using one way ANOVA and Sidaks multiple comparison test. Significant increases in CD69 expression were observed in patients 3, 12, 13 and 16 following treatment with PPD.

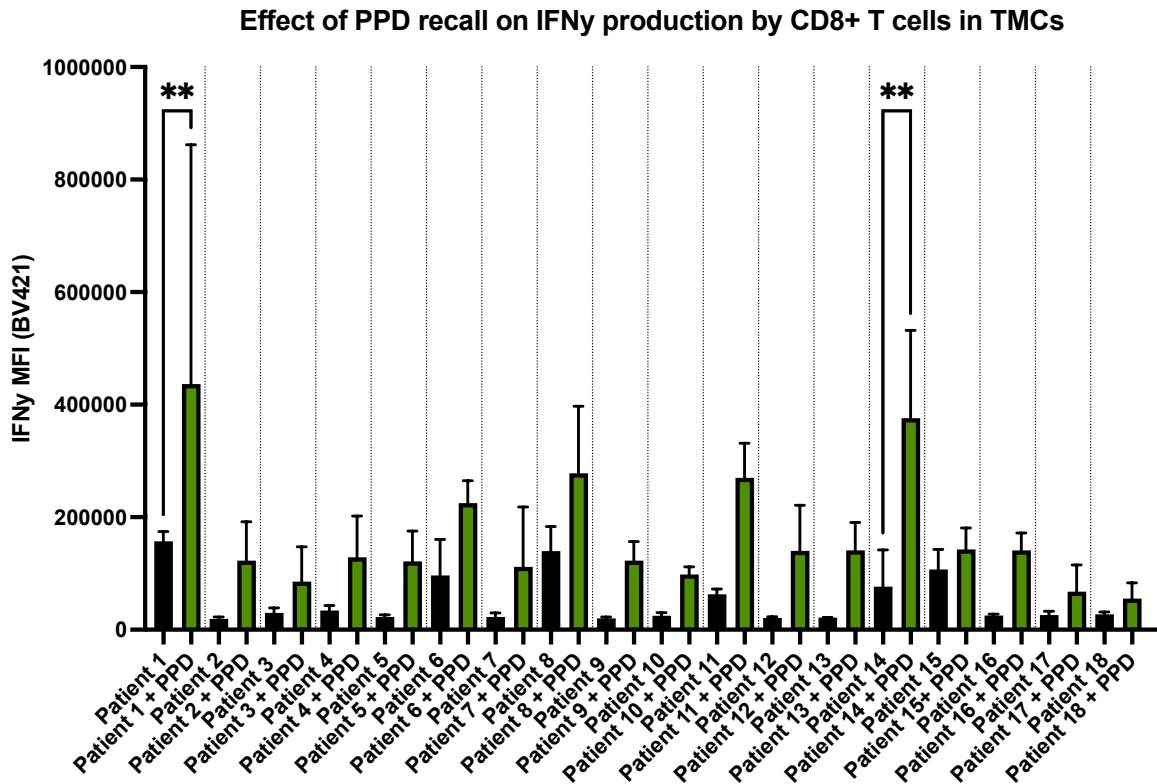


Figure 4.10.6. Effect of PPD recall on IFN γ production by tonsillar CD8 $^{+}$ T cells. Triplicate wells of 96 well plates were seeded with 1×10^5 TMCs. These were incubated for 6 days with 5TU of PPD. Flow cytometry was used to identify CD8 $^{+}$ T cells. Intracellular production of IFN γ was measured. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . The data was analysed using one way ANOVA and Sidaks multiple comparison test. Significant increases in IFN γ production were seen in patients 1 and 14 stimulated with PPD.

4.11 ACE 2 receptor expression on tonsillar cells.

The COVID 19 pandemic developed into a significant public health concern during this project. This study investigated whether the angiotensin converting enzyme 2 (ACE 2) receptor was expressed on tonsillar tissue. The ACE 2 receptor has a variety of physiological roles. In the lungs, it is involved in protecting alveolar epithelial cells from death and injury (Samavati & Uhal, 2020). The ACE 2 receptor is used by the SARS-CoV-2 virus to gain entry into human cells (Li, W. et al., 2003).

If tonsillar cells were shown to express ACE 2 receptor, it would be possible to develop an *in vitro* infection model for SARS-CoV-2 using tonsil cells. There is already an infection model based on lung epithelial cells. A tonsil based infection model would complement this. TMCs contain a heterogeneous mixture of primary cells isolated from mucosal lymphoid tissue. This would be very relevant to COVID 19 infection and might provide insights into the infection of different cell subsets. Flow cytometry was used to measure ACE 2 expression on CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells from TMCs. ACE 2 expression was also measured on whole tonsil and THP1 cells.

There was a minimal expression of ACE 2 receptor on CD8+ T cells. Figure 4.11.1 shows that the FITC MFI of stained CD8+ T cells was only 1.25 fold higher than the negative control. FITC MFI was 268,420 in the negative control, compared to 337,398 in the ACE2 bound CD8+ T cells. 18.5% of negative control cells were FITC+ whilst 25.6% of CD8+ T cells were FITC+. This was only a difference of 7%. These results show that a minority of CD8+ tonsillar T cells express the ACE 2 receptor. This agrees with newly published work that shows ACE2 expression on CD8+ T cells. The SARS-CoV-2 spike protein is able to bind to ACE2 on CD8+ T cells, suppressing their ability to form immunological synapses and limiting their ability to kill viral infected cells (Baldari et al., 2023).

There was no detectable ACE2 expression on TMC derived CD4+ T cells. This is seen in figure 4.11.1.

ACE 2 receptor expression on tonsillar cells

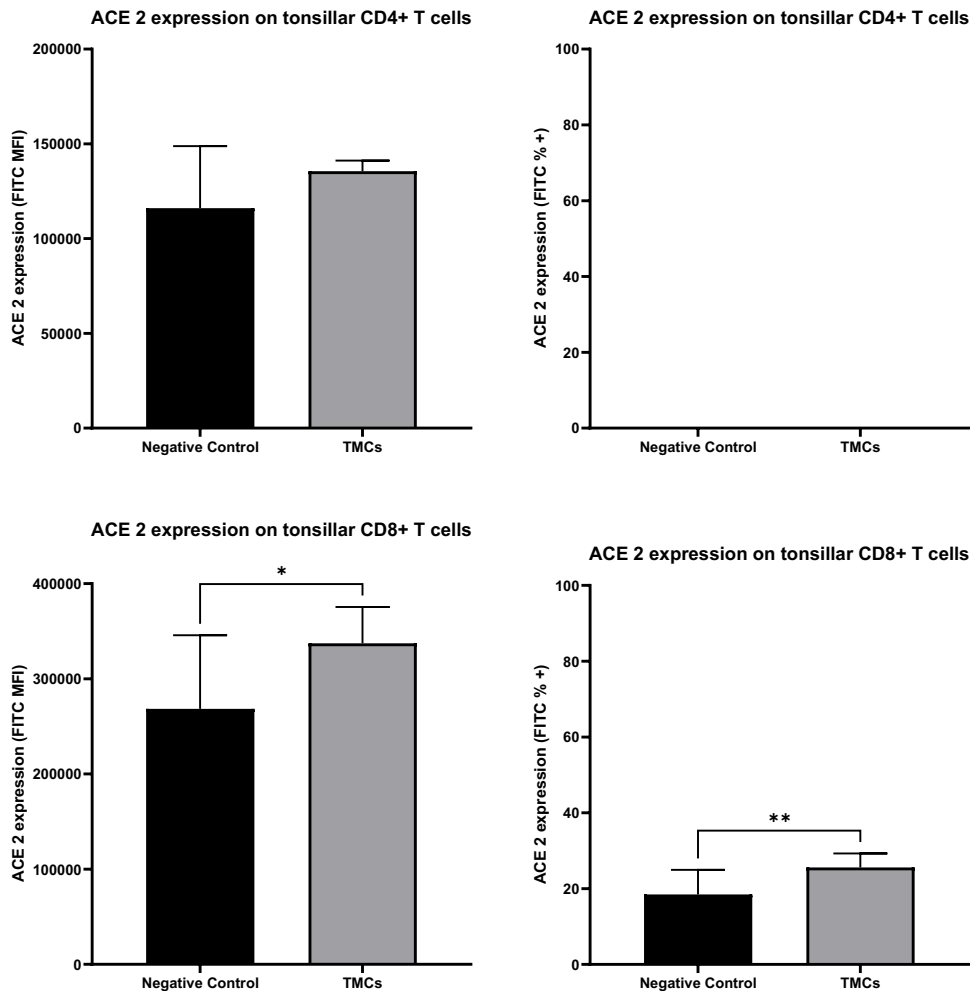


Figure 4.11.1. ACE 2 receptor expression on CD4+ and CD8+ tonsillar T cells. TMCs were seeded into triplicate wells on 96 well plates at a density of 1×10^6 cells/ml. These were stained with a primary antibody anti ACE 2, and then a secondary detection antibody bound to FITC. Cells expressing ACE 2 receptors were FITC+. The MFI of FITC was measured, along with the percentage of FITC+ cells. Flow cytometry was used to identify CD4+, CD8+ T cells (n=3). Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 and ** of ≤ 0.01 . These results were analysed using a T test. There was no detectable ACE 2 expression on CD4+ T cells. ACE 2 was expressed on a minority of tonsillar CD8+ T cells at a low level.

Figure 4.11.2 shows that there is no ACE2 expression on tonsillar CD14+ monocytes or CD19+ B cells. This corresponds well with previous findings that there is little ACE2 expression on PBMCs. ACE 2 is not expressed on CD4+, CD8+, NK cells, B cells, neutrophils, monocytes or DCs (Song et al., 2023a) (Wang, Yiliang, Wang, Luo, Huang, Xiao, Li, Qin, Song, Wu, & Zeng, 2020a). ACE 2 expression has not been detected in the thymus, spleen or lymphoid organs, with the exception of some limited expression on sinus endothelial cells (Hamming et al., 2004a).

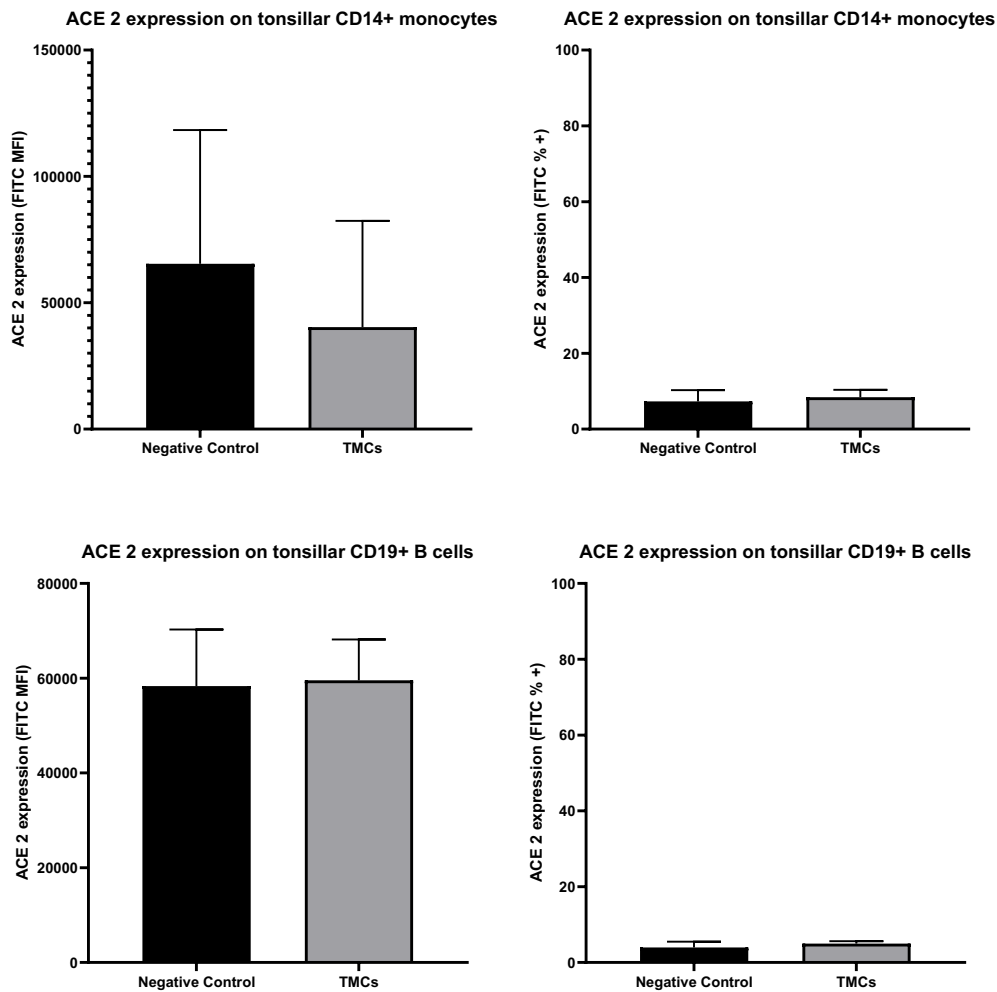


Figure 4.11.2. ACE 2 receptor expression on tonsillar monocytes and B cells. TMCs were seeded into triplicate wells on 96 well plates at a density of 1×10^6 cells/ml. These were stained with a primary antibody anti ACE 2, and then a secondary detection antibody bound to FITC. Cells expressing ACE 2 receptors were FITC+. The MFI of FITC was measured, along with the percentage of FITC+ cells. Flow cytometry was used to identify CD14+ and CD19+ cells (n=3). Mean values and $\pm$ standard deviations are shown. These results were analysed using a T test. There was no detectable ACE 2 expression on CD14+ monocytes or CD19+ B cells.

TMCs are prepared by FICOLL density centrifugation. This excludes some cell types. Whole tonsil preps were used in this experiment to determine whether there was any ACE2 expression in any cell type present within tonsils. These whole tonsils would contain endothelial cells and epithelial cells. When the whole cell cultures were tested, no ACE 2 expression was detected. This is shown in figure 4.11.3.

Previous studies have examined the expression of ACE 2 on a variety of human cell types and tissues. The ACE 2 receptor is highly expressed on M1 alveolar macrophages, Kupfer cells and microglial cells (Song et al., 2023a). ACE2 expression has also been observed on pneumocytes, lung epithelial cells, gut enterocytes, smooth muscle cells and endothelial cells (Song et al., 2023a) (Hamming et al., 2004a).

Previous studies have found no ACE 2 receptor expression on T lymphocytes (Hamming et al., 2004b) (Song et al., 2023b; Wang, Yiliang, Wang, Luo, Huang, Xiao, Li, Qin, Song, Wu, & Zeng, 2020b).

This study shows that tonsillar CD8+ T cells express low levels of ACE 2 receptor. Low level ACE2 expression has been shown on Jurkatt cells and some primary T cells from primary donors PBMCs (Welch et al., 2022a). SARS- CoV-2 is capable of binding to T lymphocytes and infecting them. Viral entry into T lymphocytes was not accompanied by replication of the virus (Welch et al., 2022a).

The absence of ACE 2 receptor expression makes tonsil cells unsuitable for developing a COVID 19 infection model.

ACE 2 receptor expression on whole tonsillar cells

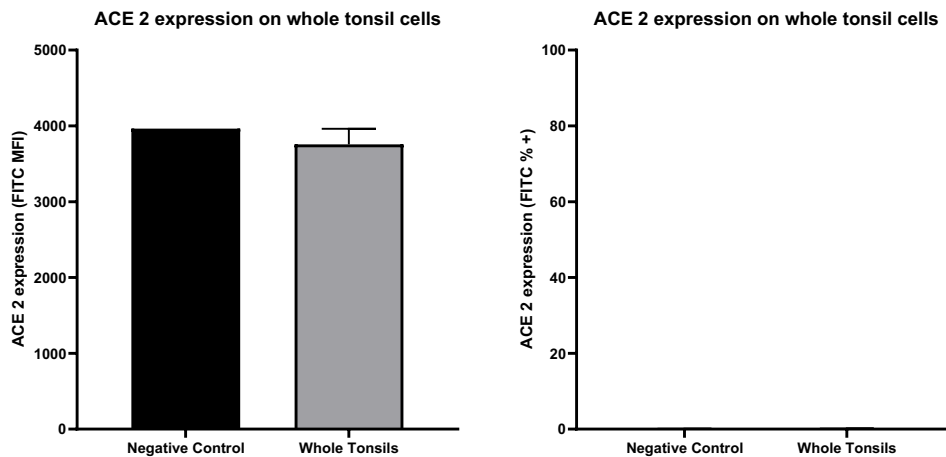


Figure 4.11.3. ACE 2 receptor expression on whole tonsillar cells. Whole tonsil cells were seeded into triplicate wells on 96 well plates at a density of 1×10^6 cells/ml. These were stained with a primary antibody anti ACE 2, and then a secondary detection antibody bound to FITC. Cells expressing ACE 2 receptors were FITC+. The MFI of FITC was measured by flow cytometry, along with the percentage of FITC+ cells. (n=2). Mean values and $\pm$ standard deviations are shown. These results were analysed using a T test. There was no detectable ACE 2 expression on whole tonsil cells.

Figure 4.11.4 shows that undifferentiated THP1 monocytic cells do not express the ACE 2 receptor. Differentiated THP1 cells have previously been used to examine the effect of SARS CoV-2 on TLR signalling (Pantazi et al., 2021) but THP1 cells have not previously been examined for ACE 2 receptor expression (Zhang, D. et al., 2020). It is possible to induce expression of ACE2 using PMA and Ionomycin stimulation (Welch et al., 2022b). Alternatively, IFN- β can be used to induce ACE 2 expression (Ziegler et al., 2020). It may be possible to use *in vitro* stimulation of tonsil cultures with PMA / Ionomycin or IFN- β to induce ACE 2 expression. This could allow tonsillar tissue to be used for a SARS CoV-2 infection model. Further work could be done to explore this possibility.

ACE 2 receptor expression on THP1 monocytes

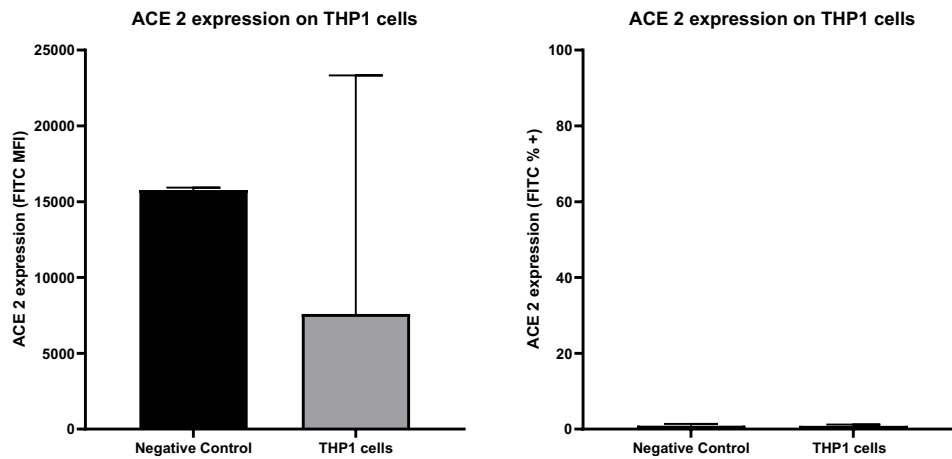


Figure 4.11.4. ACE 2 receptor expression on THP1 monocytes. THP1 monocytes were seeded into ten repeat wells on 96 well plates at a density of 1×10^6 cells/ml. These were stained with a primary antibody anti ACE 2, and then a secondary detection antibody bound to FITC. Cells expressing ACE 2 receptors were FITC+. The MFI of FITC was measured by flow cytometry, along with the percentage of FITC+ cells. (n=3). Mean values and $\pm$ standard deviations are shown. These results were analysed using a T test. There was no detectable ACE 2 expression on THP1 cells.

Key Findings

Phenotype of tonsil mononuclear cells

TMCs were composed of 60% B lymphocytes and 20% T lymphocytes. Of these, 60% were CD4+ T cells and 30% were CD8+ T cells. 1% of TMCs were monocytes and 2% were myeloid cells.

Long term viability of TMC cultures

TMCs maintained a high viability over 12 days of *in vitro* culture.

The percentage of myeloid, monocyte, CD4+ and CD8+ T cells and B cells remained relatively stable over 12 days of culture.

Identification of memory and naïve T cells.

Approximately 20% of tonsillar CD4+ and 30% of CD8+ T cells are naïve cells.

Effect of cytokines on long term viability of TMCs.

Culturing TMCs with THP1 conditioned media, IL-2, IL-21 and BAFF together induces proliferation.

Ability of TMCs to respond to immunogenic stimulation

PMA / Ionomycin upregulates IFN γ , IL-17 and IL-2 production and induces proliferation. This makes it a suitable positive control for a TB immunogenicity panel.

Con A can be used to induce IL-4 and IL-10 production by T helper cells

Con A can be used to induce IFN γ , IL-4, IL-2 and IL-10 production by B cells.

Key Findings

Mycobacterial antigen recall

PPD recall responses were observed in CD4+ T cells and CD8+ T cells from TMCs.

It may be possible to identify patients with LTBI using tonsil cells.

11% of patients were identified as being potentially latently infected with TB on the basis of PPD recall responses.

Suitability of the tonsil model for a COVID 19 infection model

There was no detectable expression of ACE 2 by CD4+ T cells, B cell, monocytes or myeloid cells.

Approximately 5% of tonsillar CD8+ T cells expressed the ACE2 receptor.

Tonsillar cells are not suitable for the development of a SARS COV2 infection model.

Chapter 5 *in vitro* vaccine immunogenicity models

Abstract

The development of novel vaccine candidates benefits from robust *in vitro* models. Here, TMCs have been used to establish an *in vitro* model to test the immunogenicity of novel vaccines. This model recapitulates the complex interactions between different immune cells in primary human tissue.

The TMC model was first used to test vaccines against TB. The BCG vaccine and 5 other vaccine candidates; Spore FP1, YC NaMa FP1, Liposome FP1, IgA ICM and IgG ICM were tested. Flow cytometry was used to measure the production of IFN γ , IL-17 and IL-2 by CD4 $^{+}$ and CD8 $^{+}$ T cells. The proliferation of these cells was also examined. BCG vaccination induced IFN γ , IL-17 and IL-2 responses as well as proliferation. IgA ICM generated a similar immunogenic profile making it a promising candidate for further work. In contrast, the IgG ICM variant was not immunogenic.

Spore FP1 generated IFN γ responses in CD4 $^{+}$ T cells. Liposome FP1 induced IFN γ and IL-17 responses. YC NaMa FP1 induced IFN γ , IL-17 and proliferative responses in CD4 $^{+}$ T cells, making it a promising TB vaccine candidate.

The TMC *in vitro* model was also used to investigate the immunogenicity of novel Dengue vaccine candidates. Four dengue vaccine candidates; D-PIGS, Modified D-PIGS, DERICS and My42J generated IFN γ , IL-4, TNF α and IL-10 responses in T cells. D-PIGS, Mod D-PIGS and My42J generated TNF α , IFN γ and IL-4 responses in CD4 $^{+}$ T cells. DERICs generated IFN γ and IL-4 responses in CD4 $^{+}$ T cells. D-PIGS, Mod D-PIGS and My42J generated IL-4 responses in CD8 $^{+}$ T cells. The findings of this study contribute to our understanding of the immune responses generated by each of these vaccine candidates.

Aims

Test the immunogenicity of BCG using tonsillar *in vitro* model.

Measure IFN γ , IL-17 and IL-2 production by CD4 $^{+}$ T cells.

Measure the proliferation of tonsillar T cells using Ki67 expression upon stimulation with vaccine candidates.

5.1 Immunogenicity of the BCG vaccine in TMCs

The Bacillus Calmett Guérin vaccine (BCG) is an attenuated strain of *Mycobacterium bovis*. It has been used as a vaccine against TB since 1921 and is still the only effective TB vaccine in use. The immunogenic effects of BCG are well characterised. BCG generates a predominantly Th1 CD4 $^{+}$ T cell driven response. IFN γ is necessary for protection against TB as previously discussed. BCG also elicits IL-10, IL-23 and IL-17 responses (Gopal et al., 2012)., BCG specific Th17 cells have been shown to offer protection against TB in a mouse model (Wozniak et al., 2010). BCG induced IL-17 memory responses are also known to be necessary for the migration of CD4 $^{+}$ IFN γ^{+} T cells to the lungs during infection with TB (Khader et al., 2007). BCG is known to elicit antigen specific IFN γ responses in humans (Black et al., 2002) and induce the production of TNF α and IL-2 by CD4 $^{+}$ T cells in macaques (White et al., 2015). Proliferation of CD4 $^{+}$ T cells is also observed following BCG vaccination (Mauch et al., 2020).

This study seeks to develop an *in vitro* model which can be used to assess the immunogenicity of novel TB vaccines. In principle, this tonsil immunogenicity model would simply replace the *in vivo* mouse models which are currently used. The first step in validating this model is to use the *in vitro* model to assess the immunogenicity of the current vaccine, BCG.

The immunogenic effects of BCG on tonsil cells have not been previously characterised. In spite of this, because the BCG vaccine has been extensively studied, it is possible to anticipate that BCG would

generate IFN γ and IL-17 responses in tonsillar CD4 $^{+}$ T cells. In addition, it would be likely that proliferation of CD4 $^{+}$ T cells would be observed. If it is possible to detect the immunogenic effects of BCG using the *in vitro* TMC model, this would help validate the concept of testing immunogenicity of other vaccine candidates using the same model.

5.2 Immunogenicity of the BCG vaccine in tonsillar CD4 $^{+}$ T cells.

BCG vaccination generated IFN γ and IL-17 responses in tonsillar CD4 $^{+}$ T cells. It also elicited production of the proliferative cytokine IL-2. The CD4 $^{+}$ T cells proliferated themselves as shown by elevated Ki67 levels. These results are shown in figure 5.2.1. The TMCs were stimulated with BCG at 3 concentrations; 1:10, 1:1 and 10:1. Stimulation with BCG at an MOI of 10:1 was immunogenic whilst the lower multiplicities of infection were not sufficient to measurably activate the CD4 $^{+}$ T cells. IFN γ levels increased in CD4 $^{+}$ T cells following stimulation with BCG at an MOI of 10:1. The percentage of IFN γ^{+} CD4 $^{+}$ T cells rose from 20.6% to 29% ($p=0.0027$). Stimulation with BCG at an MOI of 10:1 also raised intracellular IL-17 levels in CD4 $^{+}$ T cells. Intracellular IL-17 was here measured as APC Cy7 MFI. This rose from 771.5 in unstimulated cells to 930.75 MFI with BCG at 10:1 MOI ($p<0.0001$). The Th1 and Th17 responses noted here are characteristic of the BCG vaccine and are associated with the immune responses required for protection against TB.

The percentage of proliferating CD4 $^{+}$ T cells doubled following BCG stimulation, from 6% to 12% ($p=0.0011$). This was only seen with BCG at an MOI of 10:1. Lower doses of BCG did not alter the proliferation of the tonsillar CD4 $^{+}$ T cells. The proliferation of the CD4 $^{+}$ T cells was accompanied by an increase in the intracellular expression of the proliferative cytokine IL-2. IL-2 levels rose with BCG stimulation at an MOI of 10:1. PE MFI rose from 390 MFI to 587 MFI ($p<0.0001$). These proliferative responses would contribute to the development of a robust and sustained immune response to BCG.

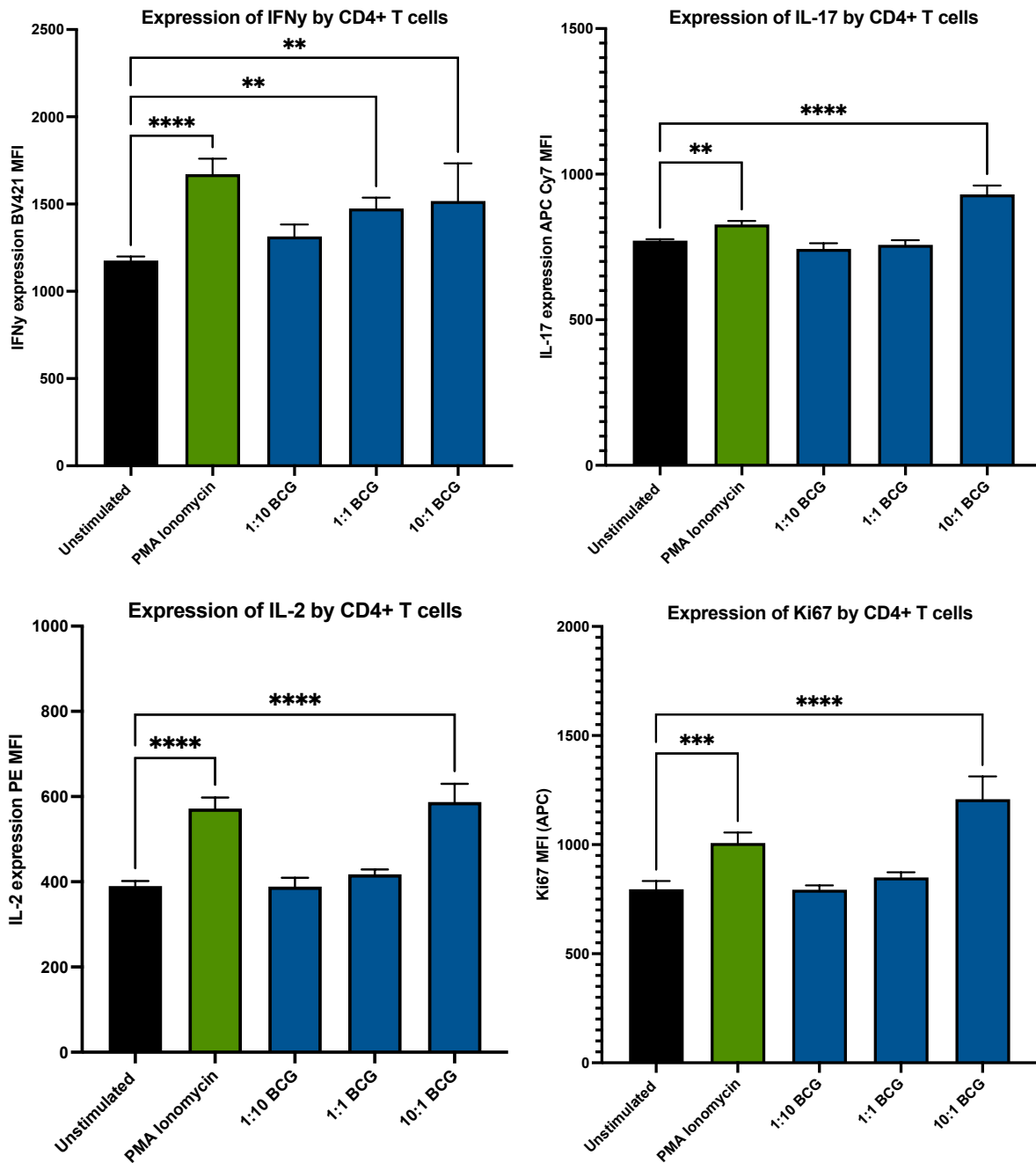


Figure 5.2.1. Immunogenicity of the BCG vaccine in tonsillar CD4+ T cells. TMCs from patients 8, 13, 15 and 16 were seeded in triplicate at 1×10^5 cells/well in 96 well plates ($n=4$). These were infected with BCG Pasteur at an MOI of 1:10, 1:1 and 10:1 for 5 days. After 5 days, the cells were stained with the TB immunogenicity flow cytometry panel. Flow cytometry was used to identify CD4+ T cells and measure the intracellular expression of A) IFN γ , B) IL-17 and C) IL-2, along with D) Ki67 expression. MFI values are shown. Data was analysed using one way ANOVA and Dunnetts multiple comparison test.

5.3 Immunogenicity of the BCG vaccine in tonsillar CD8+ T cells.

The BCG vaccine is known to generate responses in both CD4+ T cells and CD8+ T cells. In CD8+ T cells, BCG vaccination has been shown to cause proliferation, degranulation and IFN γ secretion by CD8+ T cells (Kumar et al., 2021) (Murray et al., 2006). BCG vaccination induces polyfunctional CD8+ T cells which express TNF α , IL-2 and IFN γ in response to mycobacterial antigens (Ritz et al., 2016). Delivery of BCG via the airways generates polyfunctional antigen specific CD8+ T cell responses within the lungs compared with systemic vaccination (Kaveh et al., 2020).

It is also important to note that vaccination with live BCG is known to induce the formation of regulatory CD8+ T cell populations. These CD8+ T cells have a suppressive activity. CD8+ T cells stimulated with live BCG have been shown to inhibit the antigen induced proliferation of a reporter Th1 clone (Boer et al., 2014). This regulatory function of CD8+ T cells is thought to limit the immunogenic response to BCG and hinder the development of protective immunity.

This study has investigated the effect of BCG stimulation on tonsillar CD8+ T cells. Stimulation with BCG induced expression of IL-17 and IL-2 by tonsillar CD8+ T cells. BCG also induced the proliferation of CD8+ tonsillar T cells. The data in figure 5.2.2 reveals that stimulation with BCG at an MOI of 10:1 led to enhanced IL-17 and IL-2 expression. The effect of BCG on the proliferation of CD8+ T cells is shown in figure 5.2.2. A lower dose of BCG, at an MOI of 1:1 enhanced CD8+ T cell IFN γ production. Stimulation with BCG at a MOI of 1:1 increased the percentage of IFN γ + CD8+ T cells from 63% up to 69.5% ($p=0.0230$). This was 10x lower than the concentration of BCG required to elicit IFN γ responses in CD4+ T cells. This suggests that the CD8+ T cells were more sensitive to BCG stimulation than CD4+ T cells. A higher MOI of BCG, 10:1 did not significantly increase the percentage of IFN γ + CD8+ T cells. The higher dose of BCG may have exhausted the capacity of the CD8+ T cells to generate IFN γ .

BCG stimulation provoked IL-17 responses in CD8+ T cells. Intracellular IL-17 was measured using APC Cy7 MFI. This rose from 791 in the unstimulated cells to 1,112 following BCG stimulation ($p<0.0001$). IL-17 production by CD8+ T cells has been previously documented but it was not previously known

that BCG vaccination induces IL-17 production by CD8+ T cells. The role of IL-17+ CD8+ T cells in TB has not been explored. IL-17+ CD8+ T cells are present in a variety of inflammatory diseases, from psoriasis to HCV (Srenathan et al., 2016).

The CD8+ T cells proliferated in response to BCG at a MOI of 10:1. The level of Ki67 expression increased from 1,121 MFI in unstimulated cells to 1,915 MFI in CD8+ T cells stimulated with BCG at an MOI of 10:1 ($p<0.0001$). The proliferative cytokine IL-2 was also generated by CD8+ T cells in response to BCG at a MOI of 10:1. PE MFI was used to measure intracellular IL-2, this rose from 672 in the unstimulated TMCs to 1,115 in the BCG stimulated cells ($p<0.0001$).

The immune responses observed here in tonsillar CD4+ and CD8+ T cells matched the expected profile for BCG. The tonsil model was able to detect the Th1 responses and the Th17 responses which are critical to effective immune control of TB infection. The proliferation of the tonsillar CD4+ and CD8+ T cells could also be detected. This confirms that TMCs can be used *in vitro* to assess the immunogenicity of novel TB vaccine candidates.

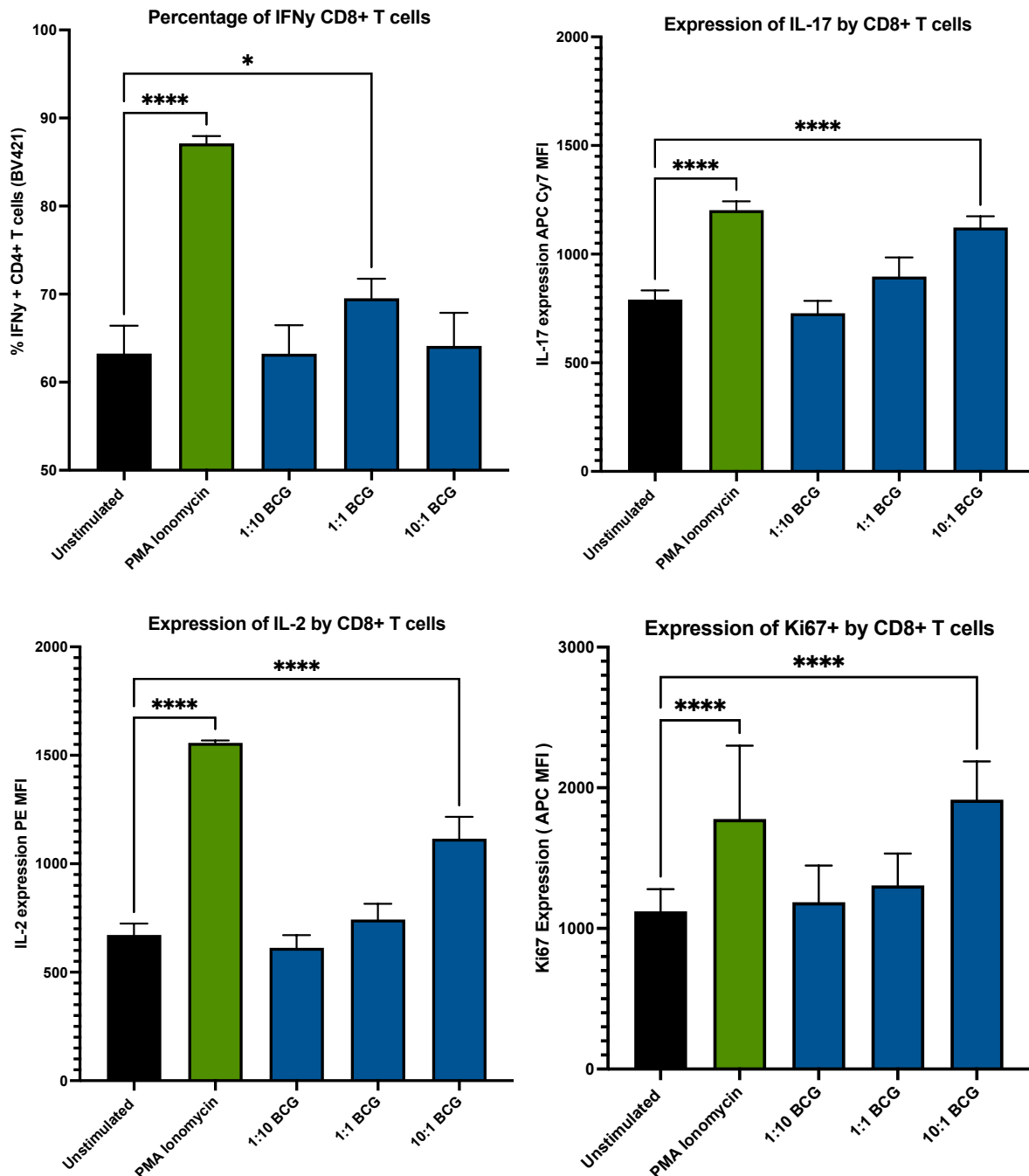


Figure 5.2.2. Immunogenicity of the BCG vaccine in tonsillar CD8+ T cells. TMCs were seeded at 1×10^5 cells/well in triplicate in 96 well plates. These cells were infected with BCG Pasteur at an MOI of 1:10, 1:1 and 10:1 for 5 days. After 5 days, the cells were stained with the TB immunogenicity flow cytometry panel. Flow cytometry was used to identify CD8+ T cells and measure the intracellular expression of A) IFN γ , B) IL-17 and C) IL-2, along with D) Ki67 expression. Mean MFI values and standard deviations are shown. Data was analysed using one way ANOVA and Dunnetts multiple comparison test. (n=4)

Key Findings

BCG stimulation at 10:1 MOI induces IFN γ , IL-17 and IL-2 production by tonsillar CD4+ T cells

Tonsillar CD4+ T cells proliferate following stimulation with BCG at an MOI of 10:1.

Stimulation with BCG at a MOI of 1:1 leads to increased IFN γ production by tonsillar CD8+ T cells

BCG at an MOI of 10:1 induces IL-17 production by tonsillar CD8+ T cells

CD8+ T cells proliferate and generate IL-2 in response to BCG stimulation at an MOI of 10:1.

This *in vitro* TMC model can be used to assess the immunogenicity of TB vaccines.

5.4 Effect of adjuvants on tonsillar lymphocytes

The tonsil model was used to assess the immunogenicity of adjuvants. TMCs were used to investigate the impact of adjuvants on B cells, CD8+ T cells and CD4+ T cell populations. Suitable adjuvants could then be incorporated into novel TB vaccine formulations. Five adjuvants were investigated; Resiquimod, Alum, peptidoglycan, Poly I:C and Quil A. These adjuvants work through different mechanisms and have all been previously incorporated into vaccines. Once TMCs had been stimulated with these adjuvants, flow cytometry was used to measure the expression of activation markers on the surface of lymphocytes. The intracellular production of important cytokines was also measured.

Aims

Investigate the immunogenicity of Alum hydroxide, Resiquimod, Peptidoglycan, Poly I:C and Quil A.

Investigate the effect of these adjuvants on CD4+ T cell activation markers and cytokines.

Investigate the effect of these adjuvants on CD8+ T cell activation markers.

Investigate the effect of the adjuvants on tonsillar B cell activation markers and cytokines.

5.5 Effect of adjuvants on CD4+ T cell intracellular cytokines

TMCs were stimulated with Alum, Peptidoglycan, Poly I:C, Resiquimod and Quil A. Flow cytometry was used to measure the effect of these adjuvants on intracellular cytokine production.

Stimulation with Quil A induced IFN γ , and IL-17 production by CD4+ T cells. This is depicted in figure 5.5.1. Quil A did not induce an increased production of TNF α . Intracellular IFN γ levels in unstimulated CD4+ T cells rose threefold from 1,316 to 4,005 in cells stimulated with Quil A ($p < 0.0001$). The level of IL-17 was slightly increased in the presence of Quil A. APC Cy7 MFI rose from 2,063 in the unstimulated cells to 2,559 in the Quil A treated cells ($p < 0.0001$).

Figure 5.5.2 shows that intracellular expression of IL-10 and IL-4 by CD4+ T cells was increased in the presence of Quil A. PE MFI was used to measure intracellular IL-10. This was 694 in unstimulated cells and increased threefold to an MFI of 2,337 with Quil A stimulation ($p < 0.0001$). IL-4 MFI levels doubled from 1,084 to 2,512 in the presence of Quil A ($p < 0.0001$). These results reveal that Quil A generates a broad cytokine profile which may be of interest in an adjuvant for a TB vaccine. It is unclear whether the effect of Quil A on IL-4 and IL-10 production would undermine the immunogenicity of a TB vaccine.

Resiquimod (R-848) is a TLR 7/8 agonist. At a concentration of 100 $\mu\text{g/ml}$ it was found to elicit IFN γ responses in CD4+ T cells. The MFI value of intracellular IFN γ doubled from 1,316 to 2,687 MFI ($p < 0.0001$). This finding is shown in figure 5.5.1. Resiquimod did not alter the expression levels of TNF α or IL-17. Figure 5.5.2 shows that the production of IL-4 and IL-10 by CD4+ T cells was also unaffected. The ability of Resiquimod to elicit IFN γ responses may make it suitable for further development as an adjuvant for a TB vaccine.

Alum, Peptidoglycan and Poly I:C were not effective in inducing intracellular cytokine production by CD4+ T cells. There were no detectable increases in intracellular IFN γ , IL-17, TNF α IL-4 or IL-10, following stimulation with these chemicals. In spite of this, in combination with delivery systems and antigens they might still be useful.

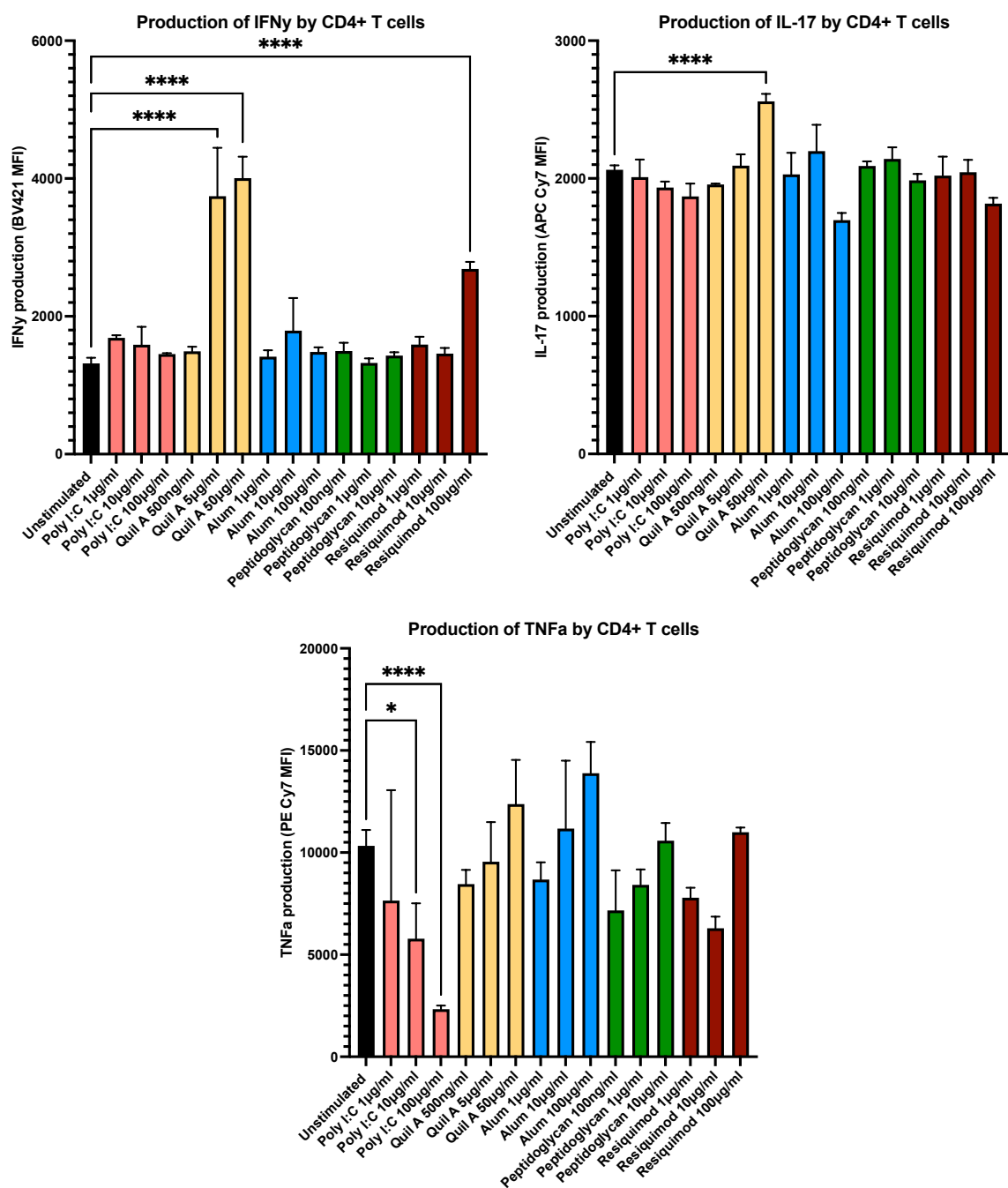


Figure 5.5.1. Effect of adjuvants on the production of proinflammatory cytokines by CD4+ T cells.

Triplicate wells were seeded with 1×10^5 TMCs. These were stimulated with Alum, Resiquimod, Peptidoglycan, Poly I:C and Quil A for 5 days. The cells were stained with Intracellular IFN γ , IL-17 and TNF α were measured by flow cytometry. Mean MFI values are shown along with standard deviations. (n=3). * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnetts test.

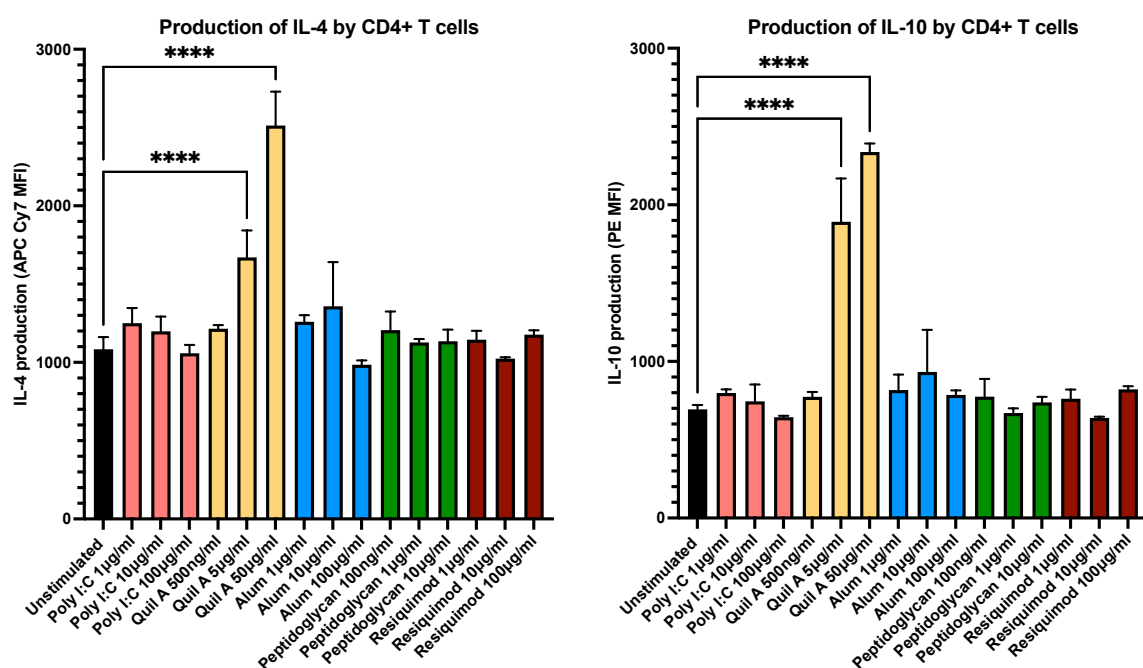


Figure 5.5.2. Effect of adjuvants on CD4+ T cell intracellular cytokines. Flow cytometry was used to measure the effect of adjuvants on cytokine expression by CD4+ T cells. TMCs were stimulated with Alum, Resiquimod, Peptidoglycan, Poly I:C and Quil A for 5 days. CD3-FITC and CD4-PerCP Cy5.5 antibodies were used to identify CD4+ T cells. Intracellular IL-4 and IL-10 were measured. Mean MFI values and $\pm$ standard deviations are shown. (n=3). * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnetts test.

5.6 Effect of adjuvants on activation of CD4+ and CD8+ T cells

The effect of adjuvant stimulation on CD69 expression by CD4+ and CD8+ T cells is shown in figure 5.6.1. Stimulation with Resiquimod activated CD4+ T cells and CD8+ T cells. On CD4+ T cells, the expression of CD69 rose from 20,395 on unstimulated cells to 32,085 in the presence of 100 µg/ml of Resiquimod ($p=0.0037$). CD8+ T cells were activated by a concentration of Resiquimod 100x lower. 1 µg/ml of Resiquimod increased CD69 expression from 4,892 to 7,218 ($p<0.0253$).

Quil A, Alum, Peptidoglycan and Poly I:C had no impact on the expression of CD69 on tonsillar T cells.

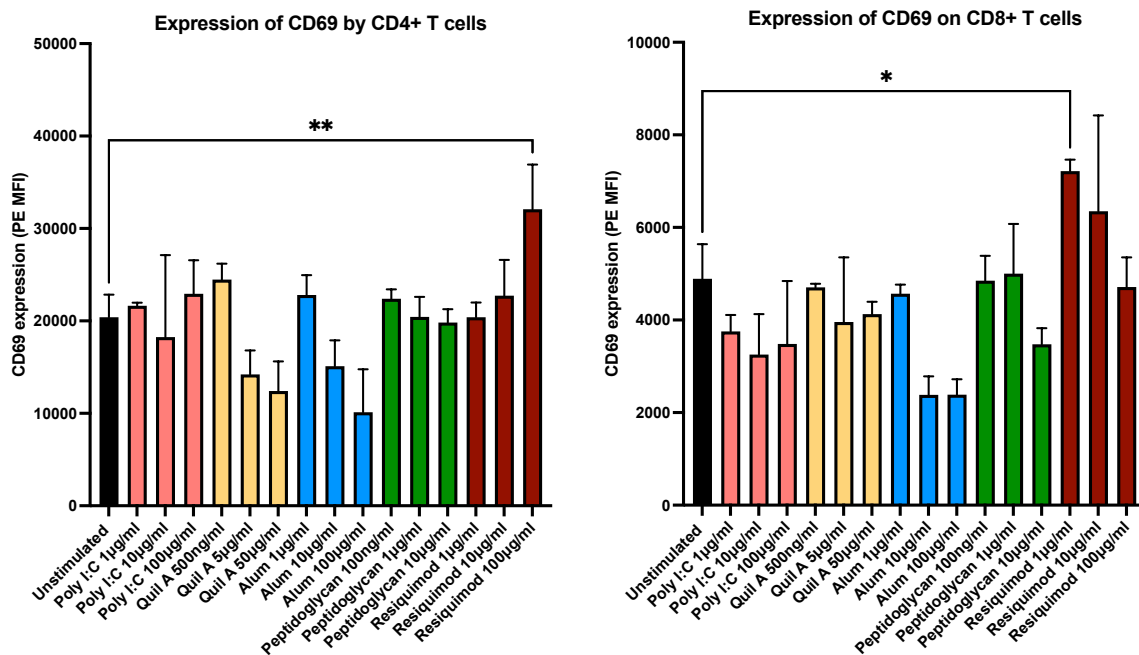


Figure 5.6.1. Effect of Adjuvants on CD4+ and CD8+ T cell activation. TMCs were seeded in triplicate wells at 1×10^5 cells/well ($n=3$). Cells were stimulated with Alum, peptidoglycan, Poly I:C, Quil A or Resiquimod for 5 days. Cells were stained with CD3-FITC and CD4-PerCP Cy5.5 antibodies to identify T lymphocyte populations. The expression of CD69 by CD4+ and CD8+ T cells was measured by flow cytometry. The mean MFI values and standard deviations are presented along with a statistical analysis which was performed using One way ANOVA and Dunnetts multiple comparison test

5.7 Expression of costimulatory molecules by CD8+ tonsillar T cells

CD40 on CD8+ T cells provides an important signal which allows activation of CD8+ T cells by DCs expressing CD40L. This activation is independent of CD4+ T cells and is used in the generation of antigen specific CD8+ T cell populations (Seah et al., 2013). Figure 5.7.1 shows the effect of Resiquimod and Quil A stimulation on CD8+ T cells. Resiquimod stimulation increased CD40 expression in CD8+ T cells. The expression of CD40 rose from 11,374 to 27,524 ($p < 0.0001$). Stimulation with Quil A also increased the levels of CD40 on CD8+ T cells. The MFI for PE Cy7 rose from 11,374 in unstimulated cells, to 21,609 in CD8+ T cells stimulated with 500ng/ml of Quil A ($p = 0.0051$). Upregulation of CD40 on CD8+ T cells might be desirable in vaccines designed to elicit strong antigen specific CD8+ T cell responses.

CD40 expression on CD4+ T cells was also investigated. The tonsillar CD4+ T cells did not express any CD40 (data not shown). This is consistent with the known biology of CD4+ T cells and confirms that the flow cytometry gating is able to distinguish between CD4+ T cells and CD8+ T cells.

Figure 5.7.1B and C show the expression of CD80 and CD86 on CD8+ T cells. CD80 expression on CD8+ T cells was increased following stimulation with Alum hydroxide. Stimulation with 10 $\mu\text{g/ml}$ of Alum raised CD80 levels from 859 to 2,383 MFI on CD8+ T cells ($p = 0.0002$). A similar effect was seen following stimulation with 10 $\mu\text{g/ml}$ of peptidoglycan. APC MFI rose 2.7 fold from 859 to 2,385 ($p = 0.0002$). CD80 is expressed on memory and effector CD8+ T cells, but not naïve cells. CD80 regulates the expansion of CD8+ memory T cells (Son & Ha, 2019). It also plays a role in the apoptosis of CD8+ T cells. PD-L1 binds to CD80 on activated CD8+ T cells resulting in their apoptosis (Rollins & Gibbons Johnson, 2017).

CD86 expression on CD8+ T cells was also elevated following exposure to peptidoglycan. BV421 MFI increased from 5,720 to 8,850 ($p < 0.0001$). This is shown in Figure 5.7.1. C

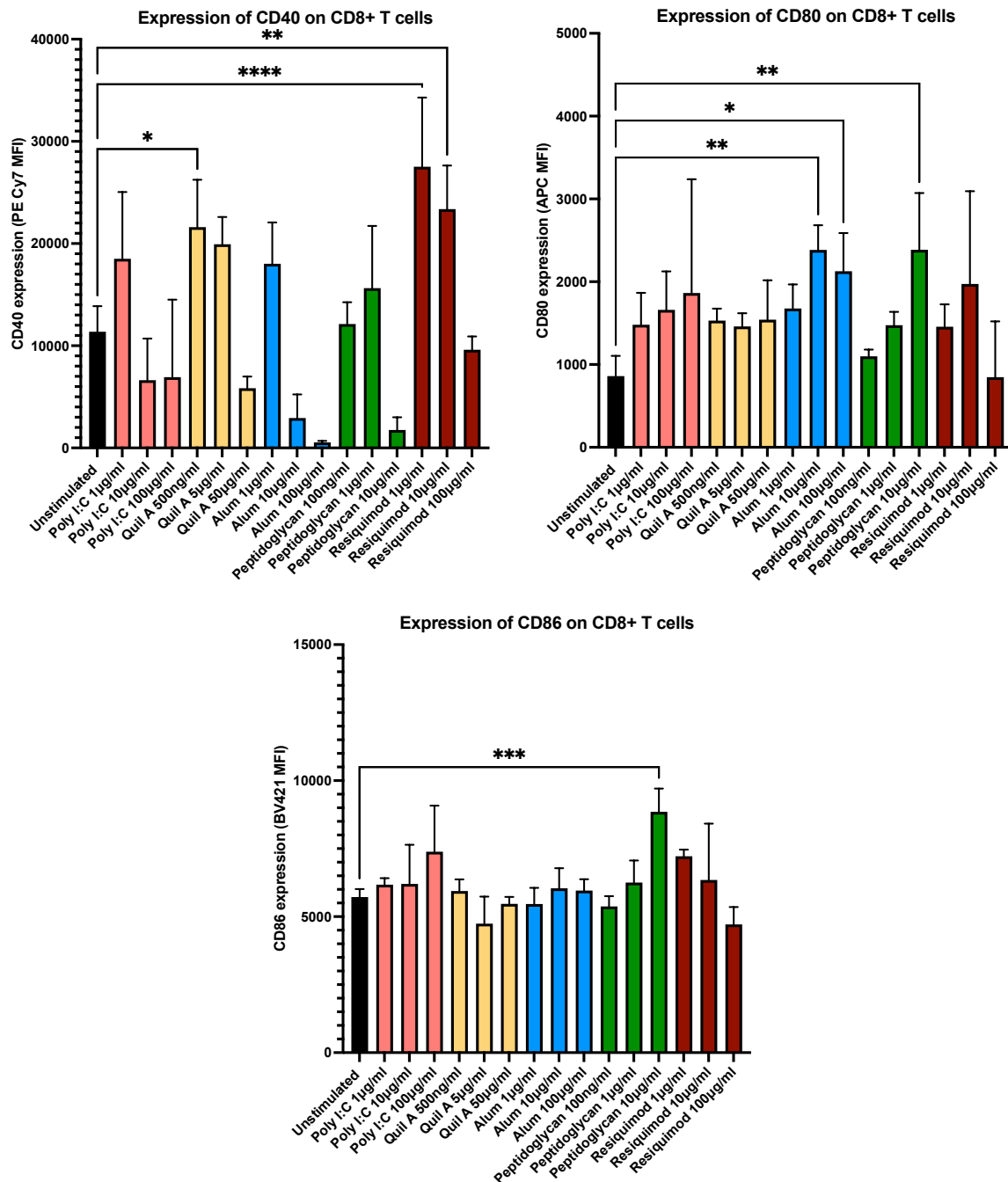


Figure 5.7.1. Effect of adjuvants on CD8+ T cell costimulatory markers. TMCs were seeded in triplicate wells at 1×10^5 cells/well ($n=3$). Cells were stimulated with Alum, peptidoglycan, Poly I:C, Quil A or Resiquimod for 5 days. Cells were stained with CD3-FITC and CD4-PerCP Cy5.5 antibodies to identify CD8+ T cells. The expression of CD40, CD80 and CD86 by CD8+ T cells was measured by flow cytometry. The mean MFI values are presented along with a statistical analysis. This was performed using One way ANOVA and Dunnetts multiple comparison test.

5.8 Effect of adjuvants on tonsillar B cells

One of the aims of this study is to develop *in vitro* models which are useful for the development of novel Dengue vaccines. B lymphocyte responses are critical in Dengue Fever. Neutralising antibodies generated by B cells are important for protective immunity against Dengue virus (Katzelnick et al., 2016) (Beltramello et al., 2010). Tonsillar tissue is predominantly composed of B lymphocytes. These tonsillar B lymphocytes may be useful for testing the immunogenicity of novel Dengue vaccines. These experiments investigated adjuvants which can be used to induce immunogenic responses in tonsillar B cells. TMCs were stimulated with Resiquimod, Peptidoglycan, Alum, Quil A or Poly I:C for 5 days. Flow cytometry was used to measure the impact of these chemicals on the expression of activation markers and the production of cytokines by B cells.

5.9 Effect of adjuvants on B cell activation markers

Four B cell activation markers were investigated; CD40, CD69, CD80 and CD86. CD69 is an early activation marker for B lymphocytes. CD40, CD80 and CD86 act as costimulatory molecules on B lymphocytes and contribute to B cells function as antigen presenting cells.

Figure 5.9.1 shows that stimulation with 10 µg/ml Resiquimod activated B cells, leading to increased expression of CD69. The MFI of PE increased from 1,724 up to an MFI of 2,750 ($p=0.0046$). CD86 was slightly enhanced following resiquimod stimulation. Expression of CD86 rose from 2,373 to 2,854 MFI ($p=0.0457$). The expression of the costimulatory molecule CD40 was also enhanced. PE Cy7 MFI rose fivefold from 2,214 to 12,554 ($p=0.0324$). Resiquimod had no impact on the expression of CD80 on B cells.

Resiquimod is known to activate dendritic cells and upregulate the expression of CD80, CD86, CD40 and HLA-DR (Ahonen et al., 1999). These experiments provide evidence that Resiquimod is also capable of activating tonsillar B cells. It is possible to use Resiquimod as a positive control in experiments which investigate B cell activation markers.

Resiquimod at 10 µg/ml was capable of activating tonsillar B cells and induced expression of each of these activation markers. This concentration was ten-fold lower than the concentration of Resiquimod needed to activate T lymphocytes, indicating that tonsillar B lymphocytes are more responsive to TLR7/8 signalling. TLR7 signalling is important for the development of B cells. It is required for the development of spontaneous germinal centres by B cells. It also contributes to the proliferation and survival of B cells (Soni et al., 2014).

Three of the tested chemicals had no effect on the activation markers or costimulatory molecules of B cells. Alum, Peptidoglycan and Poly I:C had no impact on the expression of CD40, CD69, CD80 or CD86 on tonsillar B cells.

Quil A did not enhance the expression of CD40, CD69, CD80 or CD86 on B cells. Instead it decreased the expression levels of the two B7 molecules CD80 and CD86. This is shown in figure 5.9.1.

CD80 expression on B cells decreased in the presence of 50 µg/ml of Quil A. Unstimulated B cells had an APC MFI of 1,516. This fell to 1,340 on cells treated with 50 µg/ml of Quil A ($p=0.0008$).

Quil A downregulated the expression of CD86 on B cells. 50 µg/ml of Quil A reduced the expression of CD86 from 2,373 to 1,311 ($p<0.0001$). The biological significance of this is unknown but it may limit the ability of B cells to deliver costimulatory signals whilst they present antigen.

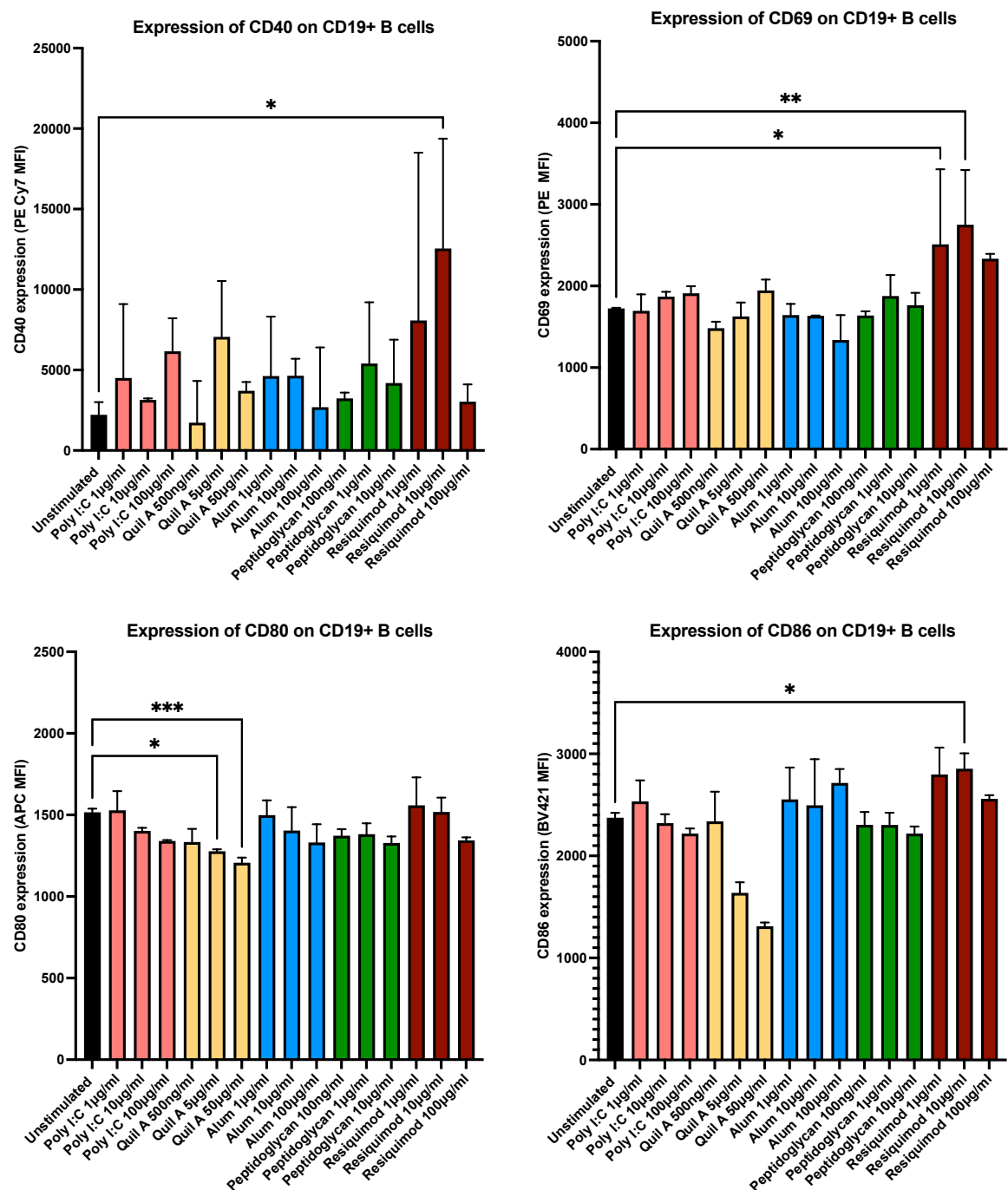


Figure 5.9.1. Positive controls for B cell activation markers. Triplicate wells were seeded with TMCs at 1×10^5 cells/well ($n=3$). The TMCs were stimulated with Alum, peptidoglycan, Poly I:C, Quil A or Resiquimod, for 5 days at three concentrations. B lymphocytes were identified by flow cytometry and their expression of CD40, CD69, CD80 and CD86 was measured. Mean values and $\pm$ standard deviations are shown. Statistical analysis was performed using One way ANOVA and Dunnetts multiple comparisons test.

5.10 Effect of adjuvants on cytokine expression by B cells.

Be-1 effector B cells produce IFN γ and drive the development of Th1 responses (Lund, 2008). Figure 5.10.1 shows that Resiquimod stimulation elicited IFN γ responses from tonsillar B cells. 3% of unstimulated B cells were IFN γ . Stimulation of B cells with Resiquimod increased the percentage of IFN γ + B cells twelvefold, from 3% to 43%. Resiquimod stimulation also induced IL-2 production, with a three fold increase in the percentage of IL-2+ B cells, from 3% to 10% of B cells ($p=0.0019$). Resiquimod elicits IFN γ and IL-2 responses from B cells and drives them towards a Be-1 phenotype.

Quil A was an effective adjuvant for eliciting Be-2 cytokine responses. This can be seen in figure 5.10.2. Quil A upregulated the Intracellular expression of IL-4, IL-10 and IL-2 by B cells. Quil A increased the percentage of IL-4+ B cells fivefold from 2.8% to 17.6% ($p<0.0001$). The percentage of IL-10+ B cells increased from 3.7% to 22% with Quil A ($p<0.0001$). IL-2 levels were also increased by Quil A, it raised the percentage of IL-2+ B cells from 3% to 13.5% ($p<0.0001$). Production of IL-4 and IL-2 is associated with a Be-2 effector phenotype. IL-10 production is a hallmark of regulatory B cell responses but IL-10 can also be generated by Be-2 and Be-1 B cells (Glass et al., 2022) (O'garra et al., 1992). Based on this data, Quil A drives B cells towards a Be-2 phenotype.

Figure 64 shows that Poly I:C induced IL-4 production by tonsillar B cells. The percentage of IL-4+ B cells doubled from 3.4% to 8.9% ($p=0.0339$).

Cytokine production by tonsillar B cells was not increased following stimulation with Alum or Peptidoglycan. The levels of IFN γ , IL-4, IL-10, IL-2, IL-12 and TNF α were found to be unaltered in B cells stimulated with alum and peptidoglycan.

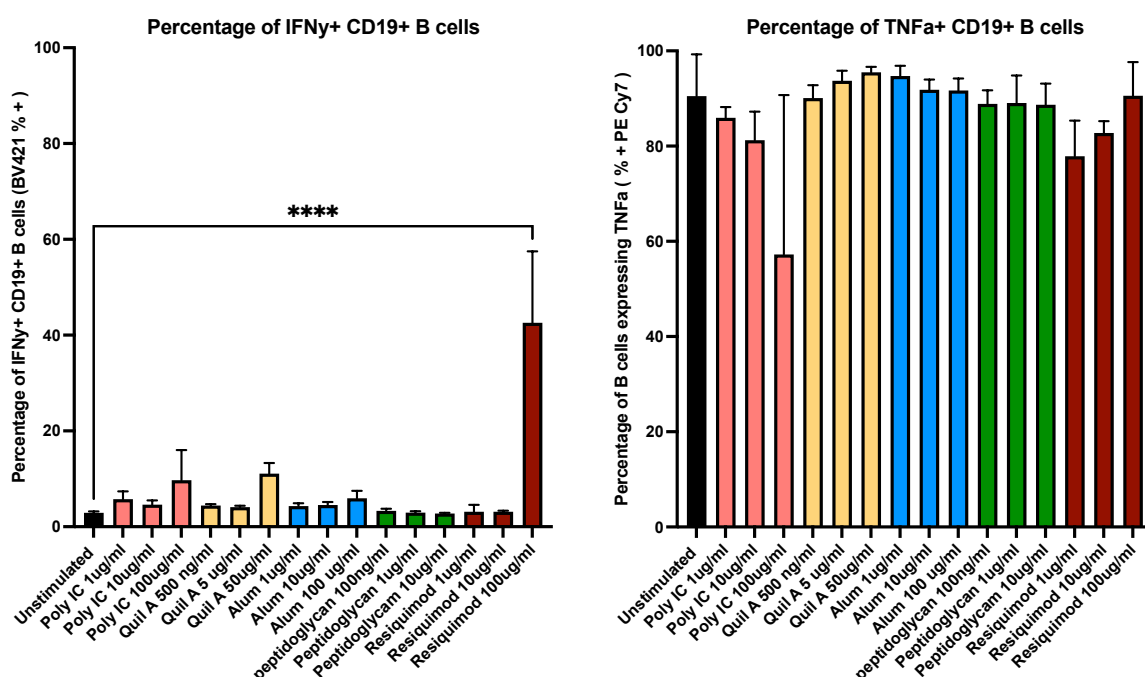


Figure 5.10.1. Effect of adjuvants on the production of proinflammatory cytokines by B cells. TMCs were cultured in triplicate in 96 well plates at a density of 1×10^6 /ml. They were stimulated for 5 days with Resiquimod, Alum hydroxide, Peptidoglycan, Poly I:C or Quil A. Flow cytometry was used to identify B cells. The percentage of B cells expressing IFN γ and TNF α was measured (n=3). Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnetts Test.

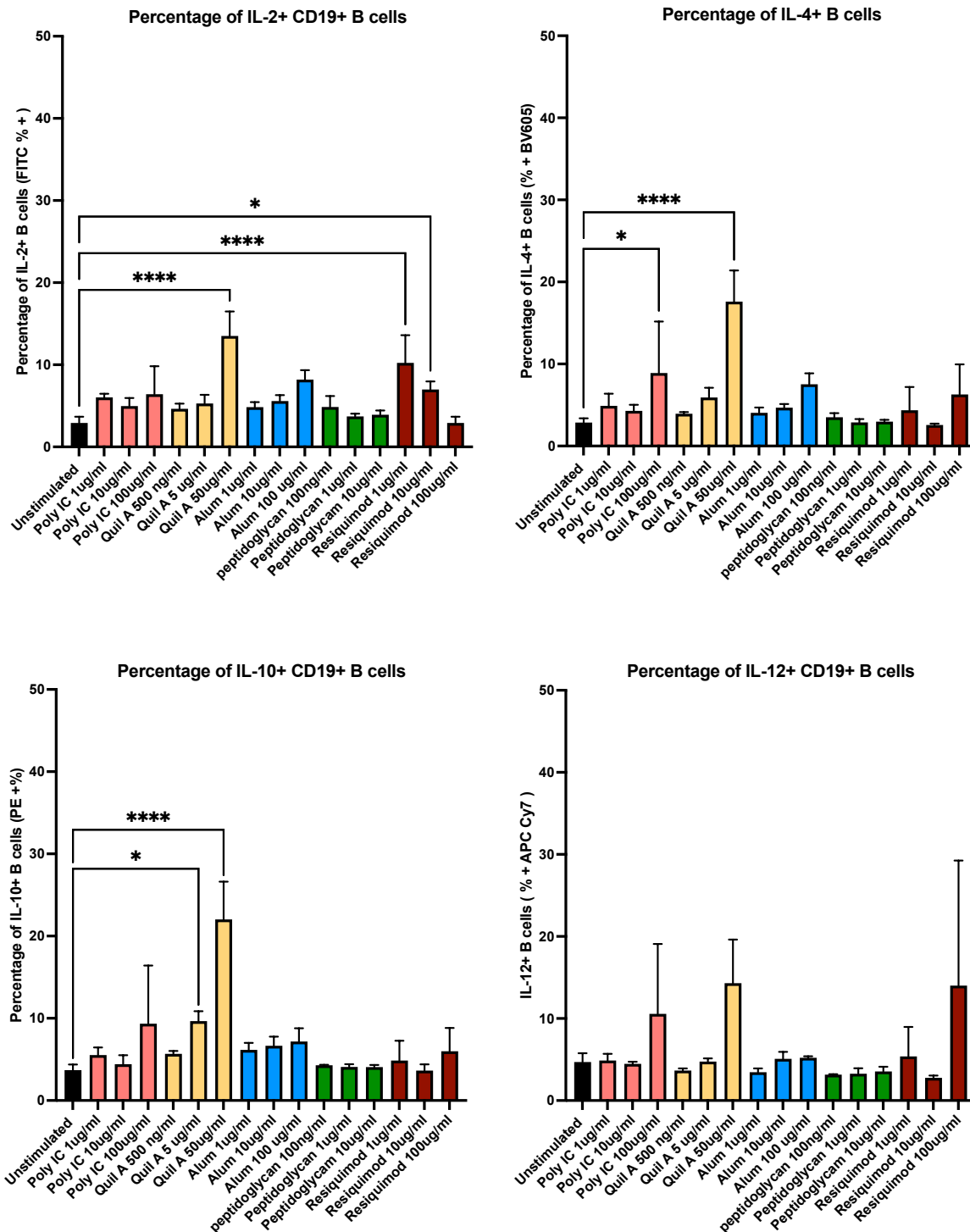


Figure 5.10.2. Effect of adjuvants on B cell intracellular cytokines. TMCs were seeded at a density of 1×10^5 cells per well and stimulated with Alum hydroxide, Resiquimod, Peptidoglycan, Poly I:C or Quil A for 5 days ($n=3$). Flow cytometry was used to measure the percentage of B cells expressing intracellular IL-4, IL-10, IL-2 and IL-12. Mean values and $\pm$ standard deviations are shown. Statistical analysis was performed using one way ANOVA and Dunnetts Test.

Key Findings

- Alum had no impact on the production of cytokines by CD4+ T cells. It also had no impact on the expression of activation markers on CD4+ T cells. Stimulation with Alum upregulated CD80 expression on CD8+ T cells. It had no effect on cytokine production by B cells or their expression of activation markers.
- Peptidoglycan upregulated the expression of CD80 and CD86 on CD8+ T cells. It had no detectable effects on CD4+ T cells or B cells.
- Poly I:C decreased the expression of TNF α by CD4+ T cells, but otherwise had no impact on their expression of cytokines. Poly I:C also had no effect on CD8+ T cells. It did increase the percentage of IL-4+ B cells.
- Quil A was the most immunogenic adjuvant in this study. It elicited IFN γ , IL-17, IL-4 and IL-10 in CD4+ T cells. Quil A increased CD40 expression on CD8+ T cells. In tonsillar B cells, Quil A induced a Be-2 phenotype increasing expression of IL-4, IL-10 and IL-2.
- Resiquimod generates IFN γ responses in CD4+ T cells. It upregulated the expression of CD69 on CD4+ T cells and CD8+ T cells. It also upregulated CD40 on CD8+ T cells. Resiquimod induces a Be-1 phenotype in B cells, with increased percentages of IFN γ and IL-2+ B cells. Expression of CD40, CD69 and CD86 on B cells was upregulated in the presence of Resiquimod.

5.11 Immunogenicity and proliferative effect of spore vaccine formulations.

Spore FP1 is known to confer protection in mice against TB as a BCG boost. This protective effect has been associated with Th1 and Th17 responses (Copland et al. 346). This study tested whether it was possible to replicate the immunogenic effects of spore FP1 in the context of a tonsil mononuclear cell culture. Poly I:C was also tested as an adjuvant to spore FP1. Spore FP1 with Poly I:C was tested *in vivo* as part of an unpublished concomitant project, EMI TB. This *in vitro* study using tonsils was conducted to support the *in vivo* study. The immunogenic effects of spore vaccine formulations on CD4+ and CD8+ T cells is shown in figure 5.11.1. This study also investigated the effect of spore vaccine formulations on T cell proliferation. The findings of these experiments are shown in figure 5.11.2. Spore FP1 was effective at generating IFN γ + CD4+ T cells responses. Spore formulations did not induce IL-17 production or proliferative responses.

Stimulation with spores alone led to a twofold increase in the percentage of IFN γ + CD4+ T cells ($p=0.0021$), as shown in figure 5.11.1. Spore FP1 stimulation also doubled the percentage of IFN γ + CD4+ T cells from 33% to 67% ($p=0.0024$). This can be seen in figure 65. In contrast, Spore FP1 did not generate IL-17 responses in CD4+ T cells (figure 5.11.1). Spore FP1 did not exert any detectable immunogenic effects on tonsillar CD8+ T cells. Stimulation with Spore FP1 for 5 days did not significantly elevate the percentage of IFN γ + CD8+ T cells.

Spore FP1 did not have any impact on the proliferation of CD4+ or CD8+ T cells, as the expression of Ki67 was unchanged (figure 5.11.2). IL-2 responses were also unaltered by stimulation with spore FP1, as can be seen in Figure 5.11.2. Spore FP1 did not induce proliferative responses in tonsillar CD8+ T cells. Neither the percentage of IL-2+ CD8+ T cells, nor the percentage of Ki67+ CD8+ T cells was significantly increased following stimulation with Spore FP1.

In this experiment, inclusion of Poly I:C as an adjuvant inhibited spore FP1 induced Th1 responses in TMCs. TMCs stimulated with Spore FP1 Poly I:C did not have a higher percentage of IFN γ + CD4+ T cells than the unstimulated controls (figure 5.11.1). IL-17 responses in CD4+ and CD8+ T cells were not

significantly increased following stimulation with Spore FP1 Poly I:C. This is seen in figure 5.11.1 B and D. The mean percentage of IL-2+ CD4+ T cells, increased 1.5 fold following stimulation with Spore FP1 Poly I:C. The percentage of IL-2+ CD4+ T cells rose from 22.9% to 34.85% in the presence of Spore FP1 Poly I:C ($p=0.0407$). This is shown in figure 5.11.2 B. This increase in IL-2+ CD4+ T cells was not seen following stimulation with spores alone or spores and FP1. Combining spore FP1 with Poly I:C did not result in an altered immunogenic profile in CD8+ T cells as shown in figure 5.11.1 as well as figure 5.11.2. Based on these findings, Poly I:C is not a suitable adjuvant to combine with spore FP1.

Stimulation with spores alone, spore FP1 or spore FP1 Poly I:C did not significantly alter the percentage of CD4+ or CD8+ T cells which were positive for IL-17. Figure 5.11.1B and D depict this result. The spore formulations were not found to induce T cell proliferation. Figure 5.11.2A shows that Ki67 expression in CD4+ T cells was not increased following stimulation.

This data establishes that the spore antigen delivery system is self adjuvanting, generating Th1 responses alone and in combination with the FP1 antigen.

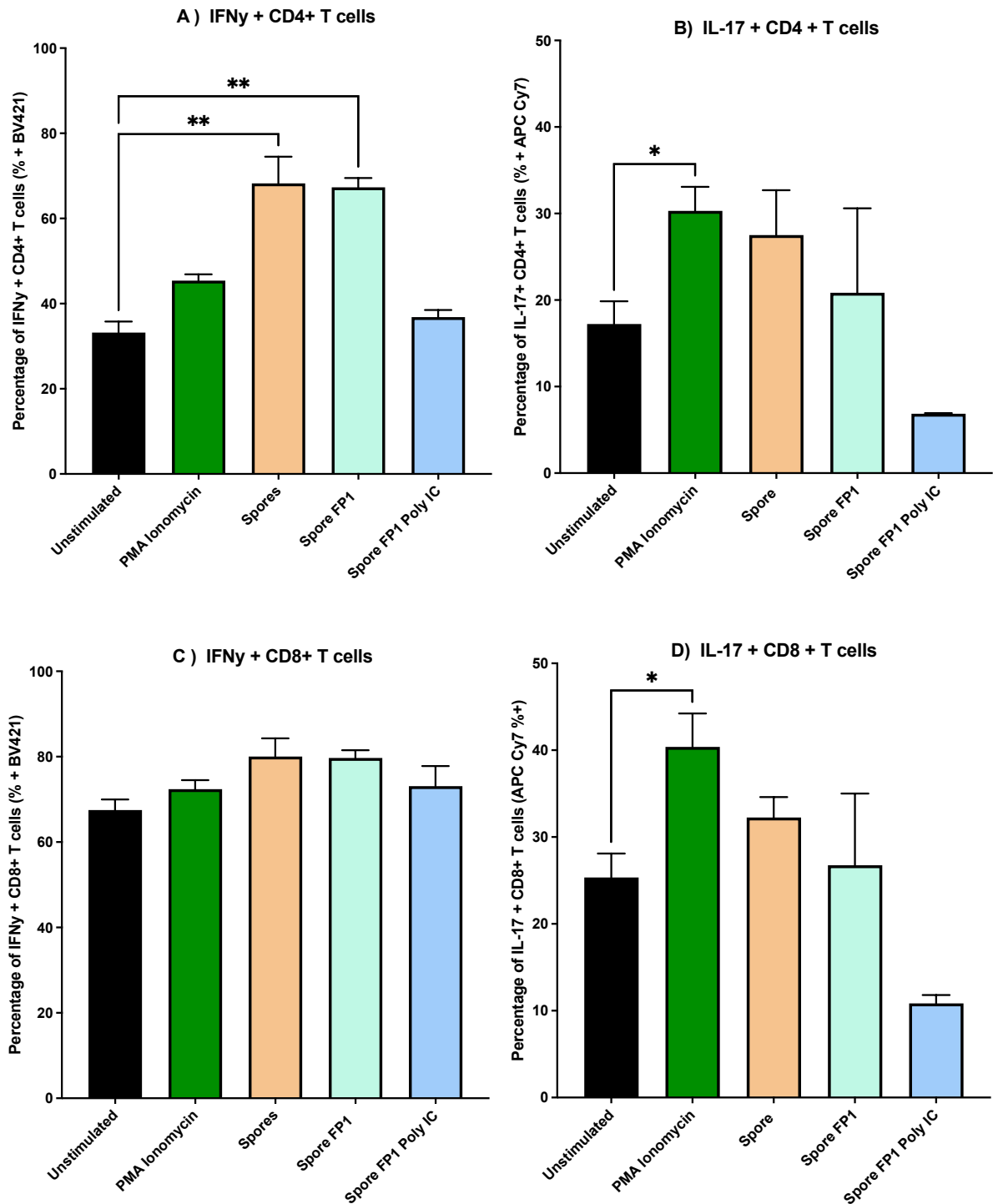


Figure 5.11.1. Immunogenicity of Spore FP1. TMCs were stimulated with *B. subtilis* spores, Spore FP1, or Spore FP1 with Poly I:C for 5 days. Cells were analysed by flow cytometry, (n=3). CD3+ T cells were gated for CD4+ and CD8+ populations. The CD4+ and CD8+ T cells were gated for IFN γ + and IL-17+ cells. Mean values and $\pm$ standard deviations are shown. Results were analysed with one way ANOVA and Dunnett's multiple comparison test.

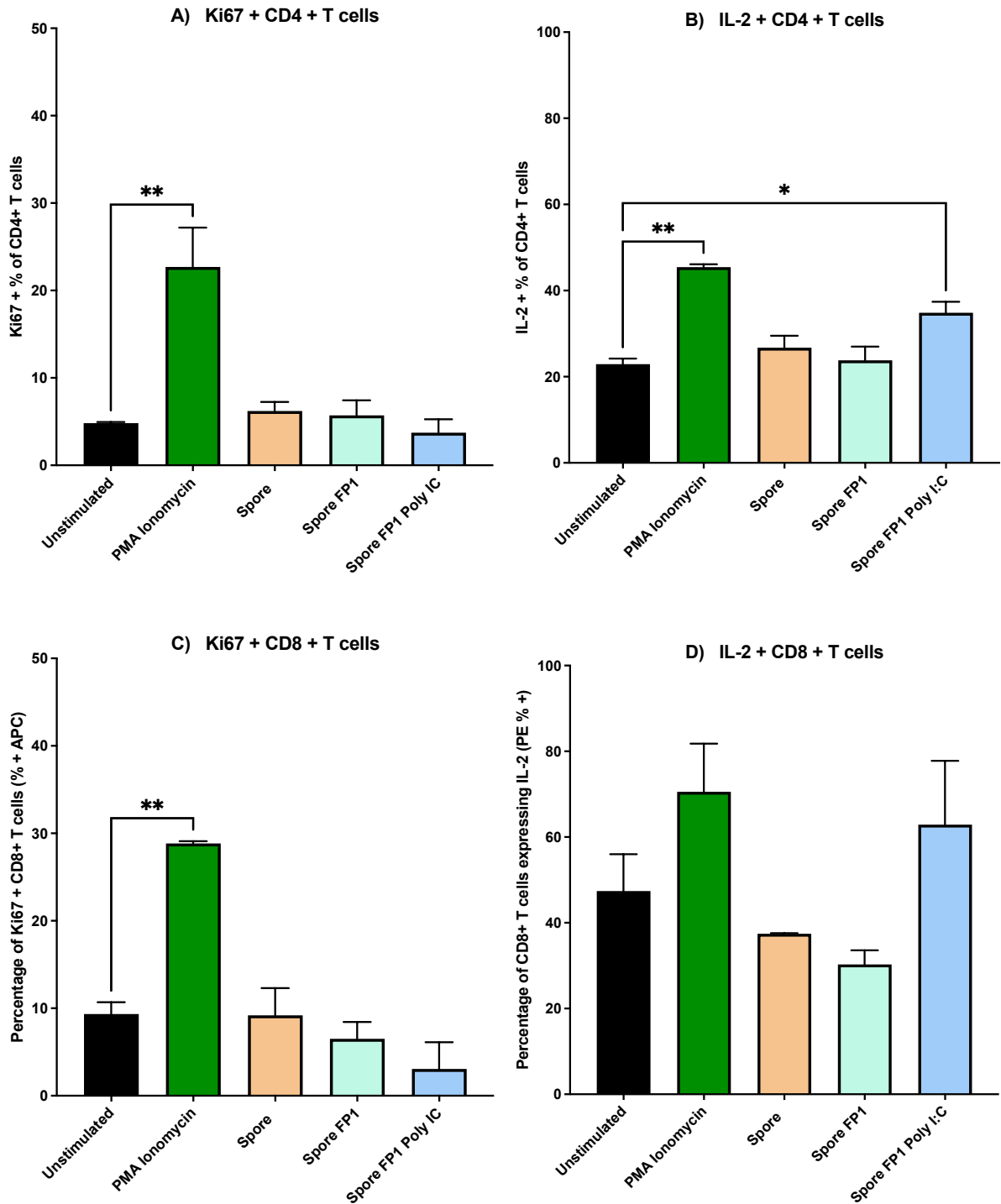


Figure 5.11.2. Effect of Spore formulations on T cell proliferative responses. Triplicate TMCs from 3 patients were stimulated with *B. subtilis* spores, Spore FP1, or Spore FP1 combined with Poly I:C for 5 days. Cells were analysed using flow cytometry, (n=3). CD3+ T cells were gated for CD4+ and CD8+ populations. The mean percentage of Ki67 and IL-2+ CD4+ and CD8+ T cells is shown with $\pm$ standard deviations. Results were analysed with one way ANOVA and Dunnett's multiple comparison test.

5.12 Immunogenicity and proliferative effect of YC NaMa vaccine formulations.

Tonsil mononuclear cells have been used as part of an *in vitro* model to determine the immunogenic effects of novel vaccine candidates. Here, CD4+ and CD8+ T cell responses were measured by flow cytometry following 5 days of stimulation with the novel vaccine candidate YC NaMa FP1. YC NaMa FP1 is known to confer protection against TB infection (Hart P. et al., 2018). This protection is associated with IRF3 signalling and TB antigen specific production of IFN γ , IL-17 and IL-10 by lymphocytes (Hart et al. 822-833). This TMC *in vitro* model builds upon these findings. Figure 5.12.1 shows the effects of YC NaMa FP1 on IFN γ and IL-17 production by T cells. Proliferation of T cells following stimulation with YC NaMa FP1 was determined by intracellular staining for Ki67. This is shown in Figure 5.12.2, along with the production of the proliferative cytokine IL-2. YC NaMa, in combination with the FP1 antigen generated IFN γ , IL-2 in CD4+ T cells and IL-17 responses in CD4+ and CD8+ T cells. These responses are predicted to be important for protective immunity against TB.

Stimulation with YC NaMa alone increased the percentage of IFN γ + CD4+ T cells from 33% to 62.9% ($p=0.0046$). YC NaMa alone did not enhance the percentage of IFN γ + CD8+ T cells. IL-2 and IL-7 expression by CD4+ and CD8+ T cells was also unaltered with the addition of YC NaMa alone.

Combining YC NaMa with the FP1 antigen generated substantial immune responses. YC NaMa FP1 increased the percentage of IFN γ + CD4+ T cells more than 1.5 fold from 33.2% to 62.7% ($p=0.0047$). YC NaMa FP1 amplified the percentage of IL-2+ CD4+ T cells from 23% to 33 % ($p=0.0187$). This can be seen in figure 5.12.2, B. YC NaMa FP1 increased the percentage of IL-17+ CD4+ T cells from 17.23% in the unstimulated cells to 34.75% ($p=0.0243$). This is seen in figure 5.12.1B. YC NaMa FP1 also induced a 1.5 fold increase in the percentage of IL-17+ CD8+ cells. 25.3% of unstimulated CD8+ T cells were IL-17+, this rose to 41.5% in the presence of YC NaMa FP1 ($p=0.0411$). This is shown in figure 5.12.1, D.

The immunogenicity of YC NaMa FP1 was altered following the addition of Poly I:C as an adjuvant. YC NaMa FP1 Poly I:C was effective at generating IFN γ responses in CD8+ T cells. The percentage of IFN γ + CD8+ T cells rose from 30.05% in the unstimulated cells, to 49.35% following stimulation with YC NaMa

FP1 Poly I:C ($p=0.0327$). These IFN γ + CD8+ T cell responses may be important in resolving intracellular infections of TB. Conversely, YC NaMa FP1 Poly I:C did not increase the percentage of IFN γ + CD4+ T cells ($p=0.9992$). There was also no induction of IL-17 or IL-2 in CD4+ or CD8+ T cells following stimulation with YC NaMa FP1 Poly I:C. In this experiment, YC NaMa FP1 induced IFN γ +CD4+ T cell responses were abrogated following addition of the TLR agonist Poly I:C. These results indicate that Poly I:C is not a suitable adjuvant for the YC NaMa FP1 vaccine.

YC NaMa, YC NaMa FP1 and YC NaMa FP1 Poly I:C were not found to induce proliferation in CD4+ T cells. There was no significant change in the percentage of Ki67+ T cells following these stimulations.

YC NaMa FP1 did not have any proliferative effects on CD8+ T cells. There was no statistically significant increase in the percentage of CD8+ T cells expressing either Ki67 or IL-2.

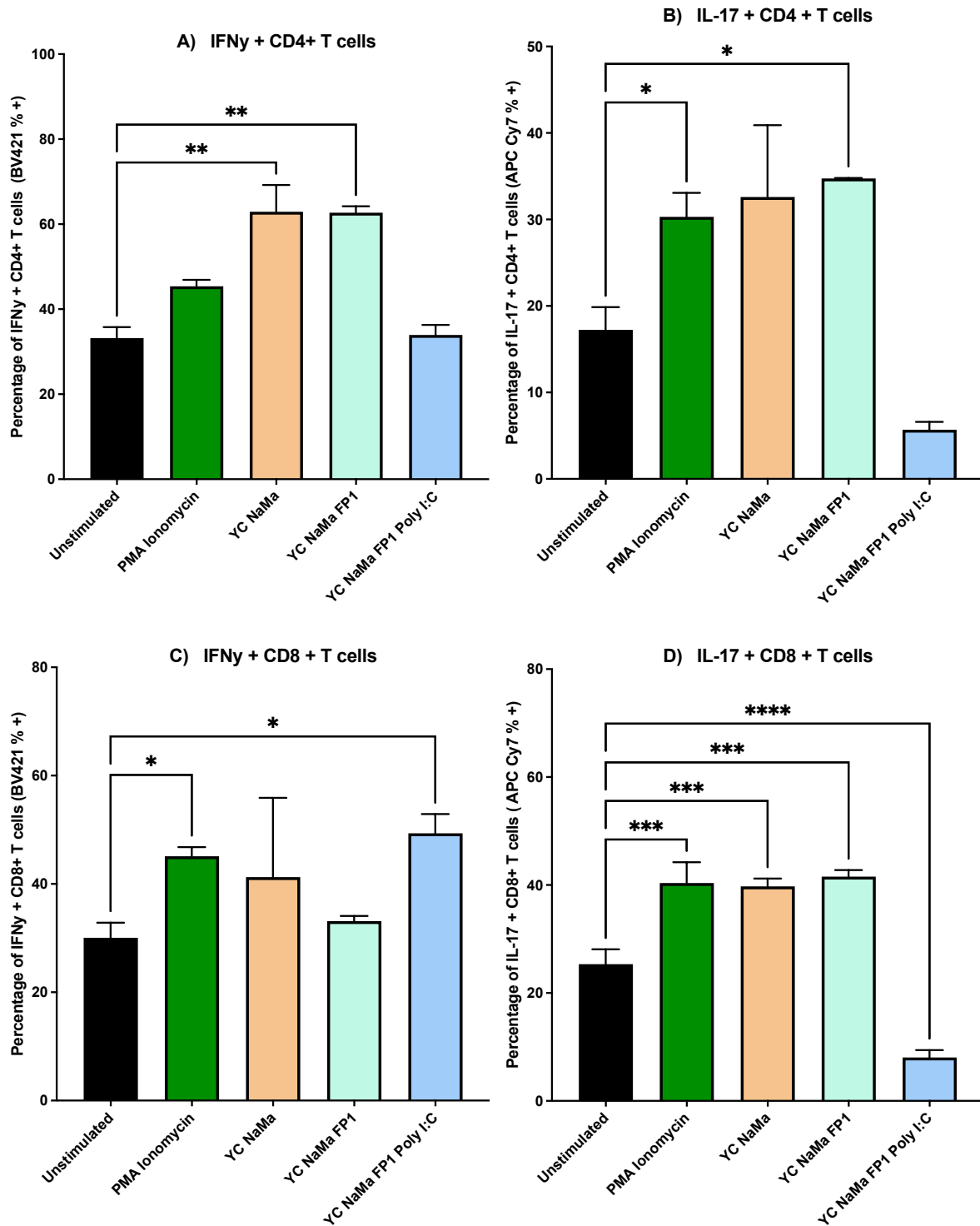


Figure 5.12.1. Immunogenicity of YC NaMa FP1 on CD8+ T cells. TMCs were seeded at 1×10^5 per well and stimulated with PMA and Ionomycin, YC NaMa, YC NaMa FP1, or YC NaMa FP1 with Poly I:C (n=3). After 5 days, CD4+ and CD8+ T cells were analysed by flow cytometry. The percentage of IFN γ +, IL-17+, IL-2+ and Ki67+ cells was determined. Mean values and $\pm$ standard deviations are shown. Results were analysed using one way ANOVA and Dunnett's multiple comparison test.

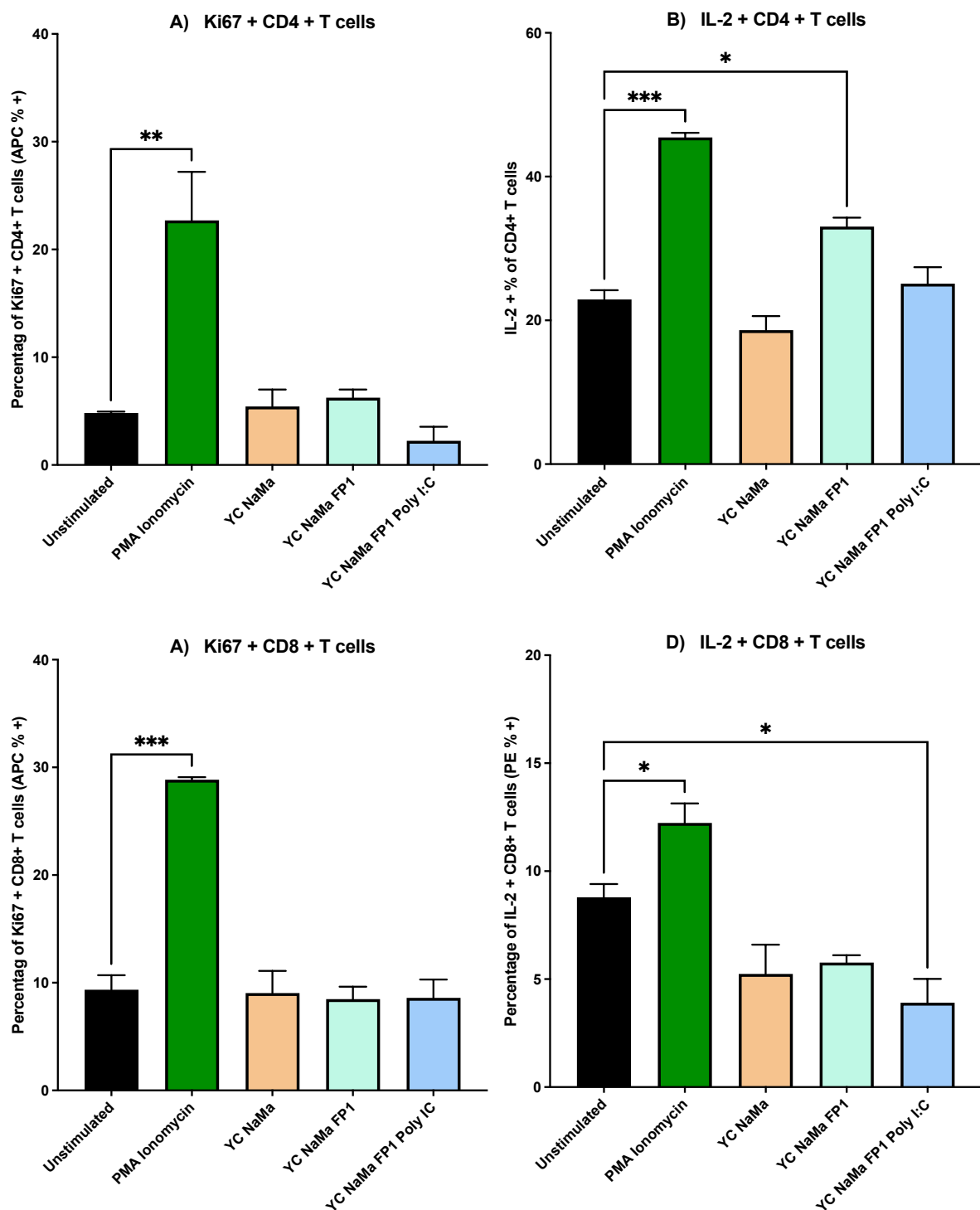


Figure 5.12.2. Effect of YC NaMa formulations on TMC proliferative responses. TMCs were seeded in triplicate at 1×10^5 per well and stimulated with PMA and Ionomycin, YC NaMa, YC NaMa FP1, or YC NaMa FP1 with Poly I:C for 5 days. (n=3). Cells were analysed by flow cytometry and the percentage of IL-2+ and Ki67+ T cells was determined. Mean values and $\pm$ standard deviations are shown. Results were analysed using one way ANOVA and Dunnett's multiple comparison test.

5.13 Immunogenicity and proliferative effect of liposome vaccine formulations.

Two vaccine formulations based upon liposomal phosphatidylserine (P/S) were tested. The first, consisted of Liposome P/S with FP1. The second, Liposome A/E was formed from equal ratio of Ag85B and ESAT6 antigen encapsulated in liposomes. Liposomal phosphatidylserine has been previously used as a component of anti-tuberculosis vaccine candidates which showed protection in mice (Greco et al. 1360). Previously published work has found that Liposome A/E has a protective effect against TB in mice. The mechanism underlying this protection was unknown. Previously, this study has demonstrated the immunogenic effects of liposome P/S on DCs. Here, the immunogenic effects of liposome P/S on CD4⁺ and CD8⁺ T cells have been examined. The effects of liposome FP1 and Liposome A/E on IL-17 and IFN γ production are shown in figure 5.13.1, whilst the effect of these formulations on T cell proliferation is shown in figure 5.13.2.

The delivery system Liposome P/S alone increased the percentage of IFN γ + CD4⁺ T cells, from 34.73% to 66.15% ($p=0.0004$). This effect was not antigen specific. Liposome P/S alone did not impact proliferation or expression of IL-2 or IL-17.

Liposome FP1 enhanced IFN γ and IL-17 responses. Liposome FP1 increased the percentage of IFN γ + CD4⁺ T cells from 34.73% to 60.4% ($p=0.0022$). Figure 5.13.1 (B) depicts the effect of Liposome FP1 on IL-17⁺ CD4⁺ T cells. Stimulation with Liposome FP1 led a more than twofold increase in the percentage of IL-17⁺ CD4⁺ T cells. 17.23% of unstimulated CD4⁺ T cells were IL-17⁺, compared to 42.1% following stimulation with Liposome FP1 ($p=0.0034$). The same effect was seen in CD8⁺ T cells. 25.3% of CD8⁺ T cells were IL-17⁺. In the presence of Liposome FP1, this rose to 45% ($p=0.0291$). CD8⁺ T cells stimulated with Liposome FP1 did not show elevated IFN γ expression.

Liposome FP1 did not increase Ki67 or IL-2 expression in CD4⁺ or CD8⁺ T cells. The effect of Liposome FP1 on CD4⁺ T cell proliferative responses is presented in Figure 5.13.2, A and B. Liposome FP1 had no proliferative impact on CD8⁺ T cells. It did not enhance the levels of intracellular IL-2. There was also

no increase in the expression of the proliferation marker Ki67. This matches the profile seen in CD4+ T cells, where Liposome FP did not generate any proliferative responses.

The Liposome A/E vaccine generated IL-17 responses in CD4+ and CD8+ T cells. It did not generate significant IFN γ production or induce proliferative responses. Stimulation with Liposome A/E increased the percentage of IFN γ + CD4+ T cells from 35% to 48%. However, this was not a statistically significant difference ($p=0.1159$). Liposome A/E induced a threefold increase in the percentage of IL-17+ CD4+ T cells. The percentage of IL-17+ CD4+ T cells increased from 17.2% to 51.5% ($p=0.0002$). Liposome A/E also doubled the percentage of IL-17+ CD8+ T cells (figure 5.13.1, B & D). 25% of unstimulated CD8+ T cells were IL-17+, compared to 57% IL-17+ in the presence of Liposome A/E. Although Liposome A/E generated IL-17 responses in CD8+ T cells, it did not elicit IFN γ production. There was also no increased expression of IL-2 or Ki67 in the CD8+ T cells stimulated with Liposome FP1.

Three vaccine formulations, Spore FP1, YC NaMa FP1 and Liposome FP1 were known to confer to protection against tuberculosis infection in mice. The antigen delivery systems of these three vaccines were different and have been demonstrated in this study to have different immunogenic effects in DCs and T cells. This TMC study has revealed that the common feature of these three vaccine candidates, is that they all generate IFN γ + CD4+ T cell responses (figure 5.11.1A, Figure 5.12.1A and Figure 5.13.2A). Earlier in this study, it was hypothesised that Spores, YC NaMa and Liposome P/S were all able to generate IRF 3 signalling by activating TRIF. IRF3 is required for the development of IFN γ and IL-17 effector functions in CD4+ T cells. IFN γ + CD4+ lymphocytes play an important role in controlling TB infection. This finding may provide an explanation for the protective effect of Spore FP1, YC NaMa FP1 and Liposome FP1.

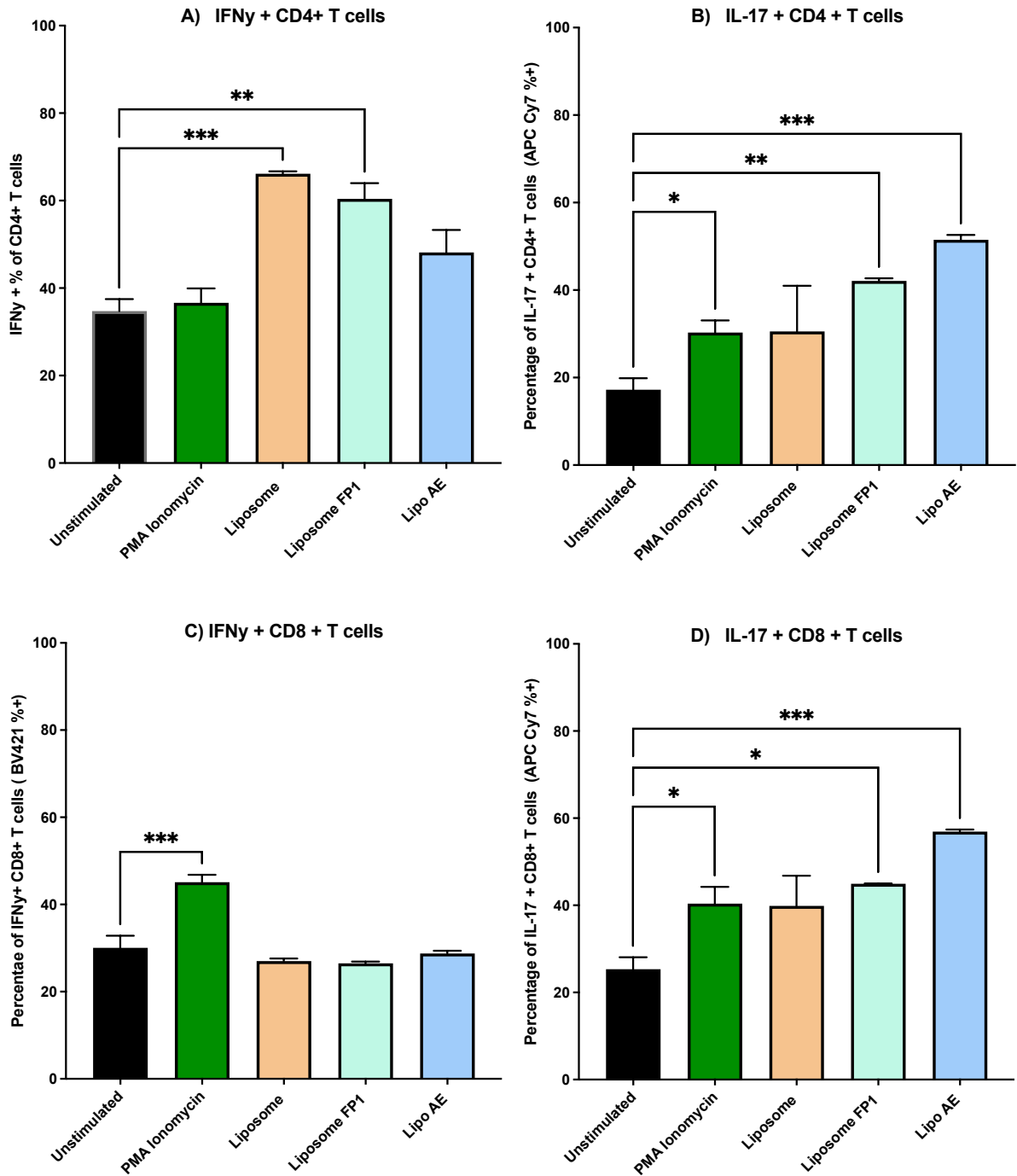


Figure 5.13.1. Immunogenicity of liposome FP1 and Liposome A/E vaccines. TMCs were stimulated for 5 days with liposome, liposome FP1 or Liposome A/E. Flow cytometry was used to identify CD4+ and CD8+ T cells producing IFN γ and IL-17. The percentage of IFN γ + and IL-17+ T cells is shown. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results were analysed using one way ANOVA and Dunnett's multiple comparison test (n=3).

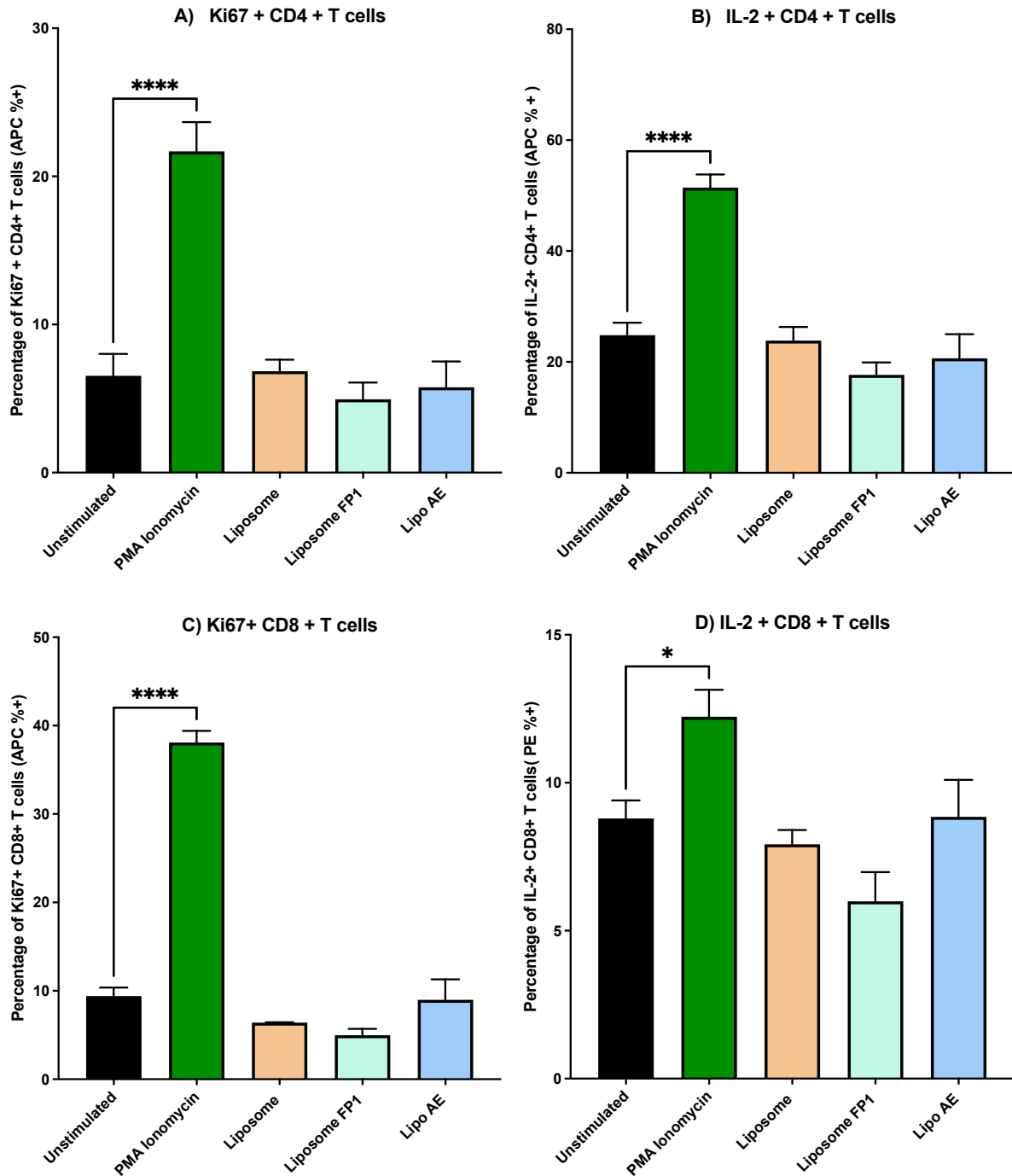


Figure 5.13.2. Effect of liposome formulations on T cell proliferative responses. Triplicate wells of TMCs were stimulated with liposome, liposome FP1 or Liposome A/E for 5 days. Flow cytometry was used to identify CD4+ T cells and CD8+ T cells which were positive for the proliferation marker Ki67 or intracellular IL-2. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results were analysed using one way ANOVA and Dunnett's multiple comparison test.

5.14 Immunogenicity and proliferative effect of α Acr IgA Immune Complex Mimic (IgA ICM) on CD4+ T cells.

This study aimed to develop the tonsil mononuclear cell (TMC) *in vitro* model and demonstrate that it can be used to test the immunogenicity of different types of vaccines. The novel anti tuberculosis vaccine candidate IgA ICM consists of polymeric structures of anti Acr IgA bound to the FP1 antigen. This anti Acr IgA has been shown to confer protection against TB (Balu et al. 3113-3119). The immunogenic effects of IgA ICM were unknown but it was anticipated that it would interact with myeloid cells which express Fc α RI receptors. This study used intracellular flow cytometry to determine the effects of this formulation on CD4+ and CD8+ T cells. The immunogenic effect of IgA ICM on CD4+ T cells is shown in figure 5.14.1. The immunogenic effect of IgA ICM on CD8+ T cells is shown in figure 5.15.1. The proliferative effect of IgA ICM on CD4+ and CD8+ T cells is shown in figure 5.14.2 and figure 5.15.2. Heat aggregated IgA was used as a positive control in this study. The effect of additional FP1 antigen was tested, to ensure that antigen was not a limiting factor in the immunogenicity of the formulation. Quil A was tested as an adjuvant for IgA ICM. IgA ICM in combination with Quil A was found to be highly immunogenic, eliciting IFN γ and IL-17 and proliferative responses.

IgA ICM enhanced IL-17 expression threefold in CD4+ T cells, but not production of IFN γ . This data is presented in figure 5.14.1. In unstimulated cells, 17.2% of CD4+ T cells were IL-17+. This increased to 50.25% IL-17+ with IgA ICM stimulation ($p=0.0040$). IgA ICM alone had no effect on IL-2 production nor proliferation of CD4+ T cells. This can be seen in figure 5.14.2. The amount of FP1 antigen was not a limiting factor in the responses elicited by IgA ICM. The immunogenic effects of IgA ICM were unaltered by the addition of more FP1 antigen to the formulation.

Combining IgA ICM + FP1 with a Quil A adjuvant led to enhanced production of IFN γ and IL-2 by CD4+ T cells. Ki67 expression in CD4+ T cells was also increased, as the cell proliferated. IL-17 responses in CD4+ T cells were retained (figure 5.14.1 B). Quil A IgA ICM FP1 stimulation led to a twofold increase

in the percentage of CD4+ T cells expressing IFN γ (figure 5.14.1 A). Unstimulated CD4+ T cells were 34.7% IFN γ +. With IgA ICM FP1 + 5 μ g/ml of Quil A, this rose to 69% IFN γ (p=0.0008).

Figure 5.14.2B shows that stimulation with Quil A, IgA ICM FP1 led to a greater percentage of CD4+ T cells expressing IL-2. The percentage of IL-2+ CD4+ T cells increased 3.5 fold from 24.8% to 87.5% (p<0.0001). The percentage of proliferating Ki67+ CD4 T cells was also increased from 6.53% to 17.55% (p=0.0130) with the combined Quil A formulation (figure 5.14.2A). When 5 μ g/ml of Quil A was added to IgA ICM FP1, the percentage of IL-17+ T cells increased twofold from 17.2% to 36.4% (p=0.1252). This was lower than the percentage of IL-17+ cells generated by IgA ICM alone. Using Quil A at a lower concentration may be more effective. With 2.5 μ g/ml of Quil A added to IgA ICM, FP1, there was a 2.5 fold increase in the percentage of IL-17+ CD4 T cells. 42.9% of the CD8+ T cells stimulated with IgA ICM FP1 + 2.5 μ g/ml Quil A became IL-17+ (p=0.0253). 2.5 μ g/ml of Quil A combined with IgA ICM FP1, enhanced IFN γ , IL-2 and Ki67 expression without limiting Th17 responses.

The IgA positive control, which consisted of heat aggregated IgA, presented a different immunogenic profile compared to IgA ICM. The IgA positive control was highly immunogenic. It induced production of IL-17 and IFN γ in CD4+ T cells (figure 5.14.1 A & B). 17.2% of unstimulated CD4+ T cells were IL-17+, when IgA positive control was added, this rose to 54.4% (p=0.0015). It also induced IL-2 production and proliferation of these T helper cells (figure 5.14.2A). Stimulation of TMCs with IgA led to an almost twofold increase in the percentage of IFN γ + CD4+ T cells, from 34.7% to 67.8% (p= 0.0010). Figure 5.14.2 A shows that the percentage of CD4+ T cells expressing IL-2, increased twofold with IgA positive control from 24.8% to 49.5%. The number of CD4+ T cells with detectable Ki67 increased fourfold from 6.5% to 29.6% (p<0.0001) (figure 5.14.2A). In these cells, IgA positive control increased the percentage of IL-17 positive CD4+ T cells threefold from 17.2% to 54.4% (p=0.0015). The IgA positive control was also immunogenic in CD8+ T cells, inducing IL-17 responses, IL-2 production and proliferation. This is shown in the following section. Crosslinking of the Fc α RI receptor by IgA is known to induce the release of proinflammatory cytokines including IL-1b, TNF α , IL-6 and IL-17 (Hansen IS. et al., 2018).

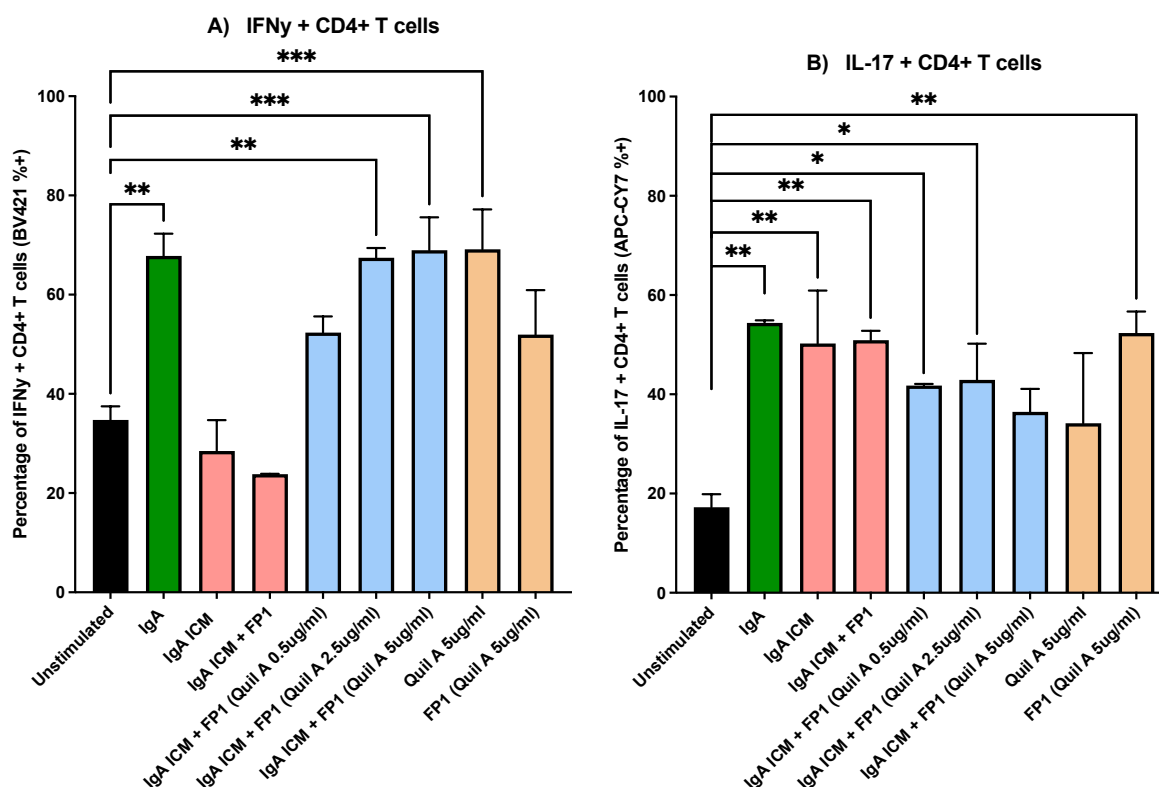


Figure 5.14.1. Immunogenicity of α Acr IgA ICM vaccines in CD4+ T cells. The effect of IgA ICM on the development of Th1 (A) and IL-17 (B) responses is shown. The percentage of CD4+ T cells which were IFN γ + increased following stimulation with IgA ICM FP1 in combination with Quil A at 5 μ g/ml. IgA ICM alone increased the percentage of CD4+ T cells which were IL-17+. Triplicate 1×10^5 TMCs were seeded per well and stimulated with IgA positive control, IgA ICM either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for IgA ICM vaccines. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IFN γ +, IL-17+ cells. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results were analysed using one way ANOVA and Dunnett's multiple comparison test.

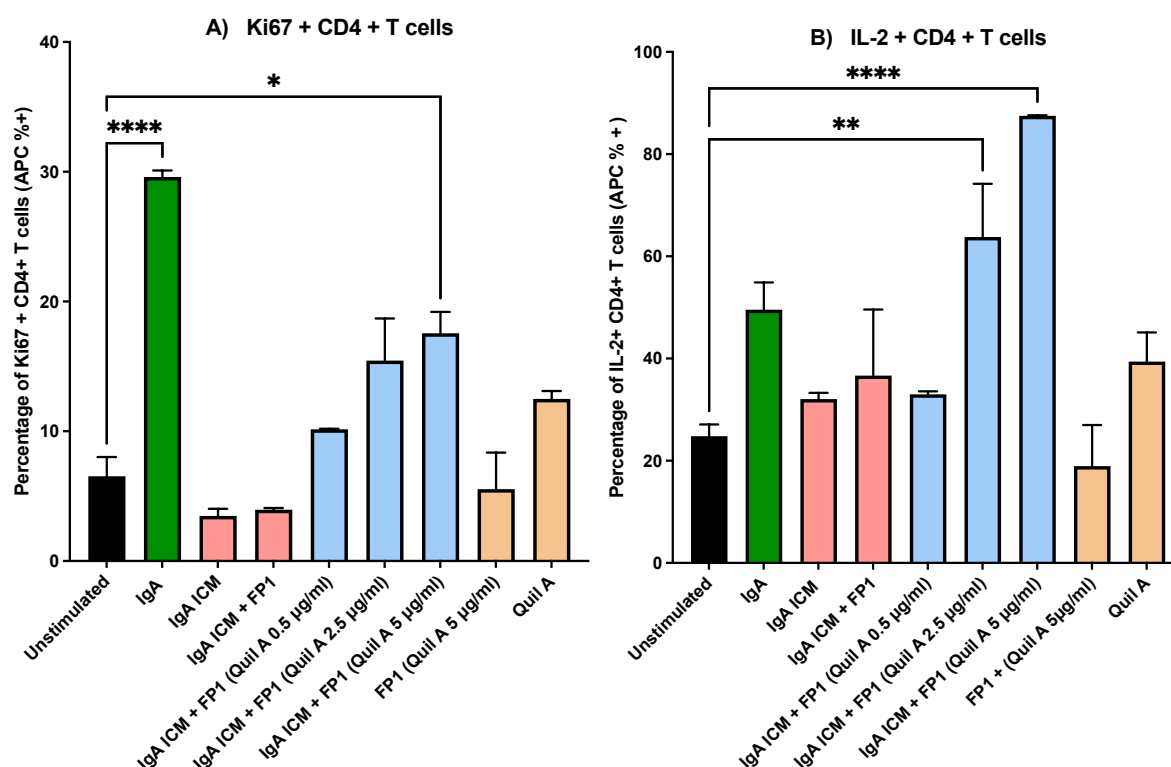


Figure 5.14.2. Effect of α Acr IgA ICM on CD4+ T cell proliferative responses. IL-2 produced by CD4+ T cells following IgA ICM stimulation is shown in graph A. The proliferative effect of IgA ICM on CD4+ T cells is shown in graph B. IgA ICM in combination with the antigen FP1 and the adjuvant Quil A, increased the percentage of Ki67+ and IL-2+ CD4+ T cells. IgA ICM alone or in combination with FP1 did not enhance IL-2 production or the proliferation of T cells. 1×10^5 TMCs were seeded per well and stimulated with an IgA positive control, IgA ICM either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for the IgA ICM vaccine. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IL-2+ and Ki67+ cells. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results analysed using one way ANOVA and Dunnett's multiple comparison test, (n=2).

5.15 Immunogenicity and proliferative effect of α Acr IgA Immune Complex Mimic (IgA ICM) on CD8+ T cells.

IgA ICM is immunogenic and generates responses in CD4+ T cells. This study also investigated CD8+ T cell responses generated by IgA ICM. The results are shown in figure 5.15.1. When IgA ICM was applied to CD8+ T cells, it elicited IL-17 after 5 days. 25.3% of unstimulated CD8+ T cells were IL-17+. Following stimulation with IgA ICM, this rose to 57.8% ($p=0.0010$). The percentage of IL-2+ CD8+ T cells rose from 8.7% to 20.7% ($p<0.0001$) (figure 5.15.2). IgA ICM did not induce a measurable increase in IFN γ production by stimulated CD8+ T cells. Without a clear IFN γ response, IgA ICM alone does not appear to be a suitable TB vaccine candidate.

The data shown in figure 5.15.1 shows that combining IgA ICM with 5 μ g/ml of Quil A resolved this problem and induced IFN γ production by CD8+ T cells. 30% of unstimulated CD8+ T cells were IFN γ +. Stimulation with IgA ICM and Quil A increased the percentage of IFN γ + cells to 51.5% ($p=0.0046$). In figure 5.15.2, the percentage of proliferating CD8+ T cells doubled in the presence of IgA ICM FP1 + Quil A. The percentage of Ki67+ cells doubled from 10.7% to 21% ($p=0.0007$). It is significant, however, that this increased IFN γ production was accompanied by a loss of IL-17 and IL-2 production in CD8+ T cells. When IgA ICM was combined with a Quil A adjuvant, it was capable of generating IFN γ and proliferative responses in CD8+ T cells. This same formulation generated IFN γ and IL-17 responses in CD4+ T cells. It also induced IL-2 production and proliferation of CD4+ T cells. This combined data indicates that IgA ICM combined with Quil A is a promising TB vaccine candidate capable of generating a sustained proinflammatory response in T cells. This work provides insight into the way in which IgA ICM engages with the immune response. Additional work can be done using TMCs to establish the effect of IgA ICM on B cell responses. IgA ICM with Quil A was highly immunogenic. IL-17 and IFN γ T cell responses have both been associated with protection against TB (Bhatt et al. 258-266; Coulter et al. 1252). Further work is needed to establish the predictive value of this TMC vaccine immunogenicity data as it relates to protection against infection by mycobacteria.

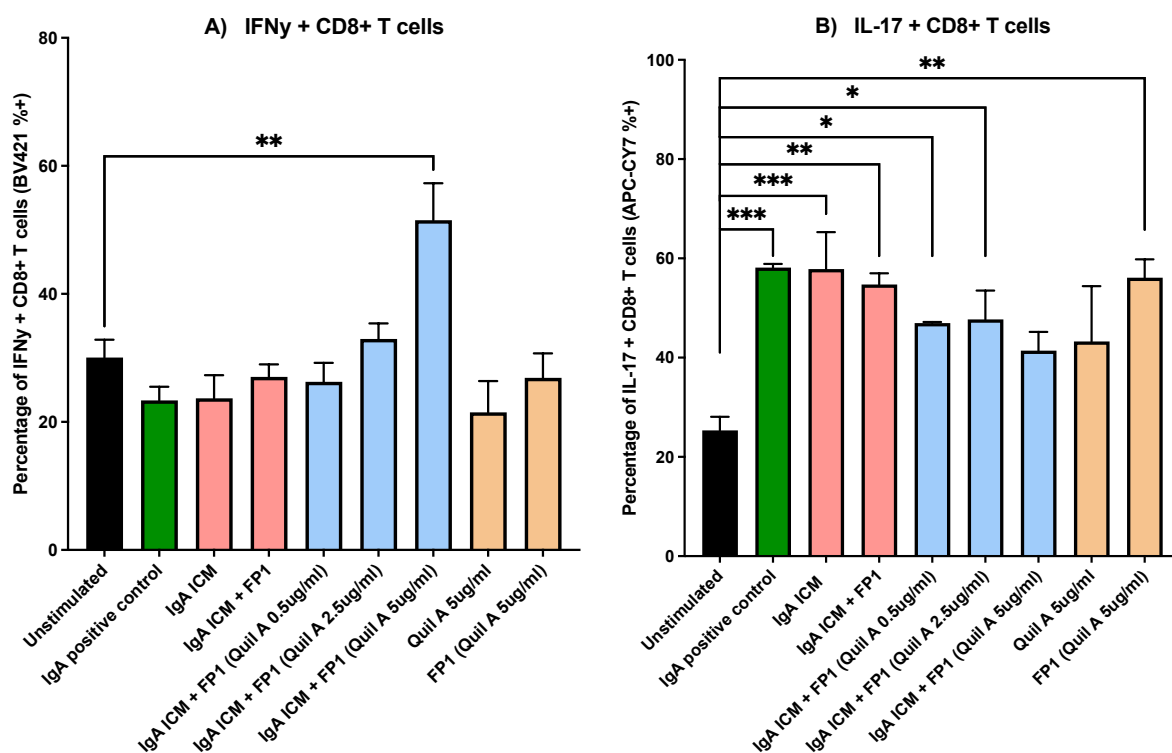


Figure 5.15.1. Immunogenicity of α Acr IgA ICM vaccines in CD8+ T cells. The effect of IgA ICM on the development of IFN γ (A) and IL-17 (B) responses in CD8+ T cells is shown. Triplicate wells were seeded with 1×10^5 TMCs. These were stimulated with IgA positive control, IgA ICM either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for IgA ICM vaccines. Cells were incubated for 5 days and analysed by flow cytometry (n=2). CD3+ T cells were gated for CD4+ / CD8+ and then IFN γ +, IL-17+ cells. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results were analysed using one-way ANOVA and Dunnett's multiple comparison test.

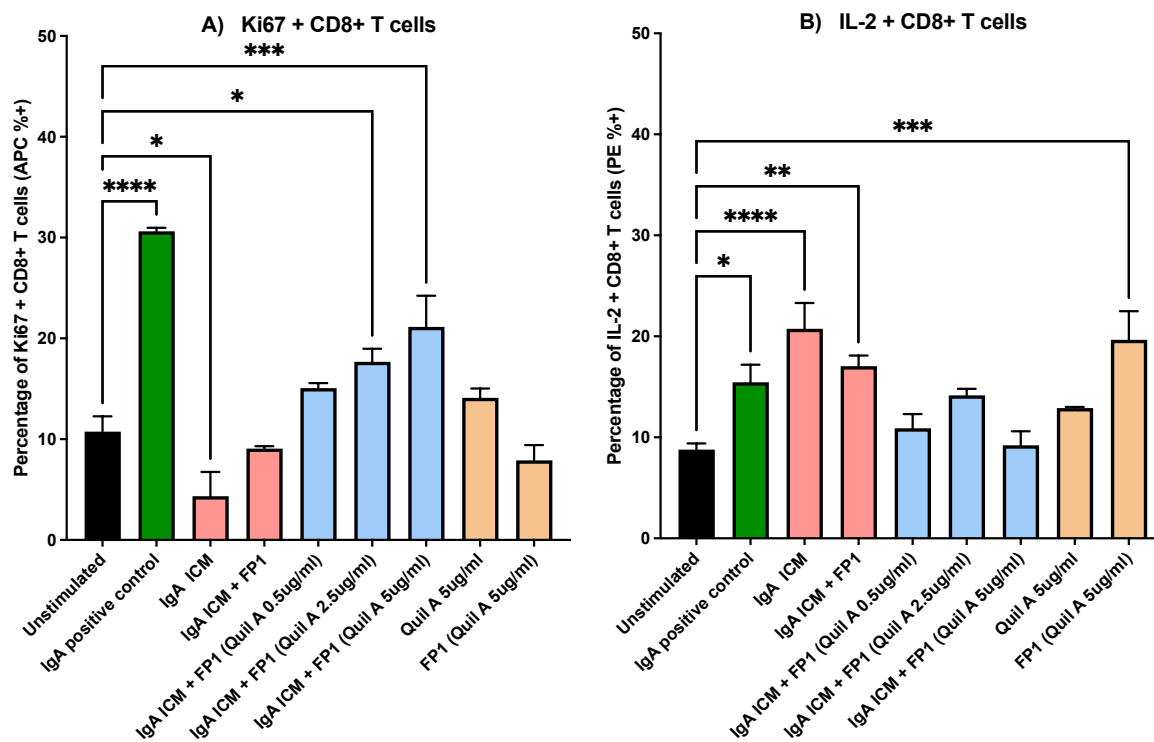


Figure 5.15.2. Effect of α Acr IgA ICM on CD8+ T cell proliferative responses. 1×10^5 TMCs were seeded per well in triplicate and stimulated with an IgA positive control, IgA ICM either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for the IgA ICM vaccine. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IL-2+ (A) and Ki67+ (B) CD8+ T cells. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results analysed using one way ANOVA and Dunnett's multiple comparison test ($n=2$).

5.16 Immunogenicity and proliferative effect of α Acr IgG Immune Complex Mimic (IgG ICM).

The previous section examined the effect of anti Acr IgA ICMs. This section investigates the immunogenicity of the IgG ICM vaccine. It is known that anti Acr IgA and anti Acr IgG bind to a different epitopes on Acr (Balu et al. 3113-3119). These immunoglobulins will also be recognised by different cellular receptors. IgA binds to Fc α RI, whilst IgG binds to Fc γ R. Figure 5.16.1 and figure 5.16.2 shows the effect of IgG ICM on IFN γ and IL-17 production in CD4 and CD8 T cells respectively. IgG ICM's proliferative effects on CD4+ and CD8+ t cells are shown in figure 5.16.3 and 5.16.4. This study did not find any immunogenic effects associated with IgG ICM. IgG ICM alone did not enhance expression of IFN γ , IL-2 or IL-17 by CD4 + T cells. It also failed to induced proliferation of CD4+ and CD8+ T cells. Adding additional FP1 antigen to IgG ICM also failed to enhance expression of IFN γ , IL-2 or IL-17 by CD4+ T cells.

Stimulation with IgG ICM, with FP1 and Quil A led to increased IFN γ + CD4+ T cell responses. It also induced proliferation in CD4+ T cells and the expression of IL-2. IL-17 expression was unaffected. Figure 5.16.1 shows IgG ICM combined with FP1 and Quil A increased the percentage of IFN γ + CD4+ T cells from 34.73% to 64.05% ($p=0.0012$). Figure 5.16.3 shows that the percentage of CD4+ T cells expressing the proliferative cytokine IL-2 doubled from 24.8% to 55.4% ($p=0.0183$). The percentage of Ki67 expressing, proliferating CD4+ T cells rose from 6.53% of unstimulated cells, to 16.7% of CD4+ T cells stimulated with IgG ICM with FP1 and Quil A. The proliferative effect was due to the combined stimulation with IgG ICM, Quil A and FP1 rather than IgG ICM.

The IgG control did not alter the proliferation of CD4+ or CD8+ T cells, nor did it increase the percentage of IFN γ + CD4 T cells. The IgG control also failed to enhance the expression of IL-2 or IL-17.

The immunogenic effect of IgG ICM on tonsillar CD8⁺ T cells was also examined. Stimulation with IgG ICM did not induce Intracellular expression of the proinflammatory cytokines IFN γ or IL-17 by CD8⁺ T cells. This can be seen in figure 5.16.2. Similarly, the expression of the proliferative cytokine IL-2 and the proliferation marker Ki67 were also unaltered following stimulation with IgG ICM. This is shown in figure 5.16.4. Combining IgG ICM with Quil A or additional FP1 antigen did not lead to significant increases in the expression of IFN γ , IL-17 or either of the proliferation markers. Instead, Quil A in combination with FP1 elicited IL-17 responses and IL-2 production by CD8⁺ T cells. When IgG ICM was included as well, this cytokine production was lost suggesting that IgG ICM actually inhibits proliferative and pro-inflammatory responses by CD8⁺ T cells.

This work strongly suggests that anti Acr IgA ICM binding to Fc α RI on myeloid cells is responsible for immunogenic effects in CD4⁺ T cells, whilst binding of anti Acr IgG ICM to Fc γ R does not generate immunogenic or proliferative effects in T cells.

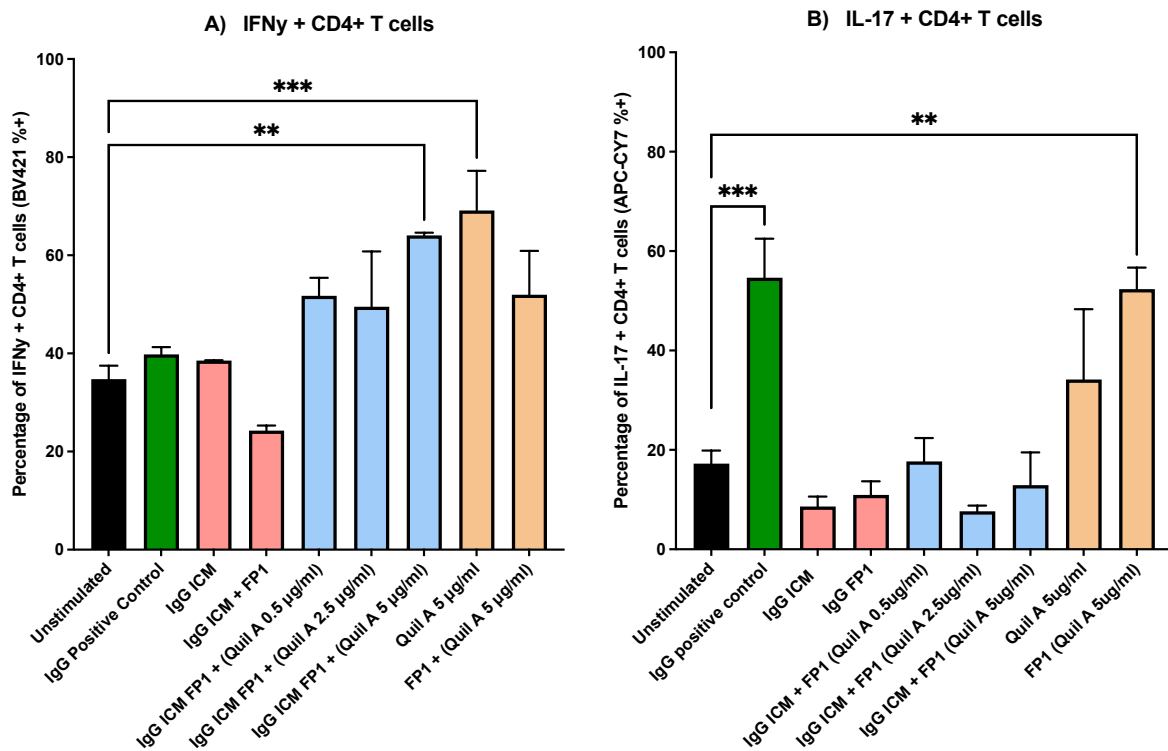


Figure 5.16.1. Immunogenicity of α Acr IgG ICM in CD4+ T cells. Th1 and Th17 responses generated by IgG ICM FP1 are shown in graphs A and B. Triplicate 1×10^5 TMCs were seeded per well and stimulated with IgG positive control and IgG ICM, either alone or in combination with additional FP1 (n=2). Quil A was also tested as an adjuvant for IgG ICM. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IFN γ +, IL-17+ cells. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Results were analysed using one way ANOVA and Dunnett's multiple comparison test.

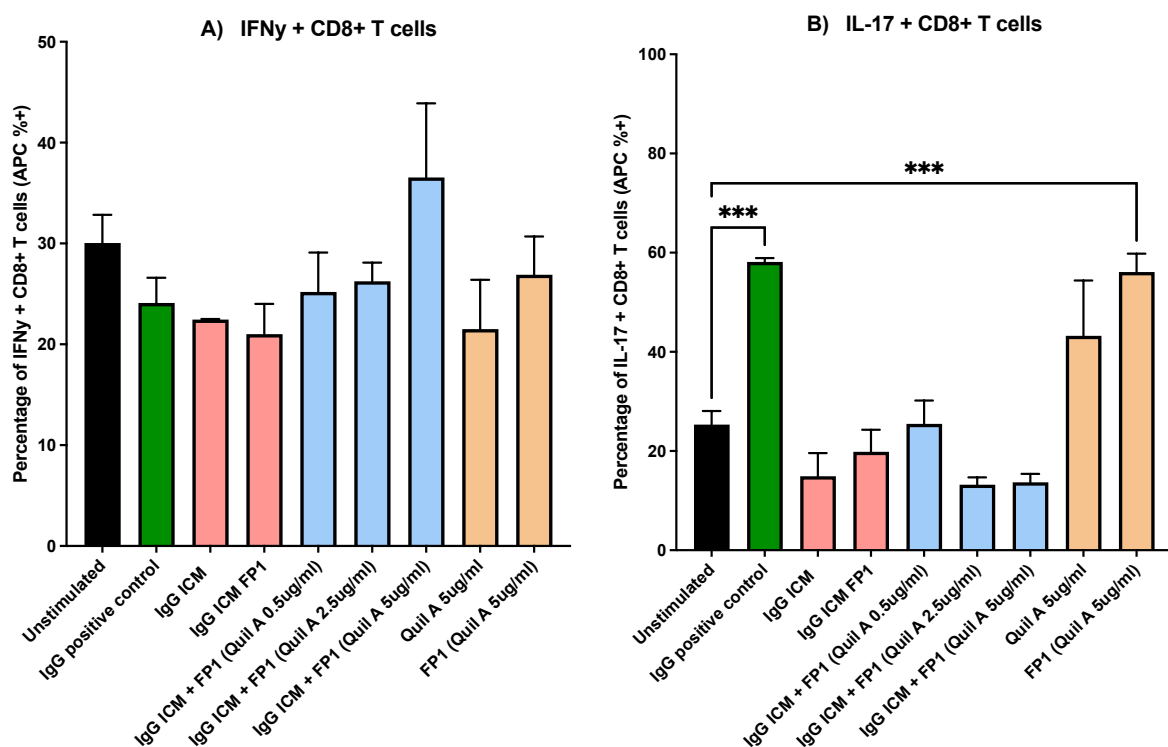


Figure 5.16.2. Immunogenicity of α Acr IgG ICM in CD8+ T cells Triplicate 1×10^5 TMCs were seeded per well and stimulated with IgG positive control and IgG ICM, either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for IgG ICM. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IFN γ +, IL-17+ cells. IFN γ and IL-17 responses generated by IgG ICM FP1 are shown in graphs A and B. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . One way ANOVA and Dunnett's multiple comparison test were used to analyse results.

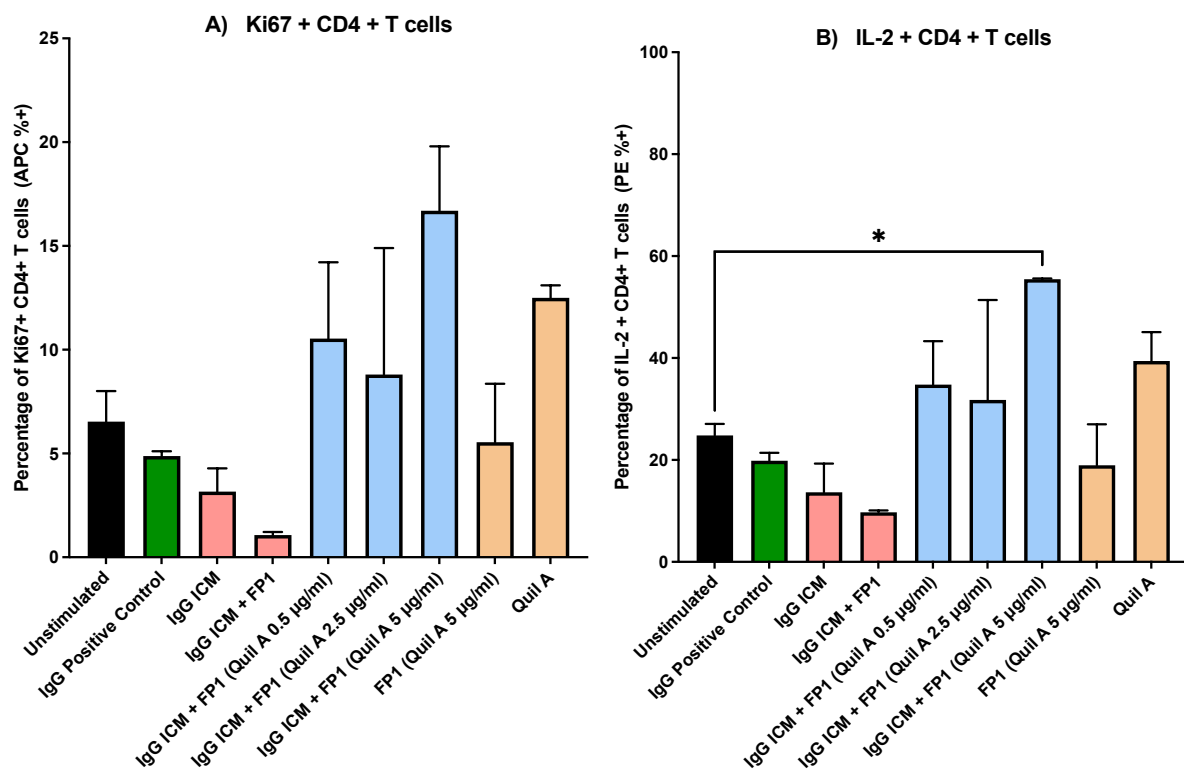


Figure 5.16.3. Effect of α Acr IgG ICM on CD4+ T cell proliferative responses. The proliferative effect of IgG ICM on CD4+ T cells is shown (A), along with the effect on IL-2 production (B). IgG ICM did not induce proliferative responses. IgG ICM with FP1 and Quil A increased the percentage of both Ki67+ and IL-2+ CD4+ T cells. Triplicate wells containing 1×10^5 TMCs were stimulated with an IgG positive control and IgG ICM, either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for IgG ICM. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IL-2+ and Ki67+ cells. Mean values and $\pm$ standard deviations are shown. These results were analysed using one way ANOVA and Dunnett's multiple comparison test.

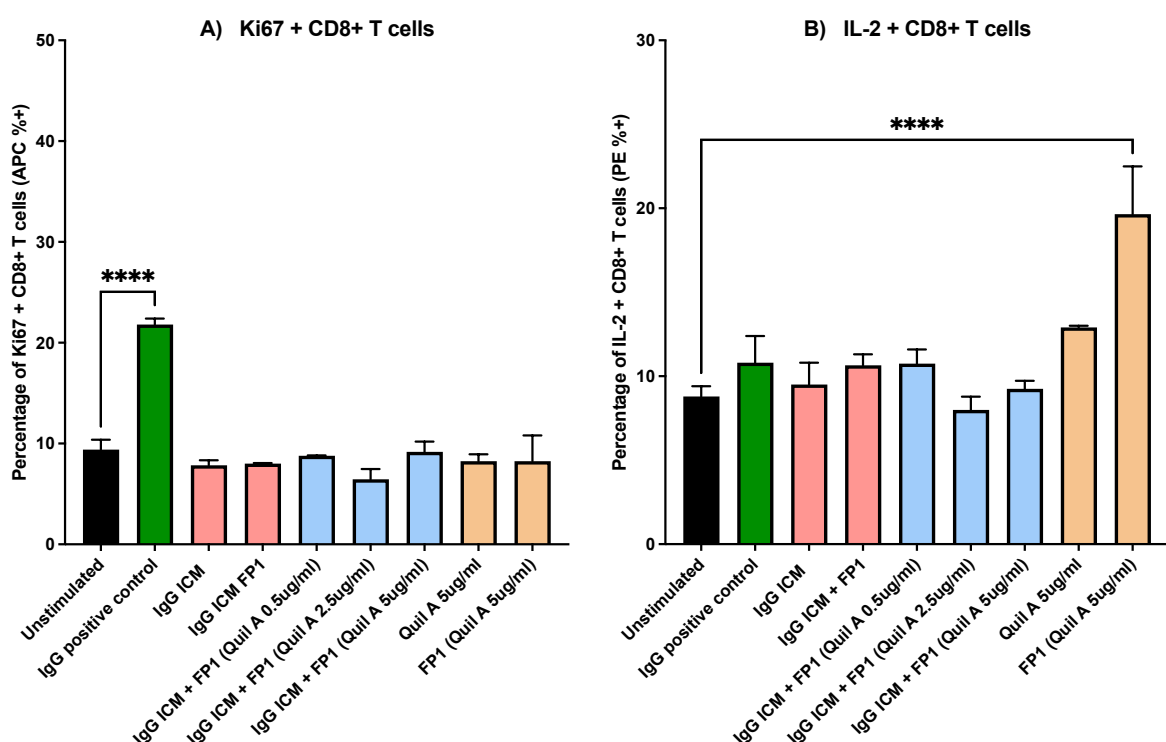


Figure 5.16.4. Effect of α Acr IgG ICM on CD8+ T cell proliferative responses. The proliferative effect of IgG ICM on CD8+ T cells is shown (A), along with the effect on IL-2 production (B). IgG ICM did not induce proliferative responses. Triplicate 1×10^5 TMCs were seeded per well and stimulated with an IgG positive control and IgG ICM, either alone or in combination with additional FP1. Quil A was also tested as an adjuvant for IgG ICM. Cells were incubated for 5 days and analysed by flow cytometry. CD3+ T cells were gated for CD4+ / CD8+ and then IL-2+ and Ki67+ cells. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . These results were analysed using one way ANOVA and Dunnett's multiple comparison test.

Key Findings

The immunogenicity of 10 TB vaccine candidates has been tested in TMCs. The results are summarised.

Spore FP1 – Induces IFN γ responses in CD4+ T cells but no IL-17 or proliferative responses.

Spore FP1 + Poly I:C –Induces IL-2 production by CD8+ T cells. No other proliferative effects were observed. Does not induce IFN γ or IL-17 production.

YC NaMa FP1 – Elicited IFN γ in CD4+ T cells. IL-2 generated in CD4+ T cells. IL-17 responses in CD4+ T cells and CD8+ T cells. YC NaMa FP1 has a similar immunogenic profile in TMCs to the BCG vaccine.

YC NaMa FP1 + Poly I:C – Induced CD8+ T cells to express IFN γ . No IFN γ or IL-17 responses were observed in CD4+ T cells. There were no proliferative effects on CD4+ T cells or CD8+ T cells.

Liposome FP1 – induced IFN γ and IL-17 responses in CD4+ T cells and IL-17 responses in CD8+ T cells.

Liposome FP1 did not have any proliferative properties.

Liposome A/E – IL-17 responses in CD4+ T cells and CD8+ T cells. No proliferative effects were seen.

IgA ICM – IL-17 responses in CD4+ and CD8+ T cells. No proliferative responses in CD4+ T cells. IL-2 produced by CD8+ T cells.

IgA ICM + Quil A - IFN γ and IL-17 responses in CD4+ and CD8+ T cells. It induced proliferation of CD4+ and CD8+ T cells. It also increased the percentage of IL-2+ CD4+ T cells. This is a similar pattern to that obtained with BCG.

IgG ICM – No immunogenic or proliferative properties.

IgG ICM + Quil A – IFN γ responses in CD4+ T cells as well as IL-2 production. These are due to the Quil A adjuvant. IgG ICM itself contributes nothing.

5.17 T cell antigen recall responses to Liposome FP1 vaccine candidate

It has been possible to use tonsil cells to assess the immunogenicity of novel TB vaccines. It is now important to determine whether these responses are antigen specific. To accomplish this, TMCs were stimulated with the BCG vaccine or the Liposome FP1 vaccine candidate for 5 days, as before. After this, the TMCs were stimulated for a further 7 days with recall antigens. Expression of IL-17, Ki67, IL-2 by CD4+ T cells and CD8+ T cells was measured by flow cytometry. It was important to demonstrate that any antigen specific responses were being created *De novo*. Prior exposure to TB would be a confounding factor. For this experiment, tonsillar tissue from patients 5, 6, 7 and 8 was used. None of these patients had previously shown reactivity to PPD.

Aims

- To demonstrate antigen recall responses to BCG in tonsillar T lymphocytes
- To demonstrate antigen recall responses to Liposome FP1 in tonsillar T lymphocytes

Figure 5.17.1 shows the effect of antigen recall on IFN γ production by TMCs stimulated with BCG or Liposome FP1. The effect of antigen recall on IL-2, IL-17 and Ki67 expression was also measured but is not shown.

Stimulation with BCG did not induce any measurable effect in TMCs using this protocol. After 12 days, there was no detectable increase in IFN γ , IL-17 or IL-2 levels. There was also no increase in the percentage of proliferating cells. BCG did not generate antigen specific responses. It was expected that antigen recall with Acr or Ag85b would induce increased proliferation or cytokine production. Instead, antigen recall had no impact on these parameters.

Stimulation with Liposome FP1 enhanced the intracellular expression of IFN γ by CD4 $^{+}$ T cells (figure 5.17.1). The MFI of BV421 was 8,079 in unstimulated cells. Following stimulation with Liposome FP1, this rose to 10,681 ($p < 0.0001$). There was no increase in intracellular IFN γ in TMCs following stimulation with the Ag85b and Acr recall antigens. Liposome FP1 did not induce T cell proliferation or IL-17 or IL-2 responses after 12 days of culture. Combining Liposome FP1 stimulation with antigen recall did not induce increased levels of IL-17, IL-2 or Ki67. It was not possible to detect any newly created antigen specific responses to either BCG or Liposome FP1 using this protocol.

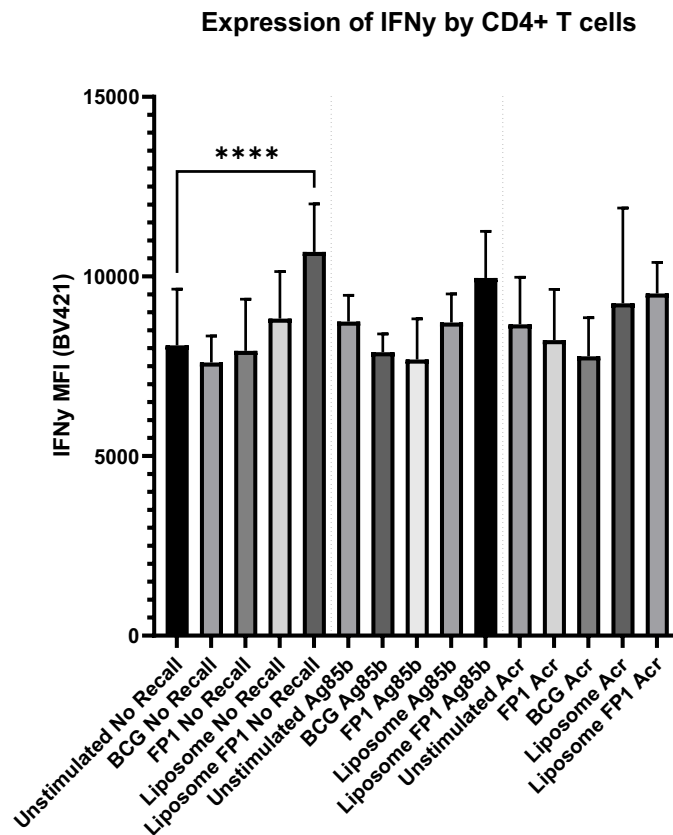


Figure 5.17.1. Antigen recall responses to BCG and Liposome FP1 in tonsillar T cells. Triplicate wells of 96 well plates were seeded with 1×10^5 TMCs. These were stimulated for 5 days with BCG Pasteur at an MOI of 1:1. Additional wells were stimulated with FP1, Liposomes or Liposome FP1. After 5 days, cells were stimulated with Ag85b or Acr or cultured with no recall antigen at all. These cells were then cultured for a further 7 days and analysed by flow cytometry. At the 12 day timepoint, intracellular expression of IFN γ , IL-17, IL-2 and Ki67 were measured in CD4 $^{+}$ T cells and CD8 $^{+}$ T cells. (n=4). Mean values and $\pm$ standard deviations are shown. Statistical analysis was performed using one way ANOVA and Sidaks multiple comparisons test.

Key Findings

It is not possible to elicit recall responses to BCG or Liposome FP1 using the tonsillar model.

5.18 TMC immunoglobulin vaccine binding study

In this section, TMCs were used to test the ability of novel immunoglobulin based vaccines to bind to human cells. Five immunoglobulin based vaccine formulations were examined. Four novel Dengue vaccines were examined; DERICS, D-PIGs, MY42J and MY45J. A novel TB vaccine MTB5J was also investigated.

It was hypothesised that immunoglobulin based vaccines designed against Dengue Fever would bind to Fc receptors and exert immunogenic effects. Before these vaccine candidates were further developed, it was important to establish whether the formulations actually bind to human cells. It was expected that IgG formulations would bind to FCyR present on monocytes. Therefore, the binding of various IgG based vaccine formulations to CD14+ monocytes in human tonsillar mononuclear cells (TMCs) was tested using flow cytometry. In this experiment, TMCs were incubated with vaccine formulations for 2 hours. They were then stained with anti human CD14 conjugated to PE and α human CD11b conjugated to APC to gate monocyte populations. Binding of IgG vaccine formulations to these cells was detected using an α human IgG bound to FITC.

Dengue – Ebola Recombinant immune complexes (DERICS) are formulated from Polymeric 6D8 IgG fused to the Dengue antigen cEDIII. This vaccine formulation bound to TMCs. Incubation with DERICS or monomeric human IgG led to increases in FITC MFI ($p=0.0050$). DERICS are shown to bind to the CD14+ monocytes in TMC cultures in figure 5.18.1A. The positive control for this experiment was polymeric D-PIGS. Polymeric D-PIGS are polymeric structures composed of IgG fused with Dengue antigen, (cEDIII). Incubation with polymeric D-PIGS increased FITC MFI from 1,799 to 2,487 ($p=0.0002$). This work supports previous papers which have shown that both DERICS and D-PIGS bind to human cells (Webster et al.) & (Kim, M. Y. et al.). A representative histogram of binding data is shown in supplemental figure 10.3.

Two novel anti Dengue formulations were also tested for their ability to bind to human cells. My42J and My45J are polymeric IgG structures combined with the J chain which is normally found in IgA and

IgM. There was no detectable binding of My42J or My45J to CD14+ TMCs, as indicated in figure 5.18.1

B. The positive control for this experiment was polymeric D-PIGS. Cells incubated with D-PIGS increased their FITC MFI from 1,799 to 2,606 ($p < 0.0001$). Unfractionated D-PIGS also bound to the cells, with FITC MFI increasing from 1,799 to 2,487 ($p < 0.0001$). Monomeric D-PIGS did not show significant binding to cells ($p = 0.443$). This showed that the polymeric structure of D-PIGS is important for binding to CD14+ cells. My42J and My45J at concentrations of 500 $\mu\text{g/ml}$, 1,000 $\mu\text{g/ml}$ and 1,500 $\mu\text{g/ml}$ did not show detectable binding to the TMCs. The addition of the J chain to the D-PIGS formulation to make My42J and My45J did not enhance binding to CD14+ cells, but rather seems to have abrogated the binding.

A novel anti tuberculosis vaccine candidate (MTB5J) was tested for its ability to bind to human monocytes. MTB5J is a formulation designed as an anti-tuberculosis vaccine candidate. It is composed of polymeric immunoglobulin scaffolds (PIGS) fused with Ag85b. MTB5J was similar to the previous two vaccine candidates, inasmuch as it consisted of a polymeric structure of recombinant IgG molecules which contained the J chain. The results shown in Figure 5.18.2 do not demonstrate any binding of MTB5J to TMCs. Incubation with the positive control, Human polymeric IgG, increased FITC MFI from 1,799 to 4,981 ($p < 0.0001$). 500 $\mu\text{g/ml}$, 1 mg/ml and 1.5 mg/ml of MTB5J were applied to TMCs but no statistically significant increase in FITC MFI was seen at any of these concentrations. This finding confirms that the addition of the J chain to IgG constructs prevents effective binding to cells.

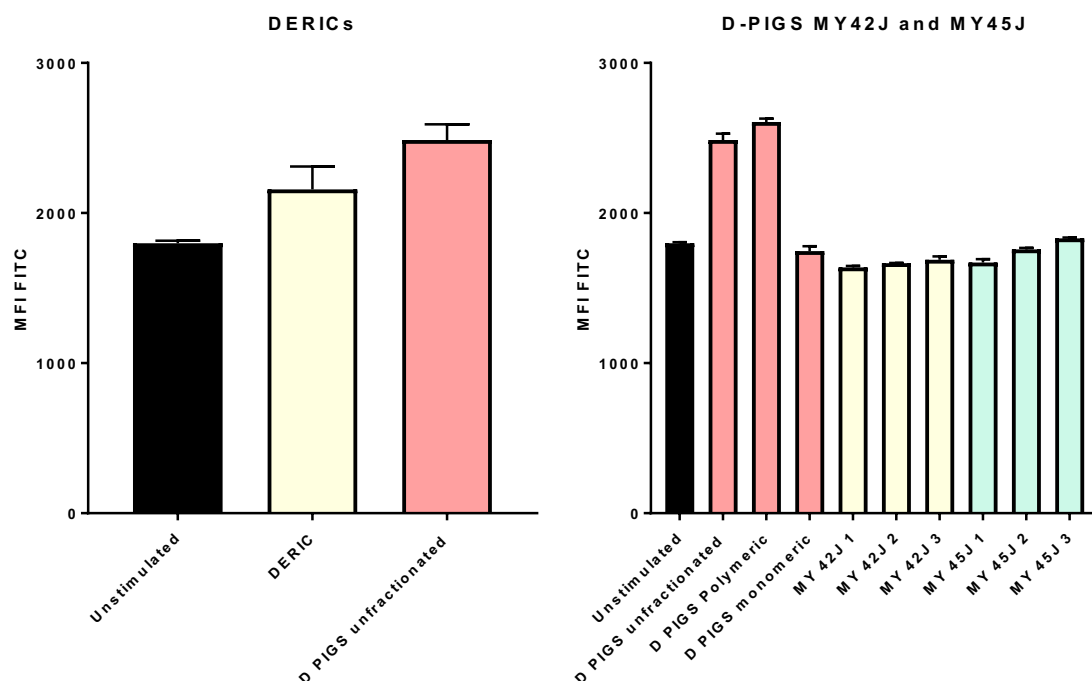


Figure 5.18.1. Binding of Dengue vaccines to human monocytes. Triplicate wells containing 1×10^6 TMCs were incubated for 2 hours at room temperature with DERICS at a concentration of 500 $\mu\text{g}/\text{ml}$ or unfractionated D-PIGS at 1mg/ml (A). Additionally, TMCs were incubated for 2 hours at room temperature with MY42J and MY45J IgG formulations at three concentrations, 500 $\mu\text{g}/\text{ml}$, 1,000 $\mu\text{g}/\text{ml}$ and 1,500 $\mu\text{g}/\text{ml}$. D-PIGS was included as a control at 1 mg/ml (B). Cells were stained with anti IgG conjugated to FITC. FITC MFI on CD14+ cells was analysed by flow cytometry. Statistical analysis was performed using one way ANOVA and Dunnetts multiple comparisons test ($n=3$). Mean values and $\pm$ standard deviations are shown. Both DERICS and polymeric D-PIGS bound to CD14+ cells. MY42 and MY45J didn't bind CD14+ cells at tested concentrations.

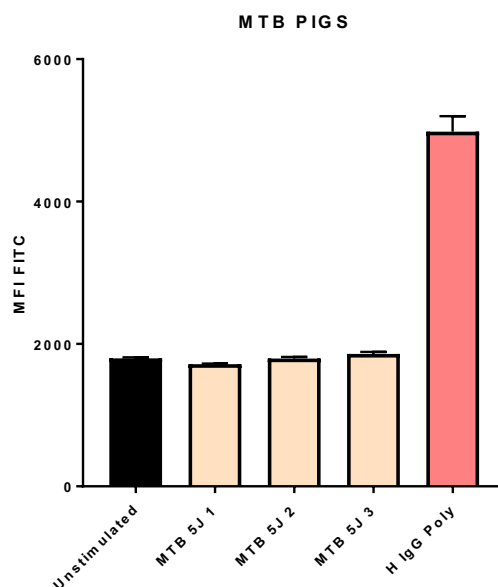


Figure 5.18.2. Binding of MTB5J vaccine formulation to human monocytes. Triplicate wells containing 1×10^6 TMCs were incubated for 2 hours at room temperature with MTB5J at three concentrations; 1 mg/ml, 1.5 mg/ml and 2mg/ml, (n=3). Cells were then stained with anti IgG conjugated to FITC. The FITC MFI of CD14+ cells was analysed by flow cytometry. Mean values and $\pm$ standard deviations are shown. The MTB45J construct did not bind to CD14+ cells at any of the three concentrations tested. The positive control, polymeric Human IgG bound to CD14+ cells.

Key Findings

- D-PIGS binds to human tonsillar monocytes.
- DERICS binds to human tonsillar monocytes.
- There was no detectable binding of My42J or My45J to CD14+ TMCs.
- The TB vaccine candidate MTB5J does not bind to tonsillar monocytes.

5.19 Immunogenicity of Dengue vaccine candidates

Neutralising antibodies and antigen specific cell mediated immunity play an important role in protection against Dengue Fever. A tonsillar *in vitro* immunogenicity model was used to screen novel Dengue vaccine candidates. This work can be used to better understand the immunogenicity of novel dengue vaccine candidates.

Using the tonsil model, it was possible to carry out a side by side comparison of four novel dengue vaccine candidates; D-PIGS, DERICS Mod-D-PIGS, My42J.

Intracellular flow cytometry was used to evaluate the cell mediated immunity generated by each of the dengue vaccine candidates. The production of IFN γ , TNF α , IL-4, IL-10 by CD4+ T cells and CD8+ T cells was measured after a five-day interval. The proliferation of these cells was investigated by measuring intracellular Ki67 levels.

Aims

Stimulate tonsillar cells with four Dengue vaccine candidates; D-PIGS, DERICS Mod-D-PIGS, My42J.

Measure the proliferation and production of IFN γ , TNF α , IL-4, IL-10 by CD4+ T cells and CD8+ T cells

5.20 Immunogenicity of Dengue vaccine candidates on tonsillar CD4+ T cells.

This experiment investigated the immune responses generated by four anti Dengue vaccines, D-PIGS, Mod D-PIGS, DERICS and My42J on tonsillar CD4+ T cells. After 5 days the levels of intracellular IFN γ , IL-4, IL-10 and TNF α were measured. Proliferation of the CD4+ T cells was also measured at the five day timepoint. Figure 5.20.1C shows that My42J induced IFN γ production in CD4+ T cells. BV421 MFI rose from 2,492 to 4,107 ($p=0.0358$). At the 5 day timepoint, the other vaccines had no detectable effect on the production of IFN γ , IL-4 or IL-10. There was also no evidence that these vaccine candidates induced T cell proliferation. These Dengue vaccines all upregulated expression of TNF α in the CD4+ T cells. Intracellular TNF α in CD4+ T cells increased with stimulations of CEDIII, D-PIGS, My42J or DERICS.

CEDIII is the dengue glycoprotein domain III. It is a consensus protein found in all dengue serotypes. Figure 83 shows that at the 5 day timepoint, stimulation with 5 $\mu\text{g/ml}$ of CEDIII increased intracellular TNF α levels from an MFI of 4,514 to 5,575 ($p=0.0192$) in CD4+ T cells. This provides evidence that the CEDIII glycoprotein alone may have immunogenic effects in human T cells. The EDIII protein has previously been shown to stimulate a proinflammatory response in the monocyte cell line THP1. EDIII stimulation induced NF- κ B signalling leading to production of IL-1 β and TNF α (Khan et al., 2019). This result provides the first evidence that EDIII is also capable of eliciting TNF α production by CD4+ T cells.

Three Dengue vaccines tested here induced TNF α responses in CD4+ T cells. This is presented in figure 5.20.2. Stimulation with D-PIGS increased TNF α levels from 4,515 to 5,664 ($p=0.0094$). A similar effect was seen with My42J. TNF α levels increased from 4,515 to 5,597 ($p=0.0161$). DERICS also increased the intracellular levels of TNF α in CD4+ T cells. The MFI of PE Cy7 rose from 4,515 to 5,504 ($p=0.0331$). PE Cy7 MFI also increased in cells stimulated with Mod D-PIGS. It rose from 4,515 to 5,246 MFI but this did not meet statistical significance ($p=0.1787$). These results confirm that D-PIGS, My42J and DERICS are all immunogenic. Each of these vaccine candidates contain CEDIII, which may explain why they show a similar pro-inflammatory profile in this experiment.

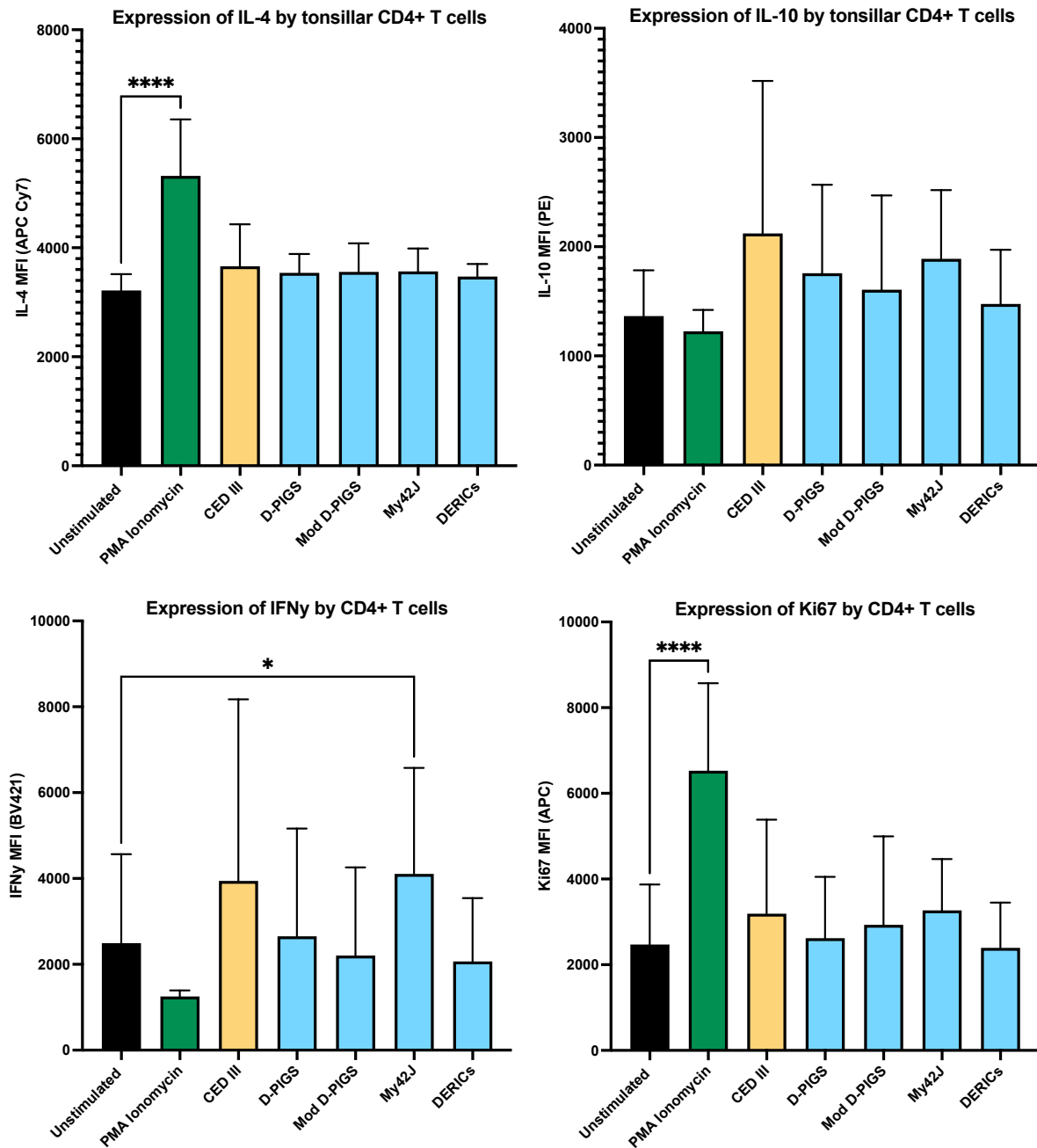


Figure 5.20.1. Immunogenicity of Dengue vaccine candidates on tonsillar CD4+ T cells. TMCs were seeded in triplicate at a density of 1×10^6 cells/ml in 96 well plates ($n=4$). The cells were stimulated with four Dengue vaccine candidates; D-PIGS, DERICS, My42J and Mod – D-PIGS at 25ug/ml. After 5 days, the cells were stained and analysed by flow cytometry. CD4+ and CD8+ T cells were identified. Ki67 expression was measured along with the levels of intracellular IFN γ , IL-4 and IL-10. MFI values and $\pm$ standard deviations are shown. Data was analysed using one way ANOVA and Dunnett's multiple comparisons test.

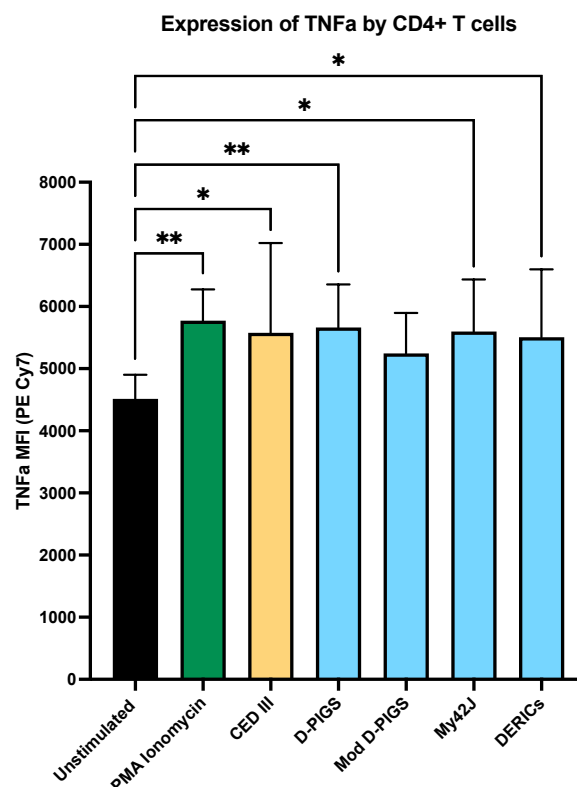


Figure 5.20.2. Immunogenicity of Dengue vaccine candidates on tonsillar CD4+ T cells. TMCs were seeded in triplicate at a density of 1×10^6 cells/ml in 96 well plates ($n=4$). The cells were stimulated with PMA / Ionomycin for 5 days. Cells were also stimulated with four Dengue vaccine candidates. D-PIGS, DERICS, My42J and Mod – D-PIGS were added to cells. After 5 days, the cells were stained and analysed by flow cytometry. CD4+ and CD8+ T cells were identified and the levels of intracellular TNF α measured. MFI values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Data was analysed using one way ANOVA and Dunnett's multiple comparisons test.

5.21 Immunogenicity of Dengue vaccine candidates on tonsillar CD8+ T cells.

This study has investigated the immunogenic effect of the dengue vaccine candidates on CD8+ T cells. CD8+ T cells are known to play an important role in the protective immune responses against Dengue. In a murine model CD8+ T cells have been shown to play a role in limiting viral replication. DENV specific CD8+ T cells drive the proinflammatory response by producing IFN γ and TNF α . They also exhibit cytotoxic activity against target cells expressing DENV peptides. Immunising animals with DENV peptides led to CD8+ T cell dependant clearance of dengue infection (Yauch et al., 2009a).

The immunogenicity of the Dengue vaccine candidates was tested. Figure 5.21.1 shows the immunogenic effect of D-PIGS, Mod D-PIGS, MY42J and DERICs on CD8+T cells. The expression of IFN γ , IL-4, IL-10 and the expression of Ki67 in CD8+ T cells was measured. There was no significant change in any of these parameters at a 5 day timepoint.

Figure 5.21.2 reveals an increase in intracellular TNF α levels in CD8+ T cells stimulated with D-PIGS. PE Cy7 MFI in these cells rose from 3,198 to 4,330 ($p=0.0306$).

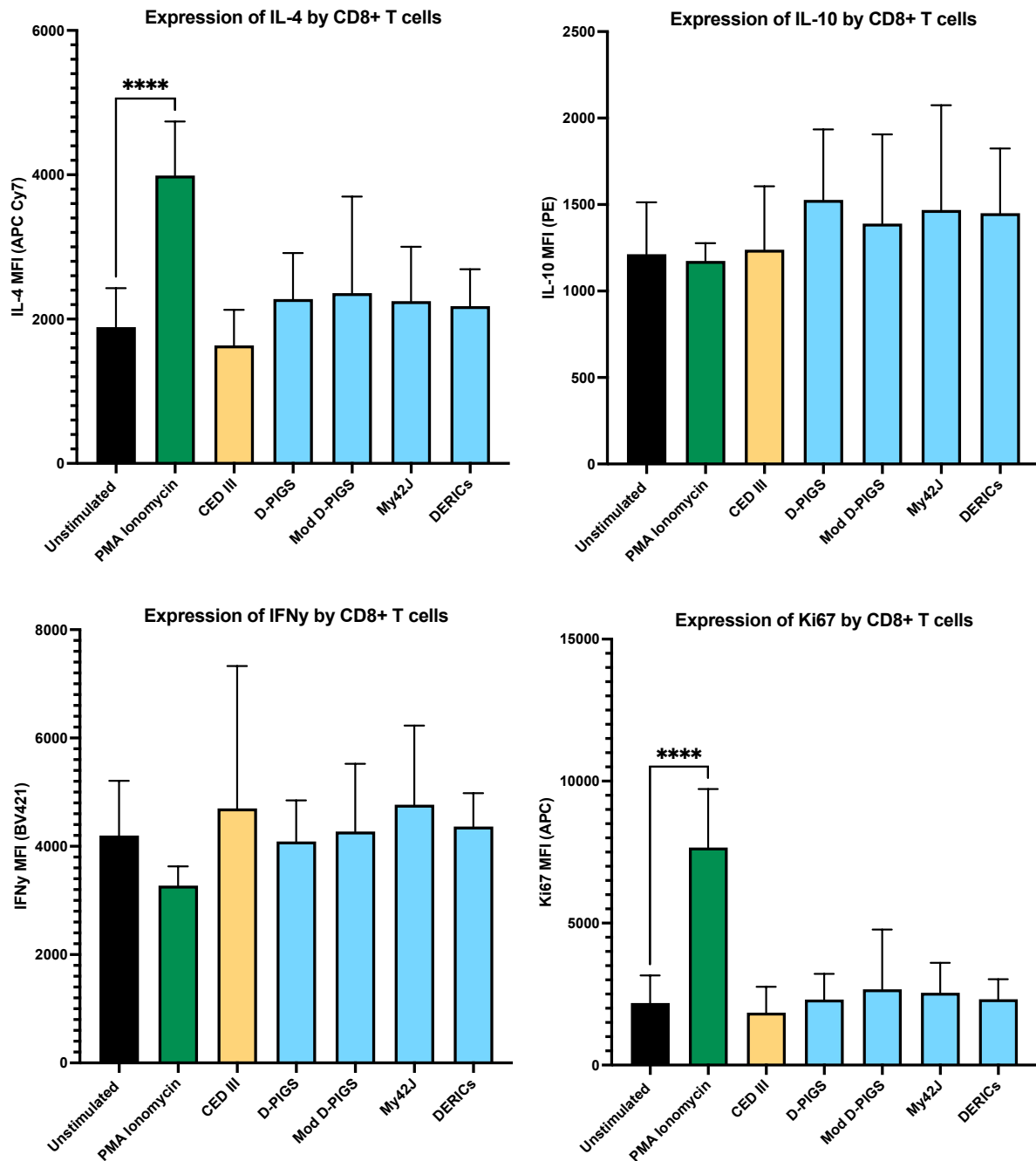


Figure 5.21.1. Immunogenicity of Dengue vaccine candidates on tonsillar CD8+ T cells. TMCs were seeded at a density of 1×10^5 cells/well in 96 well plates. The cells were stimulated with four Dengue vaccine candidates. D-PIGS, DERICS, My42J and Mod – D-PIGS were added to cells at 25ug/ml. After 5 days, the cells were stained and analysed by flow cytometry. CD8+ T cells were identified and the levels of intracellular IFN γ , IL-4 and IL-10 were measured along with Ki67 expression. The MFI values and $\pm$ standard deviations are shown. (n=4). Data was analysed using one way ANOVA and Dunnett's multiple comparisons test.

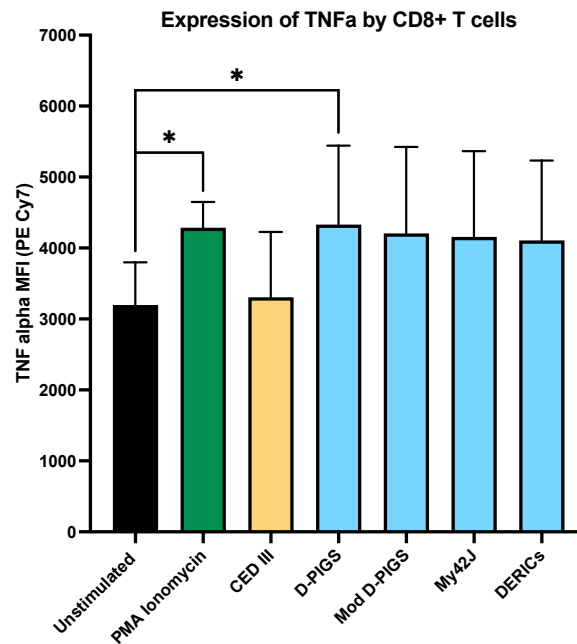


Figure 5.21.2. Immunogenicity of Dengue vaccine candidates on tonsillar CD8+ T cells. TMCs were seeded at a density of 1×10^5 cells/well in 96 well plates. The cells were stimulated with PMA / Ionomycin for 5 days. Cells were also stimulated with four Dengue vaccine candidates. D-PIGS, DERICS, My42J and Mod – D-PIGS were added to cells. After 5 days, the cells were stained and analysed by flow cytometry. CD8+ T cells were identified and the levels of intracellular TNF α were measured. The MFI values are shown. (n=4). Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Data was analysed using one way ANOVA and Dunnett's multiple comparisons test.

5.22 T cell recall responses to Dengue vaccine candidates.

Antigen specific cell mediated immunity plays an important role in the protective responses against Dengue. This experiment was designed to determine the effect of each of the dengue vaccine candidates on the development of antigen specific CD4+ and CD8+ T cells. TMCs were stimulated with Dengue vaccine candidates for 5 days. After 5 days, the TMCs were treated with 5µg/ml of CEDIII protein. The cells were incubated for a further seven days and then recall responses were measured. Intracellular production of IL-4, IFN γ , TNF α and IL-10 was measured. Ki67 expression was measured to show proliferation of these cells.

5.23 Recall responses in CD4+ T cells generated by novel Dengue vaccines

CD4+ T cells play an important role by directing and controlling the innate immune responses to Dengue infection. There is also growing evidence that cytotoxic CD4+ T cells develop during Dengue infection. These CD4+ T cells are able to induce apoptosis of infected cells. It is thought that these cells play an important role in preventing severe Dengue disease (Tian et al., 2016a). Cytotoxic CD4+ T cells expressing TNF α , IFN γ and IL-2 develop after the first week of DENV infection. Inducing these cells with DENV peptides prior to infection leads to enhanced viral clearance (Yauch et al., 2010a). IFN γ is known to play a protective role during acute Dengue infection (Gunther et al., 2011).

High frequencies of Dengue specific T cells making intracellular TNF α , IL-2 and IFN γ are associated with better patient outcomes (Hatch et al., 2011a). This immunogenicity model was designed to determine whether our novel dengue vaccine candidates were capable of generating these protective T cell responses.

The results shown in figures 5.23.1, 5.23.2 and 5.23.3 reveal that D-PIGS, My42J and Mod D-PIGS are all immunogenic. All of these vaccines generated IFN γ , IL-4 and TNF α responses in CD4+ T cells. DERICS is also immunogenic, generating IFN γ and IL-4 responses.

Figure 5.23.1 shows that after 12 days, stimulation with D-PIGS led to raised IL-4 levels in tonsillar CD4+ T cells. Intracellular IL-4 rose from 2,004 to 2,857 ($p<0.0001$). IFN γ levels also rose as can be seen in figure 5.23.1. BV421 MFI increased from 8,031 to 9,879 ($p=0.0121$). D-PIGS also induced TNF α production by CD4+ T cells. PE Cy7 MFI increased from 3,566 to 4,146 ($p=0.0067$). This is shown in figure 5.23.3.

Figure 5.25.1 reveals that stimulation with My42J generated higher levels of intracellular IL-4. APC-Cy7 MFI rose from 2,004 to 2,939 ($p<0.0001$). Figure 5.23.1 shows that IFN γ production increased as well. BV421 MFI rose from 8,031 to 10,303 ($p=0.0007$). My42J induced the proinflammatory cytokine TNF α in CD4+ T cells. In figure 5.23.3, intracellular TNF α rises from 3,566 up to 4,193 in the presence of MY42J ($p=0.0024$).

Mod D-PIGS induced IL-4 production in tonsillar CD4+ T cells after 12 days. In figure 5.23.1, Intracellular IL-4 rose from 2,004 to 2,939 MFI ($p<0.0001$). Mod D-PIGS also increased Intracellular IFN γ . BV421 MFI increased from 8,031 up to 10,176 on figure 5.23.1 ($p=0.0018$). CD4+ T cells generated TNF α in response to Mod D-PIGS stimulation. On figure 5.23.3, PE Cy7 MFI rose from 3,566 to 4,133 with Mod-D-PIGS stimulation ($p=0.0088$).

CD4+ T cells which were stimulated with DERICs generated IL-4. intracellular IL-4 rose from 2,004 to 2,576 in figure 5.23.1 ($p<0.0001$). Increased IL-4 production was accompanied by increased IFN γ production. The levels of intracellular IFN γ rose from 8,031 to 10,143 ($p=0.0023$) (figure 5.23.1). TNF α production rose from 3,566 to 3,994. This was not statistically significant ($p=0.1147$).

None of these vaccine candidates triggered CD4+ T cells to proliferate. There was no statistically significant increase in the intracellular expression of Ki67. This lack of proliferative responses would suggest that these vaccines might not produce sustained protection.

There was also no indication that these vaccines were generating significant IL-10 responses. CEDIII alone did generate IL-10. Severe dengue disease is associated with elevated IL-10 levels (Malavige et

al., 2013). The fact that our vaccine candidates did not generate IL-10 responses in CD4⁺ T cells may be a promising finding. However, it is important to note that the effect of IL-10 in the context of vaccine induced immune responses is unknown. As yet, there is not enough information to conclude whether IL-10 responses are desirable in a novel Dengue vaccine.

D-PIGS, My42J and Mod D-PIGS all generate IFN γ , IL-4 and TNF α responses in CD4⁺ T cells. The DERICS vaccine also generates IFN γ and IL-4 CD4⁺ T cell responses.

IFN γ and TNF α responses are thought to limit the severity of acute dengue infection. IL-4 responses drive the development of Th₂ populations, humoral immunity and the differentiation of B cells into plasma cells. IL-4 responses are not thought to be protective in the context of dengue disease; indeed IL-4 is a marker for the severity of dengue disease (Abhishek et al. 2017). It is unclear therefore whether the IL-4 responses generated by these vaccines would contribute towards protective immunity.

CEDIII stimulation was immunogenic in the tonsil models. CEDIII generated IFN γ , TNF α , IL-4 and IL-10 responses in CD4⁺ T cells. CEDIII was not previously known to have immunogenic effects on T cells. Intracellular IL-4 rose from 2,004 to 2,823 in CD4⁺ T cells ($p < 0.0001$). IFN γ levels rose from 8,031 up to 10,295 ($p = 0.0008$). This is shown in figure 5.23.1. CEDIII stimulation led to increased levels of intracellular TNF α . PE Cy7 MFI levels rose from 3,566 to 4,511 ($p < 0.0001$). This is seen in figure 5.23.3. The suppressive cytokine IL-10 was also induced. Intracellular IL-10 increased from 2,555 in unstimulated cells to 2,861 in CEDIII stimulated cells ($p = 0.0021$). This is depicted in figure 5.23.2

The finding that the dengue glycoprotein CEDIII is capable of eliciting IL-10 responses may be clinically significant. IL-10 levels are increased in patients with severe Dengue (Malavige et al., 2013) (Chareonsirisuthigul et al., 2007; Tsai, T. et al., 2014; Ubol et al., 2010). This IL-10 production has been theorised to limit the immune responses to Dengue virus, leading to more severe symptoms. It is important to note, however, that the role of IL-10⁺ CD4⁺ T cells in dengue disease is unclear. During acute Dengue, substantial populations of Dengue specific CD4⁺ T cells which express both IFN γ and IL-

10 develop. These cells do not seem to contribute to increased severity of disease or dengue haemorrhagic fever (Tian et al., 2019).

The recall antigen CEDIII did not elicit any antigen specific responses in this model. It proved to be impossible to detect any CEDIII recall responses using this in vitro model. The cells incubated with CEDIII for 7 days did not show any significant differences to those incubated with media alone. This protocol seems to be unsuitable for assaying the development of antigen specific responses. This may be because the cells are already saturated with antigen and adding in additional recall antigen does not have a detectable effect. Alternatively, the timing may need to be changed or there may be insufficient antigen specific naïve T cells within the TMCs. Nonetheless, these results have proved useful, as the longer timespan of this experiment revealed the development of IFN γ , IL-4 and TNF α responses in CD4 $^{+}$ T cells stimulated with the Dengue vaccine candidates.

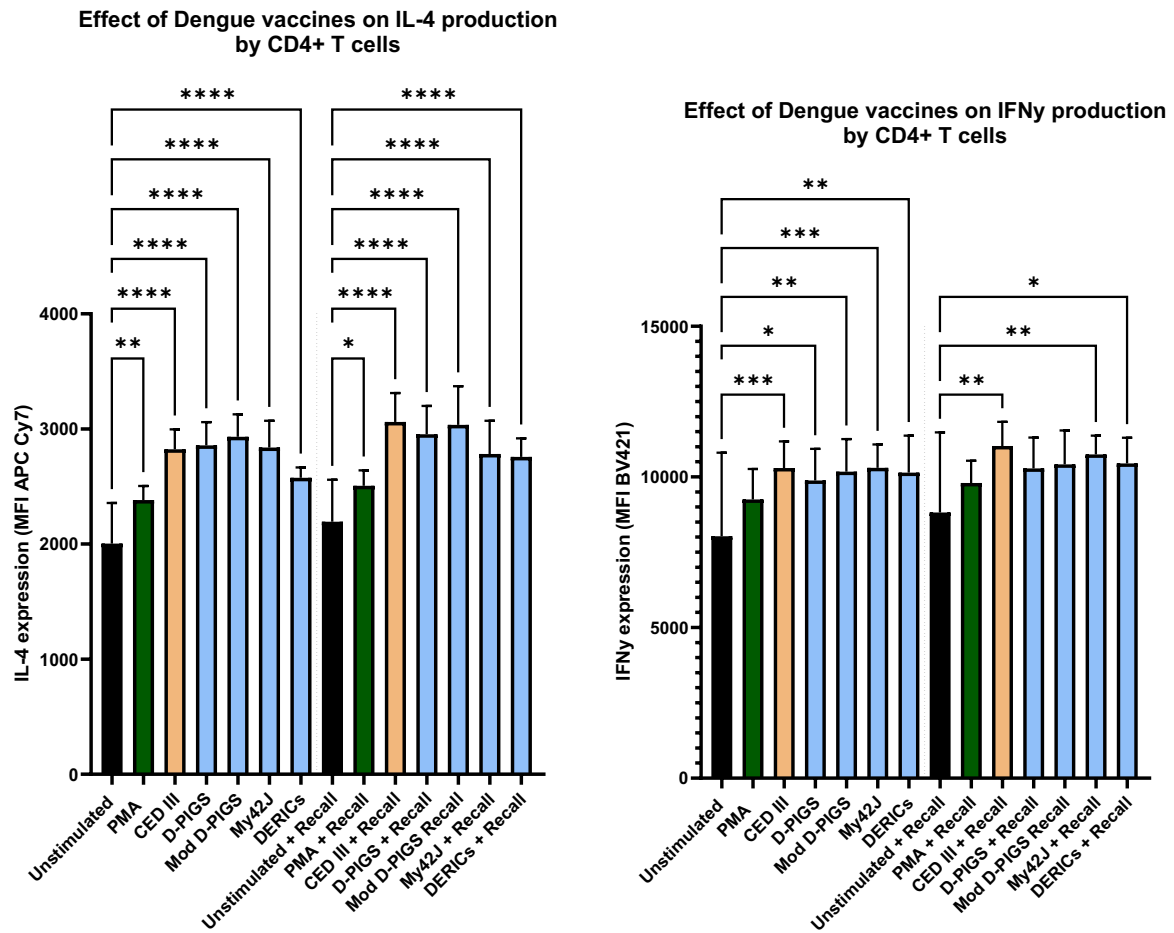


Figure 5.23.1. Recall responses in CD4+ T cells generated by novel Dengue vaccines. TMCs were seeded in triplicate at a density of 1×10^5 cells/well. They were stimulated with Mod D-PIGS, D-PIGS, My42J and DERICS for 5 days. CEDIII at $5 \mu\text{g/ml}$ and PMA Ionomycin were included as controls. After 5 days, recall CEDIII at $5 \mu\text{g/ml}$ was added to wells for a further 7 days. Flow cytometry was used to identify CD4+ T cells and measure intracellular IL-4 and IFN γ levels. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnett's multiple comparison test. (n=4)

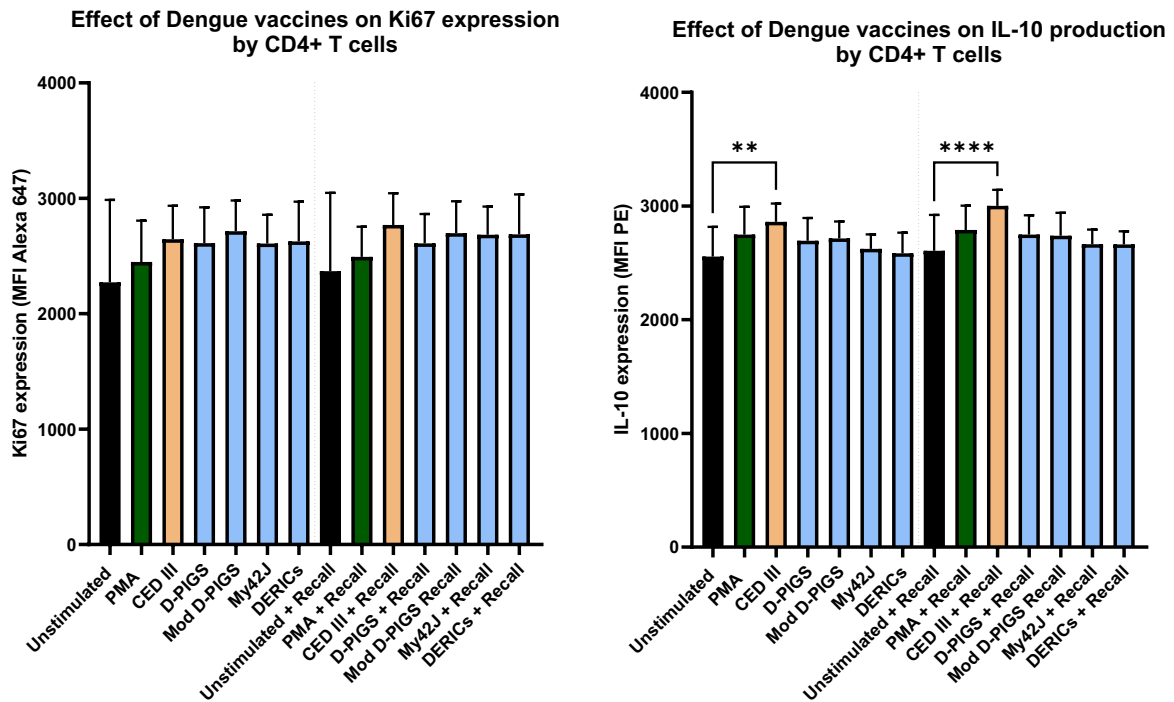


Figure 5.23.2. Recall responses in CD4+ T cells generated by novel Dengue vaccines. TMCs were seeded in triplicate at a density of 1×10^5 cells/well. They were stimulated with Mod D-PIGS, D-PIGS, My42J and DERICS for 5 days. CEDIII at $5 \mu\text{g/ml}$ and PMA Ionomycin were included as controls. After 5 days, recall CEDIII at $5 \mu\text{g/ml}$ was added to wells for a further 7 days. Flow cytometry was used to identify CD4+ T cells and measure Ki67 expression and intracellular IL-10. Mean MFI values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnett's multiple comparison test. (n=4)

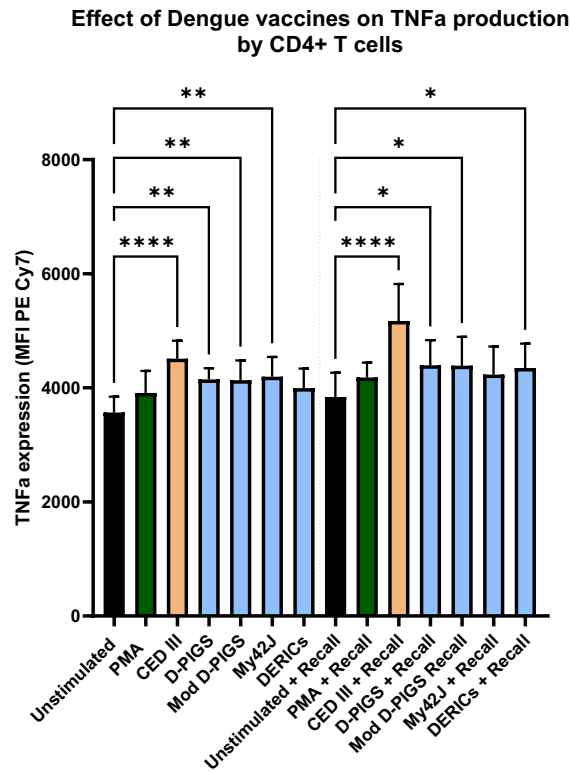


Figure 5.23.3. Recall responses in CD4 $^{+}$ T cells generated by novel Dengue vaccines. TMCs from four patients were seeded in triplicate at a density of 1×10^5 cells/well. They were stimulated with Mod D-PIGS, D-PIGS, My42J and DERICS for 5 days. CEDIII at 5 μ g/ml and PMA Ionomycin were included as controls. After 5 days, recall CEDIII at 5 μ g/ml was added to wells for a further 7 days. Flow cytometry was used to identify CD4 $^{+}$ T cells and measure intracellular TNF α levels. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnett's multiple comparison test. (n=4)

5.24 Recall responses in CD8+ T cells generated by novel Dengue vaccines

CD8+ T cell responses are important for protection during dengue infection. Dengue specific CD8+ T cells which generate IFN γ and TNF α have been shown to be protective against Dengue. The cytotoxic effect of these cells allows them to eliminate DENV infected host cells, limiting the infection (Weiskopf et al., 2013a) (Yauch et al., 2009b). This study modelled the immunogenic effect of our novel Dengue vaccines on CD8+ T cells to determine whether they would be capable of generating a suitable CMI response.

The Dengue antigen CEDIII generated IFN γ , TNF α and IL-4 responses in the tonsillar CD8+ T cells. Figure 5.24.2 shows IFN γ levels increased from 10,435 to 11,890 ($p=0.0046$). In figure 5.24.3, CED III stimulation induced TNF α expression. TNF α MFI levels increased from 4,633 to 5,700 ($p<0.0001$). The level of intracellular IL-4 also increased from 2,477 up to 3,088 with CEDIII stimulation ($p<0.0001$). This is shown in figure 5.24.1. These results contribute to the evidence that CEDIII alone is immunogenic and generates cytokine production by CD8+ T cells.

Figures 5.24.1, 5.24.2 and 5.24.3 show the effect of D-PIGS, Mod D-PIGS, My42J and DERICS on the intracellular cytokines produced by CD8+ T cells. Stimulation with D-PIGS, Mod D-PIGS or My42J resulted in increased levels of IL-4 production by CD8+ T cells. No other immunogenic effects were seen in the tonsillar CD8+ T cells.

IL-4 was induced in CD8+ T cells stimulated with D-PIGS, Mod D-PIGS and My42J. This can be seen in figure 5.24.1. APC Cy7 MFI was 2,477 in unstimulated cells. This increased to 3,096 with D-PIGS ($p<0.0001$), 3,160 with Mod D-PIGS ($p<0.0001$) and 3,017 with My42J ($p<0.0001$). Stimulation with DERICS did not significantly increase IL-4 production ($p=0.8350$). These IL-4 responses are not thought to be protective. IL-4 is associated with increased severity of Dengue disease (Abhishek et al., 2017; Butthep et al., 2012; Cui et al., 2016). Antigen presenting cells are more susceptible to dengue infection in the presence of IL-4 (Alen et al., 2009).

My42J and DERICS both downregulated the expression of intracellular IL-10 in CD8+ T cells. This may be useful for vaccine immunogenicity. IL-10 has a regulatory function in CD8+ T cells. It limits the sensitivity of antigen specific CD8+ T cells and raises the threshold required to activate them (Smith, L. K. et al., 2018).

These vaccine candidates did not elicit any other detectable immune responses in CD8+ T cells. There was no evidence of increased production of IFN γ or TNF α , which would be associated with a protective response.

Pulsing the cells with CEDIII did not alter the immune responses seen in the CD8+ T cells. Using the tonsil model, it was not possible to demonstrate that the immune responses generated by these vaccine candidates were antigen specific.

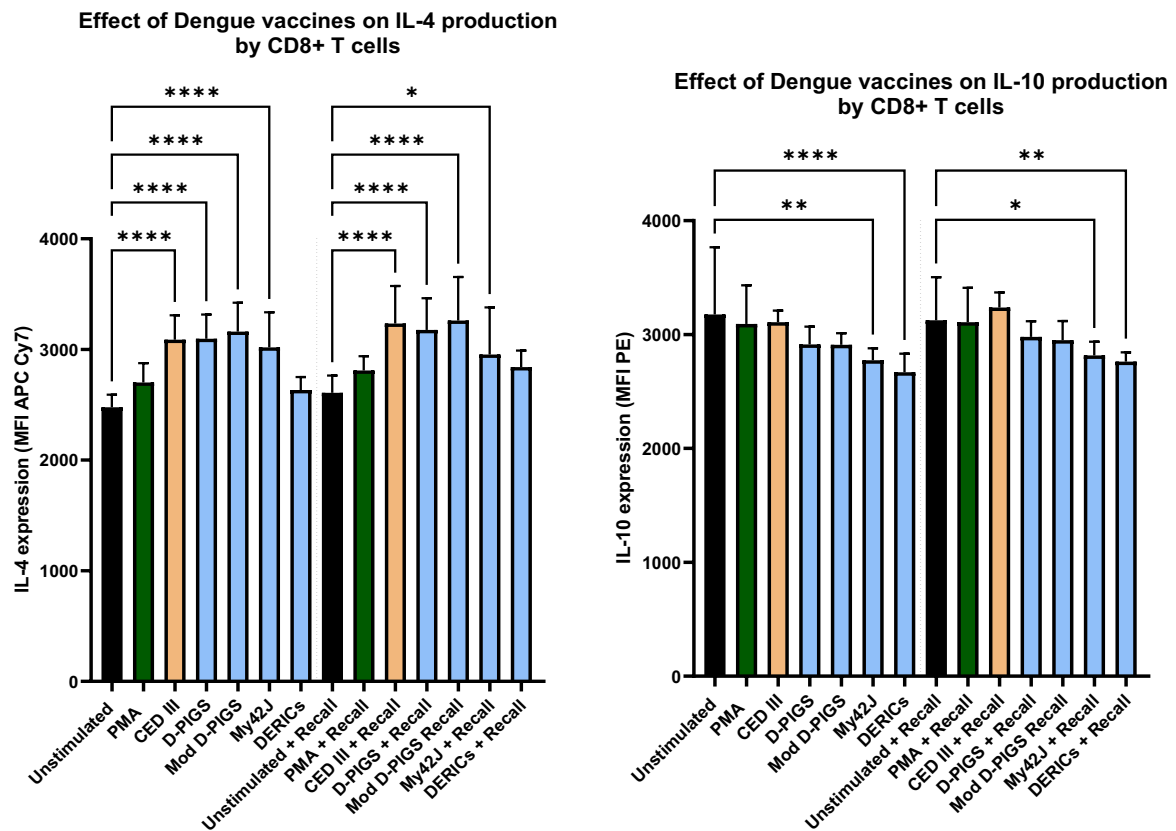


Figure 5.24.1. Recall responses in CD8+ T cells generated by novel Dengue vaccines. 96 well plates were seeded with 1×10^5 TMCs per well. Triplicate wells were stimulated with PMA / Ionomycin, 5 $\mu\text{g}/\text{ml}$ of CEDIII. The dengue vaccine candidates D-PIGS, DERICS, Mod D-PIGS and My42J were added to wells for 5 days. After 5 days, cells were either left for a further 7 days, or stimulated with 5 $\mu\text{g}/\text{ml}$ of recall CED III. Flow cytometry was then used to identify CD8+ T cells and measure intracellular expression of the cytokines IL-4 and IFN γ . (n=4) Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnetts multiple comparisons test. IL-4 responses were observed in CD8+ T cells stimulated with D-PIGS, Mod D-PIGS and My42J. No recall responses were observed. DERICS and My42J both downregulated IL-10 expression by CD8+ T cells

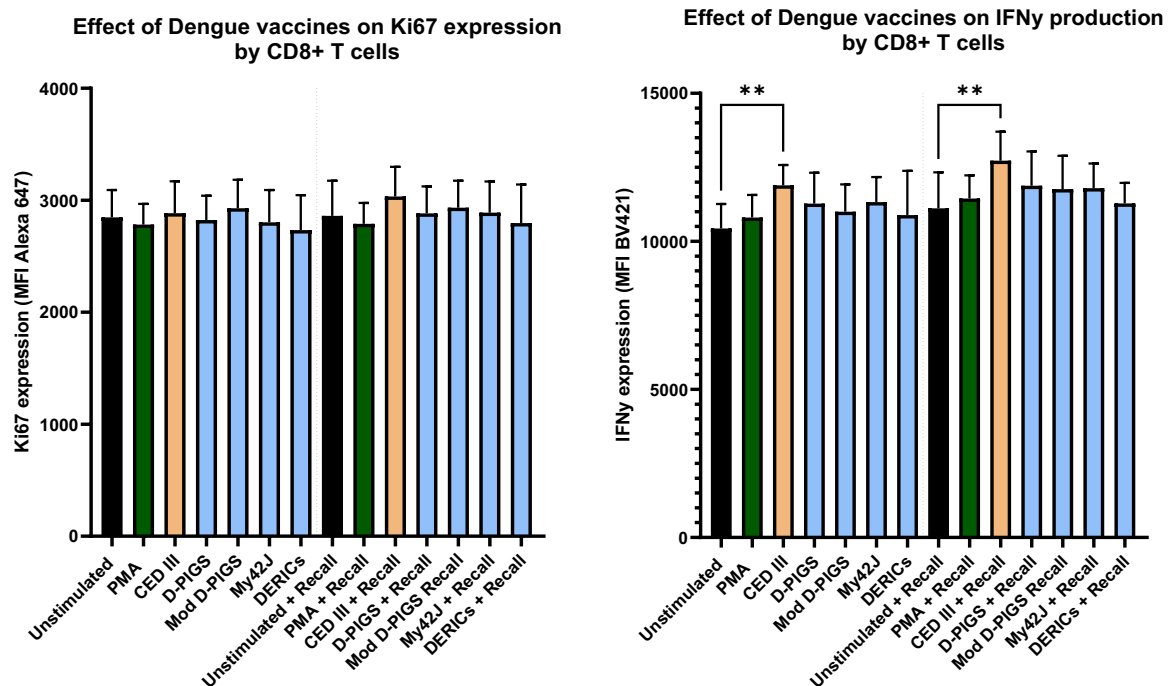


Figure 5.24.2. Recall responses in CD8+ T cells generated by novel Dengue vaccines. 96 well plates were seeded with 1×10^5 TMCs in each well. Triplicate wells were stimulated with PMA / Ionomycin, 5 μ g/ml of CEDIII. The dengue vaccine candidates D-PIGS, DERICS, Mod D-PIGS and My42J were added to wells for 5 days. After 5 days, cells were either left for a further 7 days, or stimulated with 5 μ g/ml of recall CED III. Flow cytometry was then used to identify CD8+ T cells and measure intracellular expression of IL-10 and the proliferative marker Ki67. (n=4) Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnetts multiple comparisons test. The Dengue vaccines tested did not induce IFN γ production. No proliferative recall responses were observed.

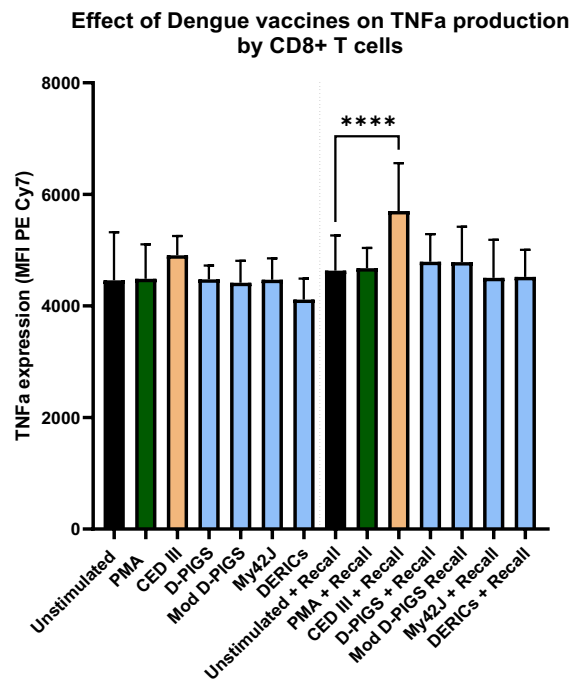


Figure 5.24.3. Recall responses in CD8+ T cells generated by novel Dengue vaccines. 96 well plates were seeded with 1×10^5 TMCs in each well. Triplicate wells were stimulated with PMA / Ionomycin, 5 $\mu\text{g}/\text{ml}$ of CEDIII. The dengue vaccine candidates D-PIGS, DERICS, Mod D-PIGS and My42J were added to wells for 5 days. After 5 days, cells were either left for a further 7 days, or stimulated with 5 $\mu\text{g}/\text{ml}$ of recall CED III. Flow cytometry was then used to identify CD8+ T cells and measure intracellular expression of TNF α (n=4). Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnetts multiple comparisons test. At the 12 day timepoint, TNF α responses were not elevated.

5.25 Antibody responses to D-PIGS

Neutralising antibodies are important for protection against Dengue virus. The D-PIGS vaccine has previously been tested in a mouse model. D-PIGS was found to generate DENV neutralising IgG responses in transgenic mice with human CD64 (Kim, Mi Young, Copland, Nayak, Chandele, Ahmed, Zhang, Diogo, Paul, Hofmann, & Yang, 2018b).

In this study we sought to use the tonsil model to replicate this finding and use D-PIGS to generate neutralising antibodies from human tonsillar B cells. TMCs were seeded in triplicate wells at a density of 10^5 cells/well. These TMCs were stimulated with D-PIGS for 14 days. The supernatant was collected and an anti CEDIII ELISA was used to detect dengue specific antibodies. No anti CEDIII antibodies could be detected. This is shown below in figure 5.25.1.

Developing a new antibody response requires a naïve B cell which is specific to the novel epitope. These antigen specific naïve B cells are rare, with a frequency of less than 0.05% of naïve lymphocytes having specificity for a given antigen (Smith, M. J. et al., 2017).

To increase the probability of finding a CEDIII specific naïve B cell, the experiment was enlarged 1,000 fold. The experiment was repeated using a large culture flask containing 2×10^8 TMCs. These were stimulated with 25 µg/ml D-PIGS for 14 days. After 14 days, viability was high, at 73%. Supernatant was collected and analysed by ELISA, but no anti CEDIII antibodies were detected. ELISA was used to detect anti CEDIII IgM and anti CEDIII IgG in the supernatant. It was not possible to generate anti CEDIII antibodies *de novo* using the tonsil model. These results strongly indicate that generating *de novo* antibody responses using tonsils is not possible. Antigen specific naïve B cells are exceptionally rare and represent a major bottleneck for generating antibody responses *in vitro*. The development of new antibody responses is a multi-step and complex process which also requires interactions with antigen specific T cells, chemotaxis and development of germinal centres. It is overambitious to expect that this can be reproduced using disassociated tonsil cells in cell culture conditions.

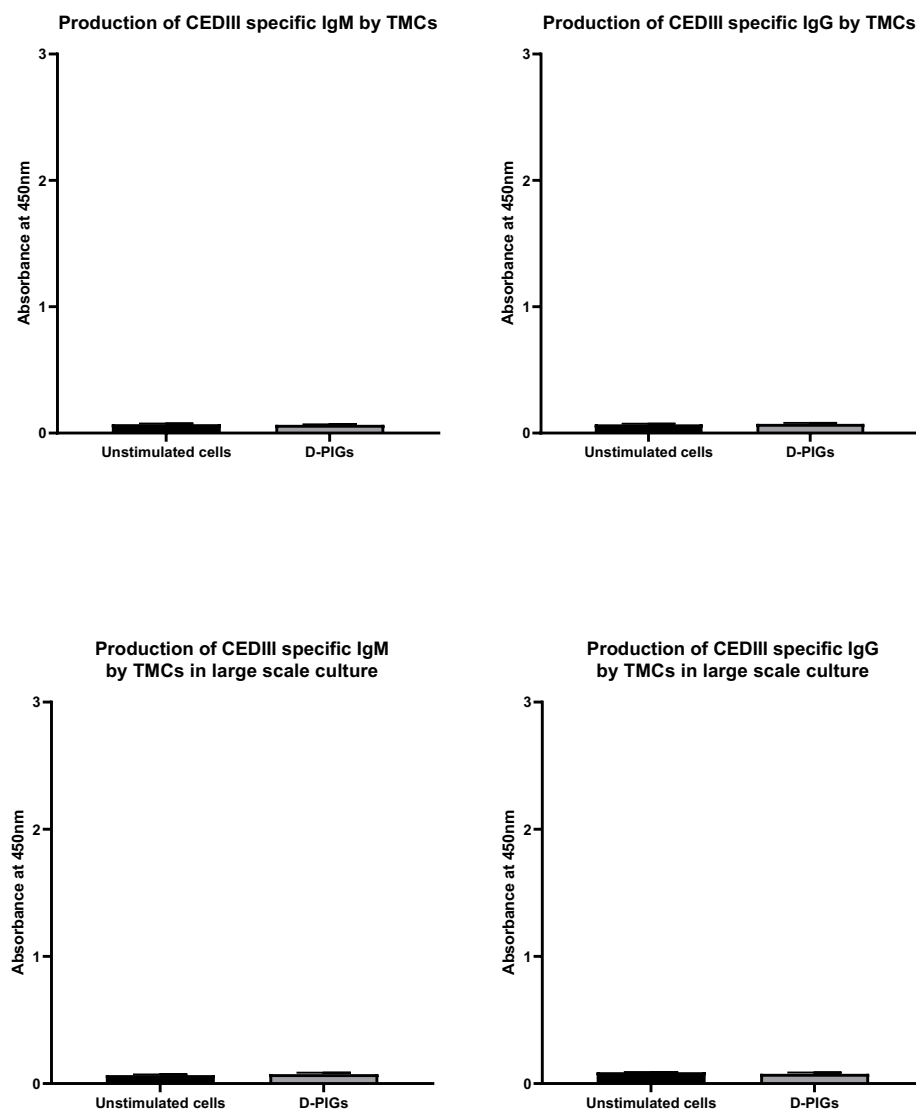


Figure 5.25.1. Production of CEDIII specific antibody responses by D-PIGS stimulated TMCs. TMCs were seeded into 96 wells plates at 1×10^5 cells per well. These cells were stimulated with D-PIGS at $25 \mu\text{g/ml}$ for 14 days. Supernatant was collected and anti CEDIII ELISAs were performed. Anti CEDIII IgM and IgG were measured. ($n=3$). This experiment was then repeated at a larger scale, with 1×10^8 cells stimulated with D-PIGS for 14 days. Mean values and $\pm$ standard deviations are shown. Supernatants from this were collected and anti CEDIII IgM and IgG were measured as before ($n=3$).

Key Findings

- Dengue vaccine candidates D-PIGS, Mod D-PIGS and DERICS bound to tonsillar monocytes
- D-PIGS, Mod D-PIGS and My42J generated TNF α , IFN γ and IL-4 responses in CD4+ T cells.
- DERICS generated IFN γ and IL-4 responses in CD4+ T cells.
- D-PIGS, Mod D-PIGS and My42J generated IL-4 responses in CD8+ T cells.
- My42J and DERICS both reduced IL-10 levels in CD8+ T cells.
- No antigen specific T cell recall responses to CEDIII were observed in TMCs.
- CEDIII specific antibodies were not detected in TMCs following D-PIGS stimulation.

Chapter 6 - Development of a mycobacterial Infection model using tonsillar tissue

Abstract

New vaccination strategies are needed to combat the spread of Tuberculosis (TB). The development of effective vaccines is hampered by a lack of known immune correlates of protection. In this study we have developed an *in vitro* model of mycobacterial infection. This model uses human tonsil cells infected with GFP BCG. This infection model can be used to test the ability of novel vaccines to limit mycobacterial infection.

We tested the protective effect of three novel vaccines, Spore FP1, YC NaMa FP1 and Liposome FP1 on mycobacterial clearance. Each of these vaccines was found to promote the clearance of mycobacterial infection in the tonsillar model. This antimycobacterial effect was not associated with an increase in nitric oxide production. In addition, these vaccines generated IFN γ and IL-17 responses in T cells during BCG infection. These responses have both been associated with protection against TB. Previous studies have established infection models for other diseases using human tonsil cells. This study uses the same approach for mycobacterial infection and demonstrates that it can be used to assess the immunogenicity and efficacy of novel TB vaccines.

6.1 Infection of tonsillar cells with BCG GFP.

Tonsillar infection models have previously been established for HIV, HHV6, EBV and influenza virus. We applied a similar approach to develop an *in vitro* infection model for mycobacterial infection. TMCs were seeded at a density of 1×10^6 cells/ml in 96 well plates and infected with BCG-GFP at an MOI of 4:1 for 24 hours. Following this, flow cytometry was used to identify CD11b+ myeloid cells, CD14+ monocytes and CD19+ B lymphocytes in addition to both CD4+ and CD8+ T lymphocytes. Infected cells were gated using FITC intensity. The results are shown in figure 6.1.1.

Myeloid cells, dendritic cells and monocytes are well established targets of mycobacteria during infection (Guirado et al., 2013) (Upadhyay et al., 2018) (Harding, J. S. et al., 2015) (Mihret, 2012) (Sampath et al., 2018). Upon infection with BCG, a large portion of tonsillar myeloid cells and monocytes became GFP positive, at 35% and 25%, respectively (Figure 6.1.1 a, b).

This study found that T lymphocytes also interact with GFP BCG, 15% of CD4+ and 17% of CD8+ T lymphocytes were positive for BCG-GFP (Figure 6.1.2 A, B). Analysis revealed a significant portion of tonsillar B lymphocytes, 40%, were associated with a BCG-GFP infection (Figure 6.1.2 C).

Using confocal microscopy, we were able to visualise the interactions between T lymphocytes and B lymphocytes with GFP BCG. This is shown in figure 6.1.3. This imaging confirmed that BCG GFP adhered to the cell surface of tonsillar B lymphocytes as well as CD4+ and CD8+ T lymphocytes

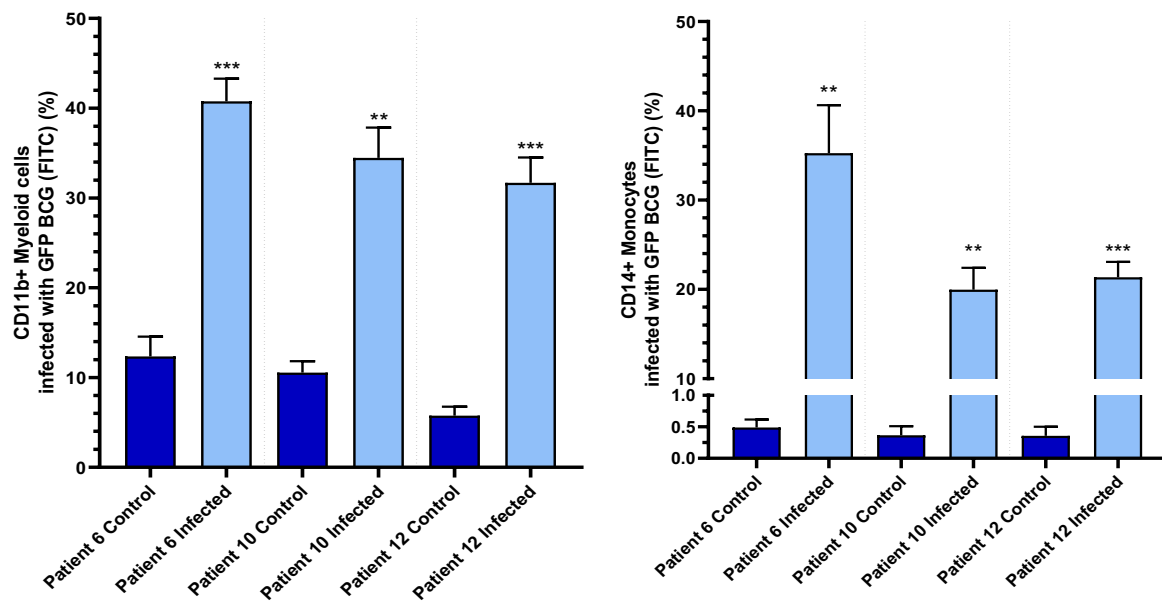


Figure 6.1.1. Infection of tonsillar myeloid cells and monocytes with BCG-GFP. TMCs were seeded in six repeat wells at a density of 1×10^6 cells/ml in 96 well plates ($n=3$). Infection with BCG-GFP was carried out at an MOI of 4:1. Following a 24-hour infection, flow cytometry was used to identify CD11b+ myeloid cells (**a**) and CD14+ monocytes (**b**). The percentage of GFP+ cells is shown along with $\pm$ SEM. Statistical significance is shown with asterisks, * indicates $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.001$ and **** is $p < 0.0001$. Statistical analysis was performed by repeated-measures one-way ANOVA with Sidak's multiple comparisons test.

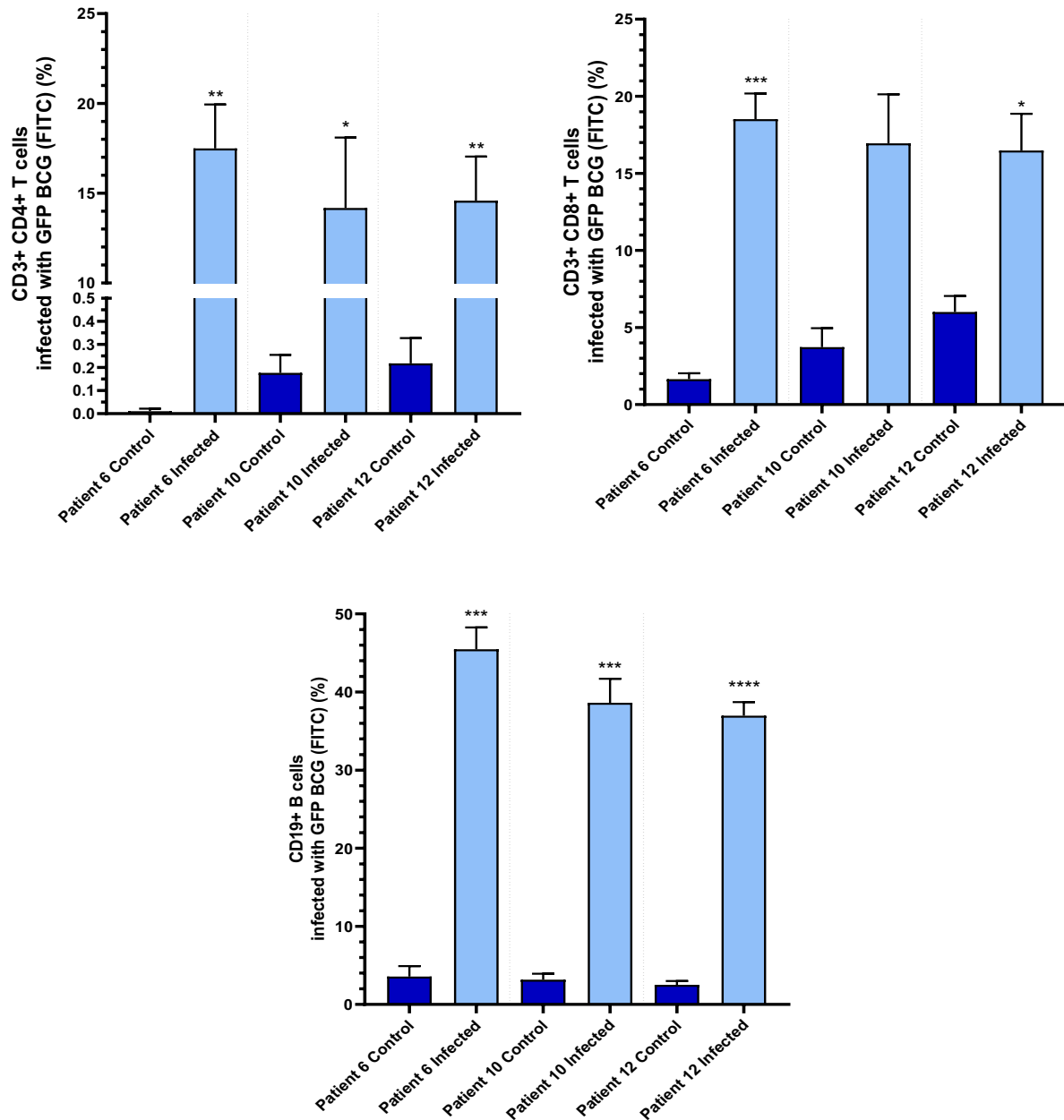


Figure 6.1.2. Infection of tonsillar T and B lymphocytes with BCG-GFP. TMCs from three patients were seeded in six repeat wells at a density of 1×10^6 cells/ml in 96 well plates. Infection with BCG-GFP was carried out at an MOI of 4:1. After 24-hours, flow cytometry was used to identify CD4+ T helper lymphocytes (A), CD8+ cytotoxic T lymphocytes (B) and CD19+ B lymphocytes (C). The percentage of GFP+ cells is shown. Statistical analysis was performed by repeated-measures one-way ANOVA with Sidak's multiple comparisons test. Statistical significance is shown with asterisks, * indicates $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.001$ and **** is $p < 0.0001$.

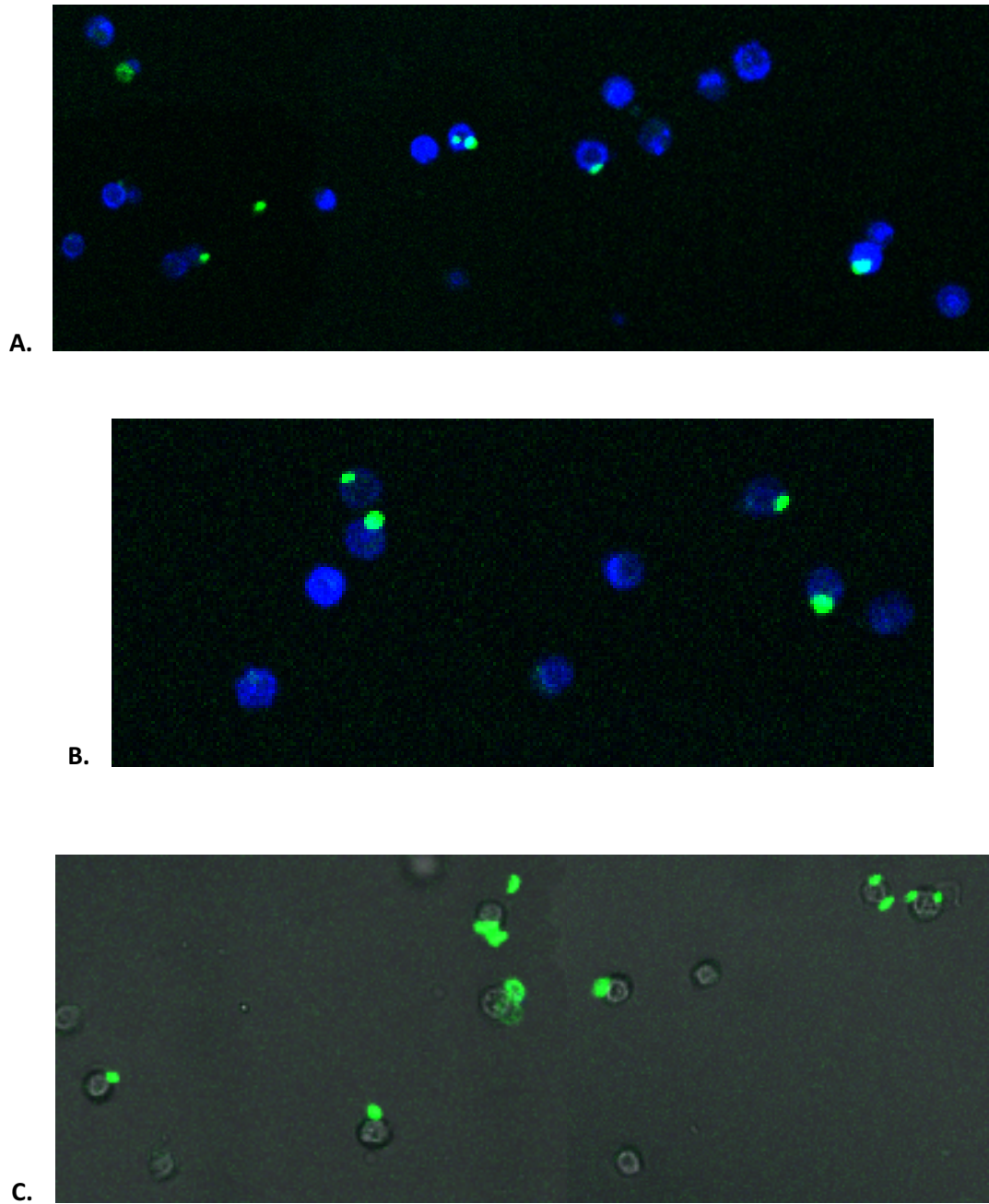


Figure 6.1.3. Imaging of interaction between tonsillar lymphocytes and BCG GFP Tonsil cells were infected with BCG GFP at an MOI of 4:1. The cells were then stained with anti CD4, anti CD8 and anti CD19 antibodies. FACS flow cytometry was used to sort CD4+ T cells, CD8+ T cells and CD19+ B cells into separate wells. These were then photographed using a NIKON AR1 confocal microscope at 200x magnification. CD4+ T cells (A), CD8+ T cells (B) and CD19+ B cells (C) are shown.

6.2 Elimination of GFP BCG by TMCs

It was hypothesised that the tonsillar infection model could be used to assess the efficacy of novel tuberculosis vaccines. We stimulated TMCs with a range of stimulations for 24 hours and then infected the cells with BCG GFP for a further 24 hours. FITC mean fluorescence intensity (MFI) was measured by flow cytometry to gauge the effect of these stimulations on the elimination of BCG GFP from the tonsillar immune cells.

TMCs were treated with BCG, the current TB vaccine, or the cytokine IFN γ , which is known to be necessary for protection against TB. Both of these stimulations led to enhanced elimination of BCG GFP from tonsillar monocytes (Figure 6.2.1). As expected, FITC MFI was highest in monocytes, which are known to phagocytose mycobacteria. The infected monocytes had a mean MFI of 29,067. Prior stimulation with BCG Pasteur led to a significant reduction in FITC MFI, down to 11,773 ($p=0.0002$). Similarly IFN γ stimulation also reduced the FITC MFI value to 20,296 ($p=0.0462$). The novel anti tuberculosis vaccine Spore FP1 also reduced FITC MFI from 29,067 to 17,281 in monocytes ($p=0.0061$). These findings demonstrate that tonsillar monocytes can be used as an *in vitro* model to assess the antimycobacterial effect of novel vaccines.

Interestingly, this effect was also observed in CD8 $^{+}$ T cells and can be seen in figure 6.2.2 B. Treatment with IFN γ or Spore FP1 reduced the FITC MFI. CD8 $^{+}$ T cells infected with BCG GFP had a FITC MFI of 2,846. In the presence of IFN γ , this dropped to 2,643 ($p=0.0261$). Prior stimulation with spore FP1 reduced the FITC MFI to 2,631 ($p=0.0176$). Activated CD8 $^{+}$ T cells are able to release perforin and granulysin to kill mycobacteria (Lu et al., 2014) (Stegelmann et al., 2005). This may explain the finding that stimulated CD8 $^{+}$ T cells had lower FITC MFI during infection than unstimulated cells.

The infection of CD11b $^{+}$ Myeloid cells and CD4 $^{+}$ T cells was unchanged by stimulation with BCG Pasteur, IFN γ or Spore FP1. The FITC MFI of infected myeloid cells and CD4 $^{+}$ T cells was not significantly different between groups. This is seen in Figure 6.2.2A and 6.2.2C.

Figure 6.2.2D shows the level of infection in tonsillar B cells. Stimulating B cells with BCG Pasteur prior to infection with GFP BCG led to a small reduction in the FITC MFI of tonsillar B cells. The mechanism underlying this effect was not known. It may be due to B cells binding to BCG Pasteur no longer being capable of binding to additional BCG GFP.

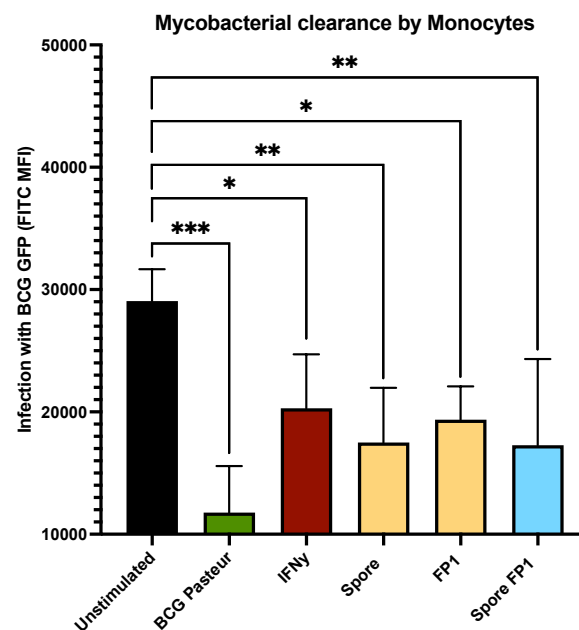


Figure 6.2.1. Elimination of GFP BCG by tonsillar monocytes TMCs were seeded in 96 well plates at 1×10^5 cells/well. TMCs were stimulated with BCG Pasteur, IFN γ , Spore FP1, Spores or FP1 alone for 24 hours. BCG GFP was then added to cultures at an MOI of 4:1 For a further 24 hours. Monocytes were stained with antibodies against CD14 and analysed by flow cytometry. Populations of monocytes were identified and their FITC MFI was measured. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test (n=4).

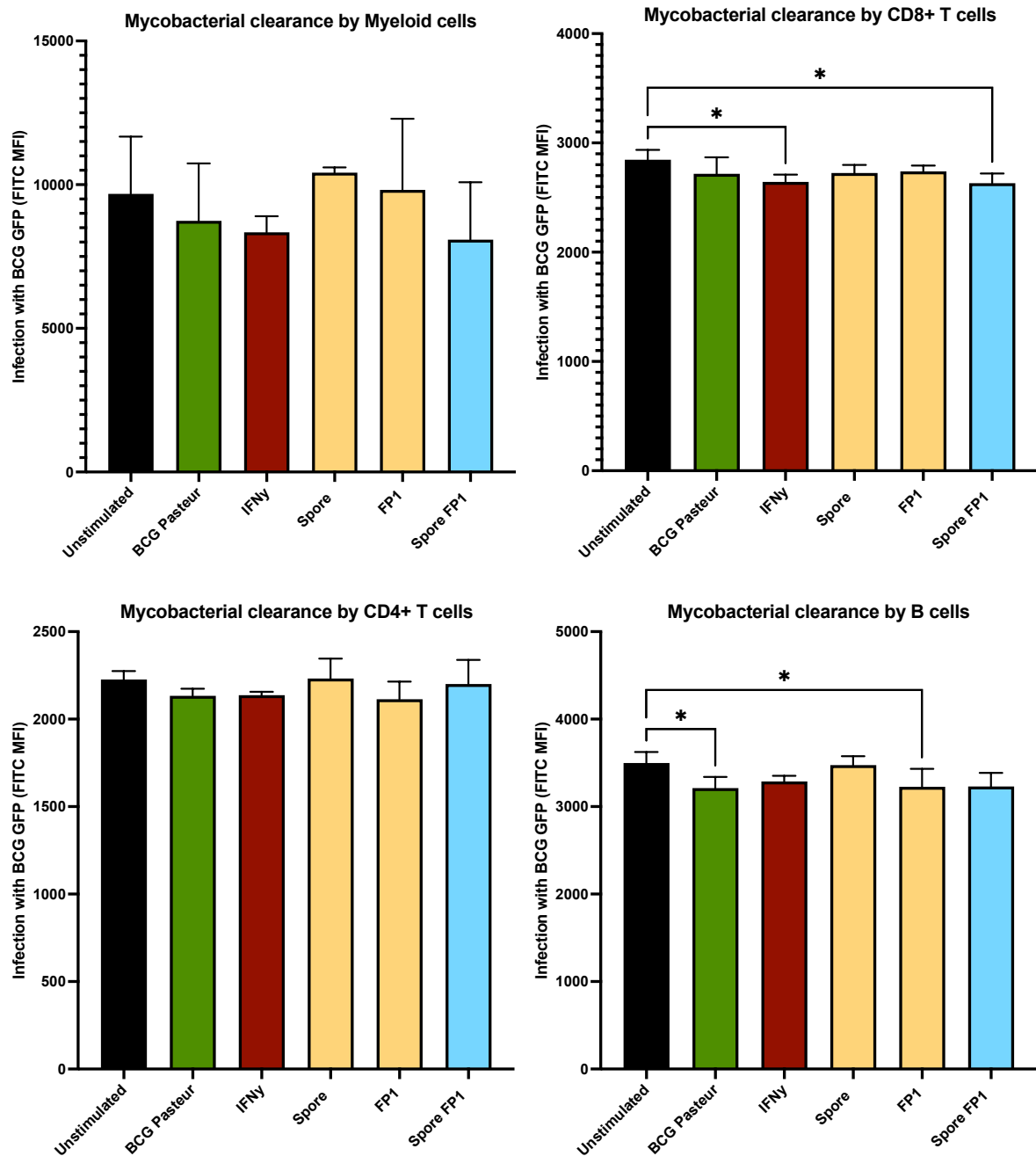


Figure 6.2.2. Elimination of GFP BCG by TMC subsets TMCs were seeded at 1×10^5 cells/well in 96 well plates. TMCs were stimulated with BCG Pasteur, IFN γ , Spore FP1, Spores or FP1 alone for 24 hours. BCG GFP was then added to cultures at an MOI of 4:1 for a further 24 hours. TMCs were stained with antibodies against CD3, CD4, CD8, CD111b and CD19 and analysed by flow cytometry. Myeloid cells (A), CD4+ T cells (B), CD8+ T cells (C), and B cells were identified. The FITC MFI and $\pm$ standard deviations are shown. (n=4). Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test

6.3 Effect of FP1 vaccines on mycobacterial clearance

Tonsillar monocytes are capable of eliminating BCG GFP after stimulation with IFN γ , BCG Pasteur or the Spore FP1 vaccine. This antimycobacterial effect may be a useful predictor of effective TB vaccine candidates. We stimulated TMCs with three FP1 vaccines, Spore FP1, YC NaMa FP1 and Liposome FP1. This experiment is shown in figure 6.3.1. All of these stimulations led to decreases in FITC MFI within monocytes. Infected monocytes which were unstimulated had a mean FITC MFI of 295,481. Prior vaccination with BCG Pasteur reduced the FITC MFI more than threefold to 78,005 ($p=0.0136$). Stimulation with Spore FP1 reduced the FITC MFI more than fivefold down to 50,415 MFI ($p=0.0058$). The greatest clearance of mycobacterial infection was seen with YC NaMa FP1 stimulation. The mean FITC MFI value in monocytes fell 22 fold down to 13,162 ($p=0.0018$). Liposome FP1 also induced clearance of mycobacterial infection. The FITC MFI fell sixfold from 295,481 to 48,048 ($p=0.0054$). This data conforms with previous work which shows Spore FP1, YC NaMa FP1 and Liposome FP1 are all able to protect against tuberculosis infection in mice (Copland et al., 2018). (Hart et al., 2018b) (Diogo et al., 2019a).

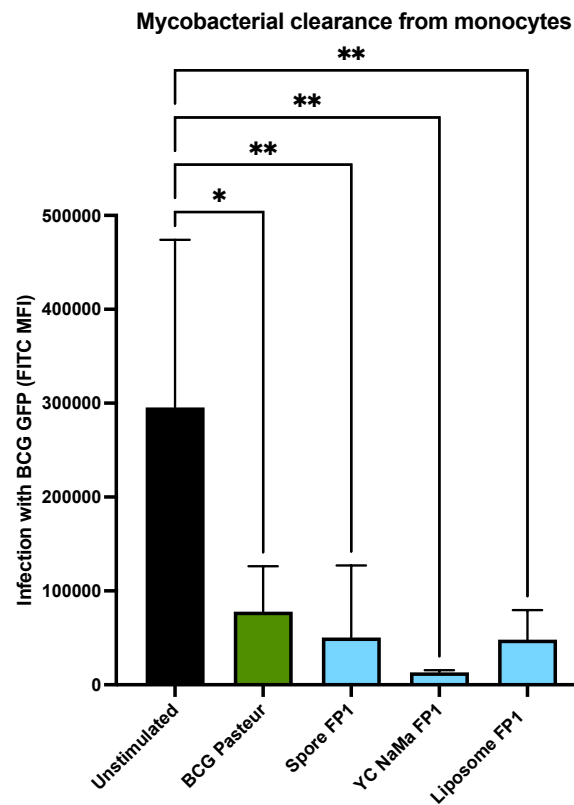


Figure 6.3.1. Effect of FP1 vaccines on mycobacterial clearance. Tonsillar cells were stimulated with BCG Pasteur at an MOI of 1:10, Spore FP1, YC NaMa FP1 or Liposome FP1 for 24 hours (n=4). BCG GFP was then added to cultures at an MOI of 4:1. After a further 24 hours, cells were analysed by flow cytometry and the FITC MFI of CD14⁺ monocytes was measured. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using One Way ANOVA and Dunnetts multiple comparisons test.

6.4 Expression of costimulatory molecules during mycobacterial infection

This section investigates the expression of four activation markers, CCR5, CD40, CD80 and CD86. MTB is known to downregulate the expression of CD40, CD80 and CD86 on APCs during infection (Bonato et al., 2001) (Saha et al., 1994). This immune subversion blocks the development of new Th1 responses and prevents existing Ag85B specific CD4+ T cells from being reactivated (Schreiber et al., 2010a).

The chemokine receptor CCR5 regulates macrophage chemotaxis and has immune functions (Desmetz et al., 2006) (Hüttenrauch et al., 2005). CCR5 upregulation on monocytes is associated with differentiation into macrophages (Tuttle et al., 1998). CCL5 binding to CCR5 also promotes macrophage survival. Upregulation of CCR5 has also been observed during infection of monocytes by MTB (Fraziano et al., 1999). This constitutes immune subversion, as the elevated CCR5 levels result in excessive IL-10 production and lowered expression of MHC II on APCs (Das et al., 2014).

Figure 6.4.1 shows the effect of mycobacterial infection on CCR5 expression by tonsillar monocytes. It was anticipated that mycobacterial infection would raise CCR5 levels. Instead, infection with BCG reduced the expression of CCR5 on monocytes. Uninfected monocytes had a PerCP Cy5.5 MFI of 12,982. This fell to 7,675 after the cells had been infected with BCG for 24 hours ($p < 0.0001$). Reduced CCR5 expression may indicate that the BCG was inhibiting the differentiation of these monocytes into macrophages. Monocytes which had been stimulated with YC NaMa FP1 prior to infection showed a higher expression of CCR5. YC NaMa FP1 increased CCR5 expression on infected monocytes from an MFI of 7,675 to 9,798 ($p = 0.0011$). Prior stimulations of Resiquimod, BCG Pasteur, IFN γ , Spore FP1 and Liposome FP1 had no impact on the expression of CCR5 during mycobacterial infection.

The coreceptor CD40 plays an important role in TB infection. CD40 knockout mice are vulnerable to TB (Lazarevic et al., 2003) and MTB is known to downregulate expression of CD40 during infection (Schreiber et al., 2010b). CD40 binding to CD40L is capable of inducing IL-12 production (Cella et al., 1996).

CD40 co-stimulation is also necessary for the formation of IL-17 responses (Perona-Wright et al., 2009). Boosting CD40/ CD40L interactions has been shown to enhance antigen specific Th17 responses in the context of TB infection, leading to improved outcomes (Sia et al., 2017).

In this study, mycobacterial infection resulted in a significant drop in CD40 expression on monocytes. The PE Cy7 MFI on uninfected cells fell from 26,732 to 15,262 on infected monocytes ($p < 0.0001$). Figure 6.4.1 shows that this effect was intensified when the TMCs were stimulated prior to infection. Stimulation with Resiquimod reduced PE Cy7 down to 8,941 ($p < 0.0001$). IFN γ reduced CD40 expression levels twofold from 15,262 to 6,883 ($p < 0.0001$). Stimulation with BCG Pasteur before mycobacterial infection caused CD40 levels to drop from 15,732 to 8,099 ($p < 0.0001$). The FP1 vaccines had the same effect of reducing CD40 expression during mycobacterial infection. Spore FP1 vaccination caused CD40 levels to fall from 15,262 to 8,501 ($p = 0.0003$). Liposome FP1 reduced CD40 levels further, from 15,262 to 3,734 PE Cy7 MFI ($p < 0.0001$). This was a fourfold reduction of monocyte CD40 during mycobacterial infection. The greatest reduction in CD40 expression was seen with YC NaMa FP1. CD40 levels fell eightfold from 15,262 to 1,929 ($p < 0.0001$).

The costimulatory molecules CD80 and CD86 play important roles in fine tuning immune responses. CD80 and CD86 are important for T cell responses, because ligation of CD80 and CD86 on APCs with CD28 provides anti-apoptotic signals to T cells (Boise et al., 1995). The costimulatory molecules CD80 and CD86 are both downregulated during MTB infection. Double knockout CD80 and CD86 mice are more susceptible to MTB infection due to a weakened Th1 response (Bhatt et al., 2009).

Figure 6.4.2 shows that Mycobacterial infection did not significantly alter the expression of CD80 or CD86. Prior stimulation with YC NaMa FP1 significantly increased CD80 expression on monocytes during mycobacterial infection. YC NaMa FP1 stimulation caused CD80 expression to rise from 1,917 to 3,187 MFI ($p = 0.0062$). Neither Spore FP1 nor Liposome FP1 had any impact on the expression levels

of CD80 during mycobacterial infection. Stimulation with IFN γ , BCG Pasteur or Resiquimod also had no effect on CD80 expression levels.

In Figure 6.4.2, the effect of each vaccine on monocyte CD86 expression is shown. YC NaMa FP1 significantly increased CD86 expression on monocytes during mycobacterial infection. BV421 MFI was 4,338 in the infected monocytes. YC NaMa FP1 stimulation raised this to 6,865 ($p<0.0001$). Resiquimod treatment also increased the expression of CD86. BV421 MFI was increased from 4,338 to 8,420 ($p<0.0001$). BCG Pasteur, IFN γ , Spore FP1 and Liposome FP1 all had no impact on CD86 expression on monocytes during infection.

The results in Figures 6.4.1 and 6.4.2 demonstrate that YC NaMa FP1 is effective at activating monocytes during mycobacterial infection and that it is capable of promoting the expression of important costimulatory molecules. YC NaMa raised CCR5 levels, counteracting the reduction in CCR5 caused by the mycobacterial infection. YC NaMa also increased expression of the B-7 molecules CD80 and CD86.

In contrast, BCG Pasteur, Spore FP1 and Liposome FP1 had no impact on the expression of CCR5 or the costimulatory molecules CD80 and CD86 on monocytes during mycobacteria infection. The common feature of each of these vaccines was that they exacerbated the reduction in CD40 expression on infected monocytes.

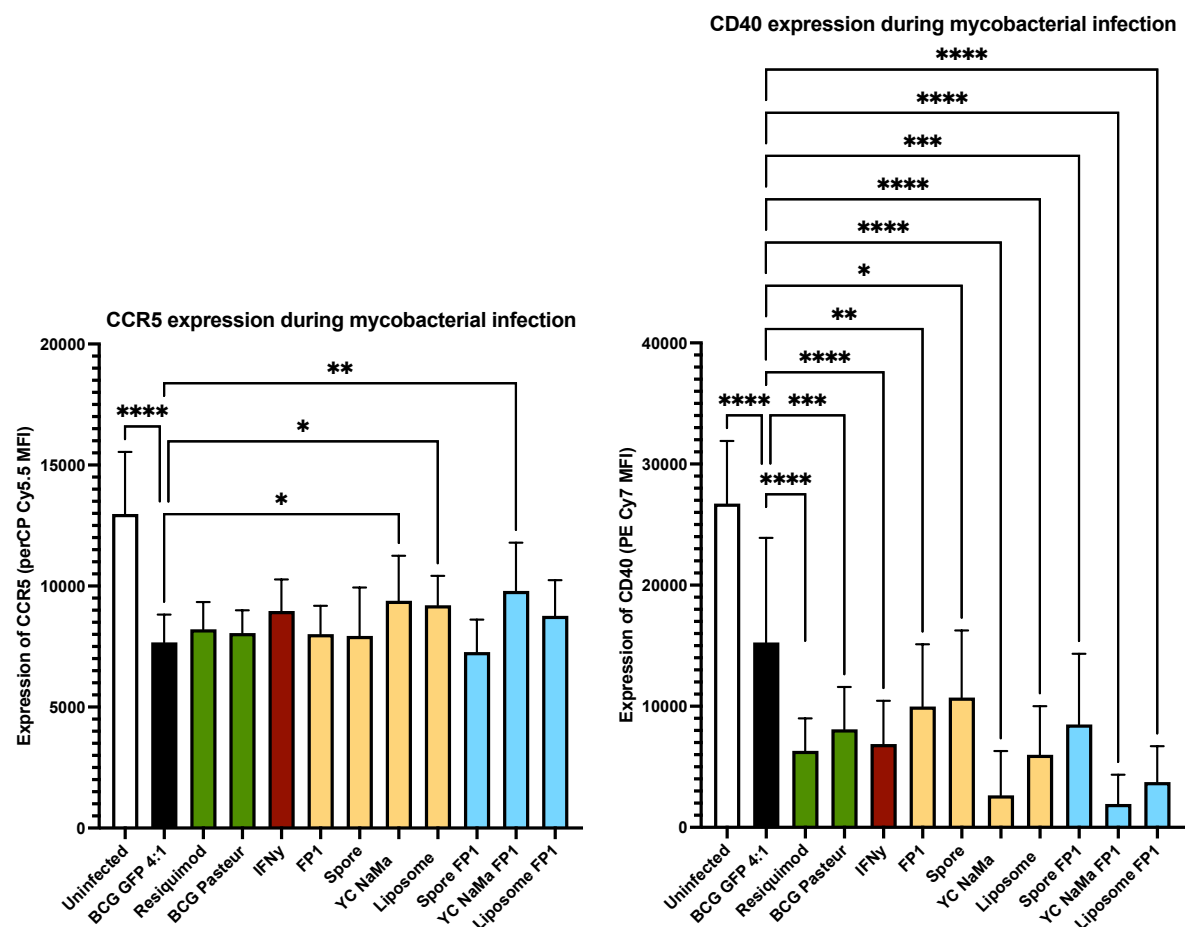


Figure 6.4.1. Expression of monocyte costimulatory molecules during mycobacterial infection. TMCs were seeded in quadruple wells at a density of 1×10^6 cells/ml. TMCs were stimulated with Resiquimod, BCG Pasteur (MOI of 1:10), IFN γ , Spore FP1, YC NaMa FP1 or Liposome FP1 vaccines for 24 hours. The TMCs were then infected with BCG GFP at an MOI of 4:1 for a further 24 hours. Flow cytometry was used to identify CD14 $^{+}$ monocytes and measure expression of the chemokine receptor CCR5 and the costimulatory molecule CD40 on tonsillar monocytes. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . One way ANOVA and a Dunnetts multiple comparisons test were used to conduct the statistical analysis (n=3).

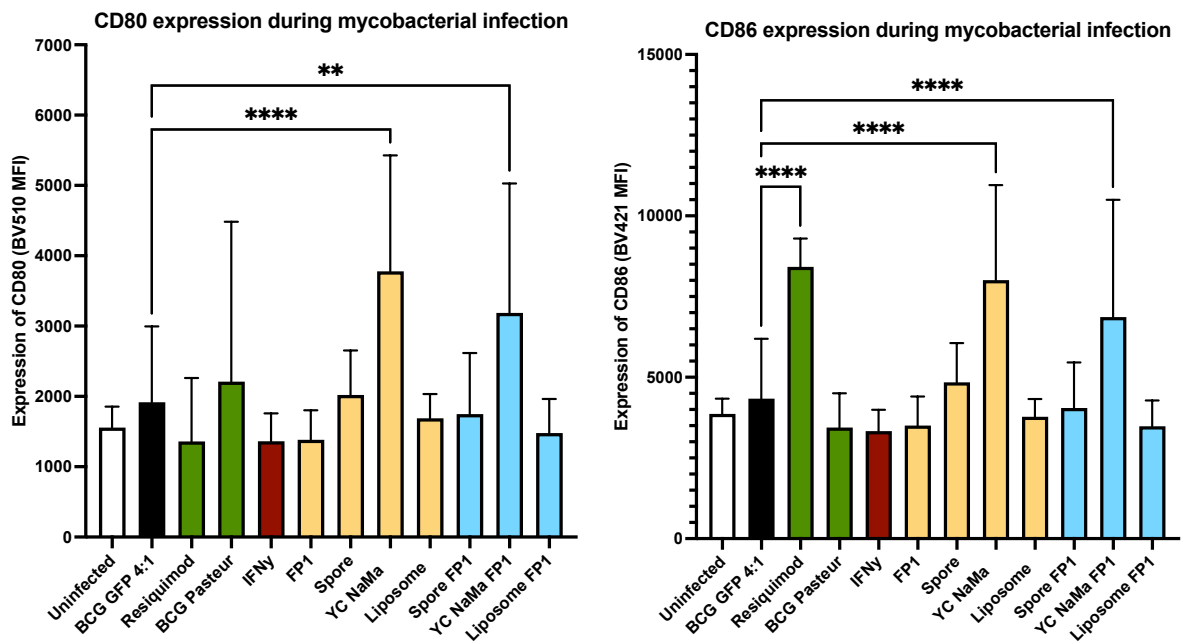


Figure 6.4.2. Expression of monocyte costimulatory molecules during mycobacterial infection TMCs

were seeded in quadruple wells at a density of 1×10^6 cells/ml. TMCs were stimulated with Resiquimod, BCG Pasteur (MOI of 1:10), IFN γ , Spore FP1, YC NaMa FP1 or Liposome FP1 vaccines for 24 hours. The TMCs were then infected with BCG GFP at an MOI of 4:1 for a further 24 hours. Flow cytometry was used to identify CD14 $^{+}$ monocytes and measure expression of the B-7 costimulatory molecules CD80 and CD86 on tonsillar monocytes. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . One way ANOVA and a Dunnetts multiple comparisons test were used to conduct the statistical analysis (n=3).

6.5 Nitric oxide production during mycobacterial infection.

Nitric oxide plays a key role in TB pathogenesis. In mice, nitric oxide produced by iNOS in macrophages has a direct bactericidal effect on mycobacteria (Chan et al., 1992). Murine macrophages stimulated with IFN γ are able to eliminate mycobacterium tuberculosis through nitric oxide induced apoptosis (Herbst et al., 2011a). The role of nitric oxide in control of mycobacterial infection in humans is less clear. IFN γ is known to control mycobacterial infection in macrophages by inducing apoptosis by a nitric oxide dependant mechanism (Herbst et al., 2011b).

A preliminary study was performed to test whether nitric oxide was involved in the vaccine induced elimination of mycobacteria by tonsillar monocytes. The results are shown in Figure 6.5.1. Stimulation with Resiquimod and IFN γ may have induced nitric oxide production but the increase was not statistically significant. Similarly, there was no statistically significant difference in the levels of nitric oxide in supernatants from unstimulated cells and those stimulated by the FP1 vaccine formulations. This finding suggests that the clearance of mycobacteria in this model is not associated with nitric oxide production.

IFN γ stimulation enhanced mycobacterial elimination by tonsillar monocytes. BCG vaccine, Spore FP1, YC NaMa FP1 and Liposome FP1 were also capable of enhancing mycobacterial elimination by monocytes. These findings are seen in figures 6.2.1 and 6.3.1.

The common feature of the BCG vaccine, Spore FP1, YC NaMa FP1 and Liposome FP1 is that they all generate IFN γ responses. This is shown in figures 5.2.1, 5.11.1, 5.12.1 and 5.13.1. This IFN γ response may be the key to understanding how these vaccines enhance mycobacterial elimination. IFN γ activation enables macrophages to successfully develop mature phagosomes which can eliminate mycobacteria (Troost et al., 2009). IFN γ has been found to enhance mycobacterial elimination through a NOs independent mechanism using LRG-47 (MacMicking et al., 2003). If nitric oxide or peroxynitrite are not responsible for the tuberculocidal effect of activated monocytes then LRG-47 might be a good candidate for further investigation.

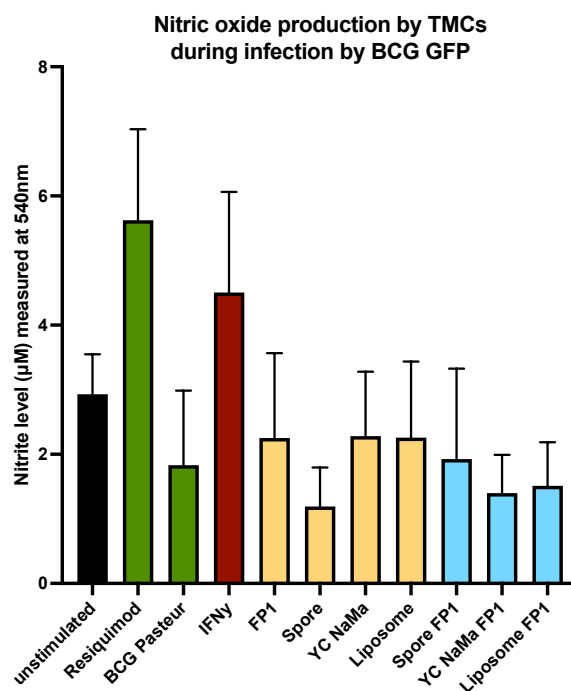


Figure 6.5.1. Nitric oxide production during mycobacterial infection. TMCs were seeded in quadruple wells at a density of 1×10^6 /ml. The TMCs were stimulated with Resiquimod, BCG Pasteur (MOI 1:10), IFN γ or three FP1 vaccines for 24 hours. After stimulation, The TMCs were infected with BCG GFP at an MOI of 4:1 for 24 hours. Supernatant was collected from wells and Greiss Reagent was used to measure nitrite levels in the supernatants. The absorbance at 560nm was recorded. A sodium nitrate standard curve was used to interpolate concentration values. Mean values and $\pm$ standard deviations are shown. Statistical analysis was performed using One way ANOVA and Dunnetts multiple comparisons test.

Key findings

- TMCs can be infected with BCG GFP to create a mycobacterial infection model
- Exogenous IFN γ stimulation led to enhanced mycobacterial clearance by monocytes.
- BCG vaccination enhanced the clearance of mycobacteria by monocytes in this infection model.
- Vaccination with Spore FP1, YC NaMa FP1 and Liposome FP1 also enhanced the clearance of mycobacterial infection
- Vaccine induced mycobacterial clearance was associated with IFN γ production by CD4 $^{+}$ T cells.

Chapter 7 – Interaction of B cells with BCG

Abstract

The mycobacterial infection model developed in chapter 6 revealed that B and T lymphocytes interact directly with BCG GFP. The interaction of B cells with mycobacteria has not been extensively studied. We sought to better characterise this interaction. Microscopy was used to visualise the infection of B cells with BCG GFP. This revealed that BCG GFP binds to the external surface of B cells. There was no evidence of BCG establishing intracellular infections within B cells.

Further investigation revealed the mechanism by which B cells and BCG interact. Antibodies contribute to the attachment of BCG to B cells. There was no evidence that phagocytosis or macropinocytosis were necessary for the interaction of B cells and BCG GFP. The complement component C1q did not impact the infection of B cells.

The functional effect of BCG binding to B cells was unknown. This study determined that BCG exerts a profound suppressive effect on the function of B cells. In RAJI B cells, BCG infection led to reduced expression of the markers for chemotaxis and proliferation. Antibody production was suppressed along with the expression of HLA DR and costimulatory molecules. In tonsillar B cells infected with BCG, chemotaxis and antigen presentation markers were suppressed, but the B cells were still capable of responding to the infection with enhanced proliferation and antibody production. The mechanism by which BCG suppresses B cells has not yet been resolved but this work showed that it was not due to B cell death or anergy.

7.1 Role of phagocytosis and macropinocytosis in the interaction between BCG and B cells

B cells are known to engage in phagocytosis and macropinocytosis. We sought to determine whether these processes were involved in the interaction of B cells with BCG. The nature of the interaction of GFP BCG with B cells was investigated by pre-treating B cells with cytochalasin D, Amiloride, PMA or treatment post infection with trypsin.

In this experiment, phagocytosis was blocked using cytochalasin D. Cytochalasin D binds to actin filaments, preventing phagocytosis.

Macropinocytosis was blocked using amiloride. Amiloride is able to inhibit macropinocytosis by acidifying submembranes within cells. In addition, macropinocytosis is an actin driven process so it would be expected that cytochalasin D would inhibit macropinocytosis.

Conversely, PMA is known to stimulate macropinocytosis. In this study, PMA was used to induce macropinocytosis by B cells.

The FITC MFI of uninfected B cells was 1,274 compared to 2,447, in BCG infected B cells ($p < 0.0001$). This confirmed that the B cells were interacting with the GFP BCG. Stimulation with cytochalasin D, Amiloride or PMA alone did not increase FITC MFI compared to the uninfected cells. Stimulation with Cytochalasin D, Amiloride or PMA followed by BCG GFP infection did not alter the FITC MFI of the infected B cells. The FITC MFI values for these conditions were 2,509, 1,705 and 2,376 respectively. This is shown in figure 7.1.1.

The fact that neither cytochalasin D nor Amiloride inhibited the interaction of B cells with BCG strongly suggests that neither phagocytosis nor macropinocytosis of BCG is required for the interaction between B cells and BCG.

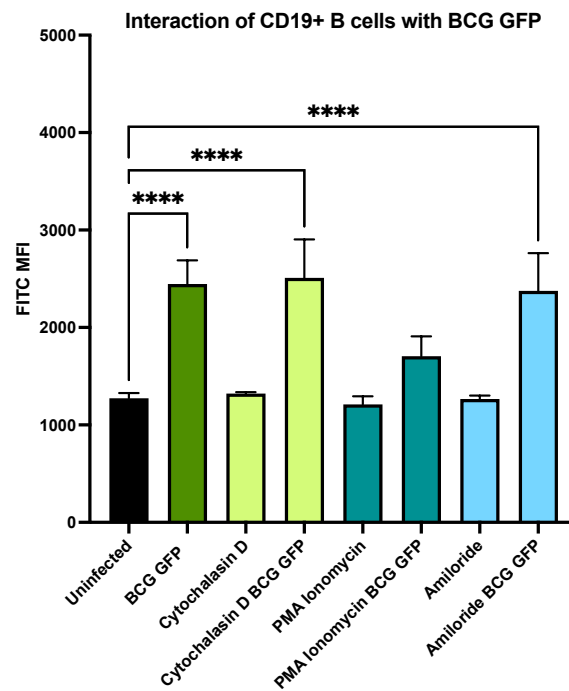


Figure 7.1.1. Effect of Cytochalasin D, PMA and Amiloride on the interaction of B cells and BCG

Triplicate wells of TMCs were prepared at 1×10^5 cells/well. Cells were treated with cytochalasin D, PMA or Amiloride for 1 hour. These cells were then infected with BCG GFP at an MOI of 4:1 for a further 3 hours. These cells were then stained for CD19 and analysed by flow cytometry. The MFI of FITC in B cells was measured. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . The results were analysed using one way ANOVA and Dunnetts multiple comparisons test (n=2).

7.2 Effect of trypsin treatment on BCG/ B cell interaction post-infection.

It was important to determine whether BCG was being internalised by B cells or whether it was simply adhering to the surface of B cells. It was hypothesised that treating infected cells with trypsin would remove any bacterial cells which were adhering to the B cell surface but leave internalised BCG within the cells. Instead, it was found that trypsin treatment of tonsillar B cells after BCG infection led to a substantial increase in the FITC fluorescence of the B cells. This data is shown in figure 7.2.1.

Uninfected B cells had a FITC MFI of 1,282. The BCG infection increased this to a mean FITC fluorescence of 2,544. Trypsin treatment after infection substantially increased the interaction of B cells with BCG GFP. B cells which were infected with BCG GFP and then treated with Trypsin had an MFI of 9,688. This was a 3.8 fold increase in the mean FITC fluorescence compared to infection alone ($p= 0.0004$).

This unexpected finding may offer an insight into the mechanism by which BCG interacts with B cells. It is possible that BCG is binding to B cells in an antibody dependant manner. The trypsin used in this experiment is able to digest IgM into individual immunoglobulin fragments (Andrew SM, 2001; Elakovich et al., 1978) (D Beale & T. Van Dort 1982). These IgM fragments can bind to the Fc receptors on B cells and T cells. Breaking pentameric IgM into monomeric antibody fragments may increase the availability of antibody which binds to BCG and B cells. If immunoglobulins are responsible for the binding of BCG to B cells, this would explain the increase in FITC MFI observed in B cells following infection and trypsin treatment. It is significant that in this experiment, the cells were not washed after they were treated with trypsin. Any monomeric fragments of IgM from the B cells would have been retained within the wells and not washed away. Further work was needed to confirm whether antibodies are responsible for the interaction of B cells and BCG. This was undertaken and the results are shown in the following sections.

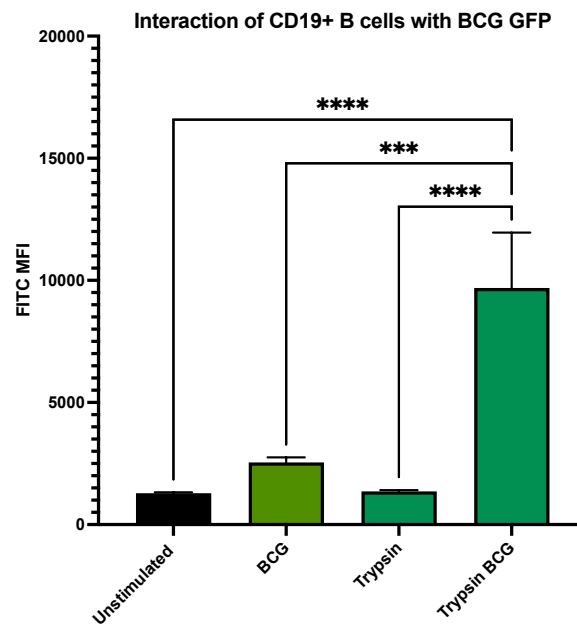


Figure 7.2.1. Effect of trypsin treatment on BCG/ B cell interaction post-infection. Triplicate wells were seeded with TMCs at 1×10^5 cells/well. These were infected with BCG at an MOI of 4:1 for 3 hours. Cells were then treated with trypsin for 5 minutes at 37°C. Cells were then stained for CD19 and the MFI of FITC in B cells was measured by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . The results were analysed using one way ANOVA and Sidaks multiple comparisons test.

7.3 The effect of pre treating B cells with trypsin before infection with BCG GFP.

The RAJI cell line was used to ascertain the effect of trypsin treatment on the interaction between B cell and BCG. RAJI cells were treated with trypsin for 10 minutes, 20 minutes or 30 minutes and then washed. The trypsin treated cells were then infected with BCG GFP at an MOI of 4:1. The results of this experiment are shown in figure 7.3.1.

Pre-treatment with trypsin, followed by a wash step led to a significant reduction in the interaction of B cells with BCG. 10% of RAJI cells interacted with BCG without trypsin pre treatment. After 30 minutes of trypsin treatment, this fell 2 fold to just 4.8% of RAJI cells ($p=0.0057$). This finding is consistent with the theory that BCG interacts with B cells in an antibody dependant manner. Trypsin treatment followed by a washing step would digest IgM into monomeric immunoglobulins. This monomeric IgM would be lost during the wash step prior to BCG infection. This would explain the reduced percentage of infected RAJI cells seen using this protocol. Following this, further experiments were carried out to test whether the interaction of B cells with BCG GFP is related to antibody binding.

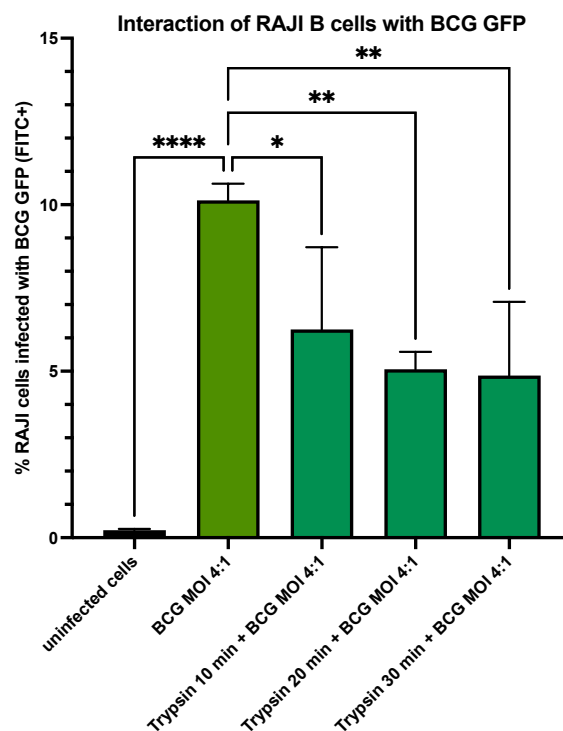


Figure 7.3.1. The effect of trypsin treatment of B cells prior to infection with BCG GFP. Triplicate wells were seeded with RAJI cells at 1×10^5 cells/well. Cells were treated with trypsin for 10, 20 or 30 minutes at 37°C . The cells were then washed with PBS. The cells were infected with BCG at an MOI of 4:1 for 3 hours. Cells were then stained for CD19 and the MFI of FITC in B cells was measured by flow cytometry. ($n=2$). Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . The results were analysed using one way ANOVA and Dunnetts multiple comparisons test.

7.4 The role of C1q in the interaction of B cells with BCG.

Previously published literature shows that complement plays a role in the infection of B cells by mycobacteria. Mycobacteria use a range of different entry mechanisms. They express heparin binding hemagglutinin adhesin (HBHA) which allows them to gain entry to non phagocytic cells (Raze et al., 2018). Mycobacteria can get into B and T cells through macropinocytosis and through the DEC 1 receptor (Castañeda-Sánchez et al., 2017). The complement 1, 3 and 4 receptors are also important entry points for mycobacteria to enter B cells (Krocova et al., 2020).

The complement component C3 assists the attachment of mononuclear cells to mycobacterium tuberculosis and their subsequent phagocytosis (Mueller-Ortiz et al., 2001). The complement component C1q is known to be present at a higher concentration in the blood of untreated TB patients (Lubbers et al., 2018). C1q has been shown to increase uptake of BCG by THP1 cells (Shwayat, 2017). It was important to determine whether C1q mediated the interaction of B cells with BCG. In this experiment RAJI cells were infected with BCG GFP in the presence of C1q. 125µg/ml of C1q was added to RAJI cells for 1 hour. These cells were then infected with BCG GFP for three hours. Infection with BCG GFP increased the percentage of infected B cells and their FITC MFI, as expected. Following infection with BCG GFP, 7.6% of the B cells became infected. The FITC MFI of B cells increased from 3,469 to 5,619 following infection with BCG GFP ($p=0.0034$). The addition of C1q to the wells did not significantly increase the FITC MFI of the infected B cells compared to infection alone. This indicates that C1q is not responsible for the observed interaction between B cells and BCG. These results are shown in figure 7.4.1.

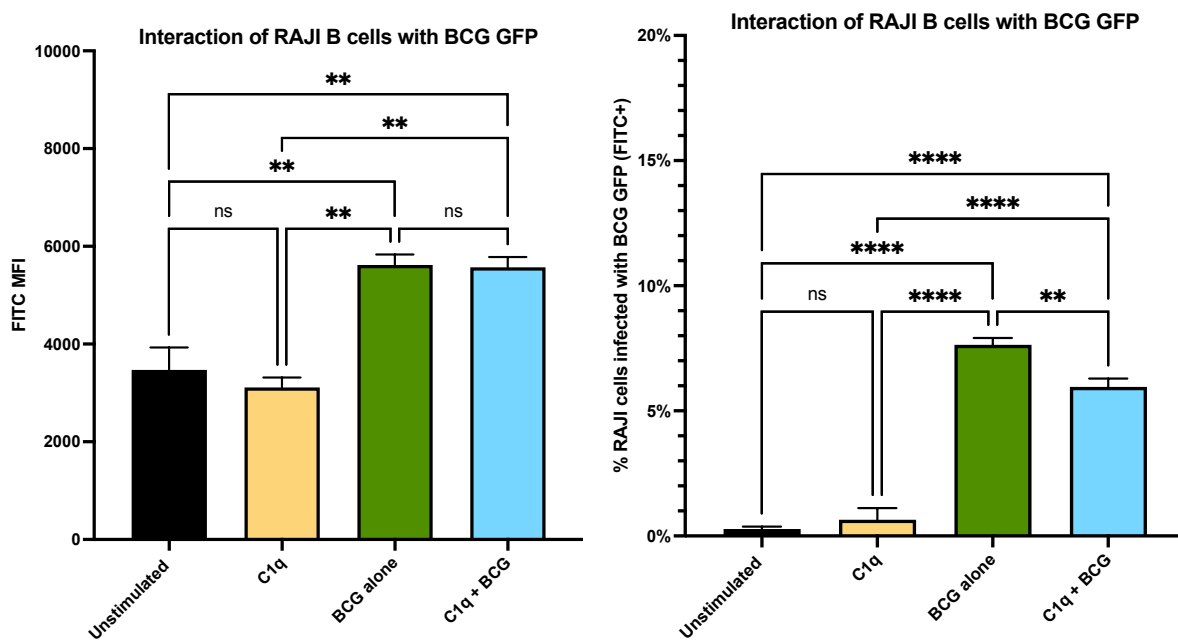


Figure 7.4.1. The role of C1q in the interaction of B cells with BCG. Triplicate wells containing 1×10^5 RAJI cells were treated with $125 \mu\text{g/ml}$ of C1q for 1 hour. These cells were then infected with BCG GFP at an MOI of 4:1 for three hours. The RAJI cells were then analysed by flow cytometry and the FITC MFI and percentage FITC + values were both measured ($n=2$). Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Tukey's multiple comparisons test. There was no significant increase in the percentage of infected B cells or the FITC MFI in the presence of C1q.

7.5 Effect of serum on the interaction of B cells and BCG.

The B cells used in this study have been grown in culture media which includes foetal bovine serum (FBS). FBS contains antibodies and complement. We sought to establish what effect the inclusion of serum in the culture media was having on the infection of B cells by BCG.

Figure 7.5.1 reveals that the interaction of B cells with BCG was significantly enhanced in the presence of foetal bovine serum (FBS). Uninfected B cells had a mean fluorescence of 2,772. After 24 hours of coculture with BCG GFP at an MOI of 4:1 without FBS, this rose to 7,388 ($p=0.041$). The addition of FBS to this infection model further increased the MFI of the RAJI cells by fourfold to 30,020. This was significantly brighter than the uninfected cells and the cells infected in the absence of FBS ($p<0.0001$).

In addition, the percentage of RAJI cells which were infected by BCG was increased in the presence of FBS. 1.4% of cells were FITC+ prior to infection. This rose 30 fold, as 44% of RAJI cells became FITC+ following infection with BCG GFP without FBS. With the addition of FBS, more RAJI cells were infected; 63.5% of RAJI cells were FITC+ after infection in the presence of 10% FBS. This increase was statistically significant ($p<0.0001$). This result shows that whilst serum is not necessary for the infection of B cells by BCG it does significantly increase the infection of B cells by BCG. The most likely mechanism for this serum dependent effect is the binding of antibodies to the surface of BCG, but further confirmation is needed.

Figure 7.5.2 shows the same experimental layout, but here, serum from BCG vaccinated rhesus macaques was used instead of FBS. When the RAJI cells were infected with BCG in the absence of serum, the FITC MFI rose from 2,772 to 7,3388 ($p=0.041$). The percentage of infected RAJI cells also rose from 1.4% to 44%. When 10% monkey serum was included, the level of infection increased from 7,388 to 24,843 ($p<0.0001$). The percentage of infected RAJI cells also increased in the presence of monkey serum, 54% of RAJI cells became infected ($p=0.0001$). Heat treatment of the monkey serum did not significantly impact the ability of the serum to enhance infection of B cells.

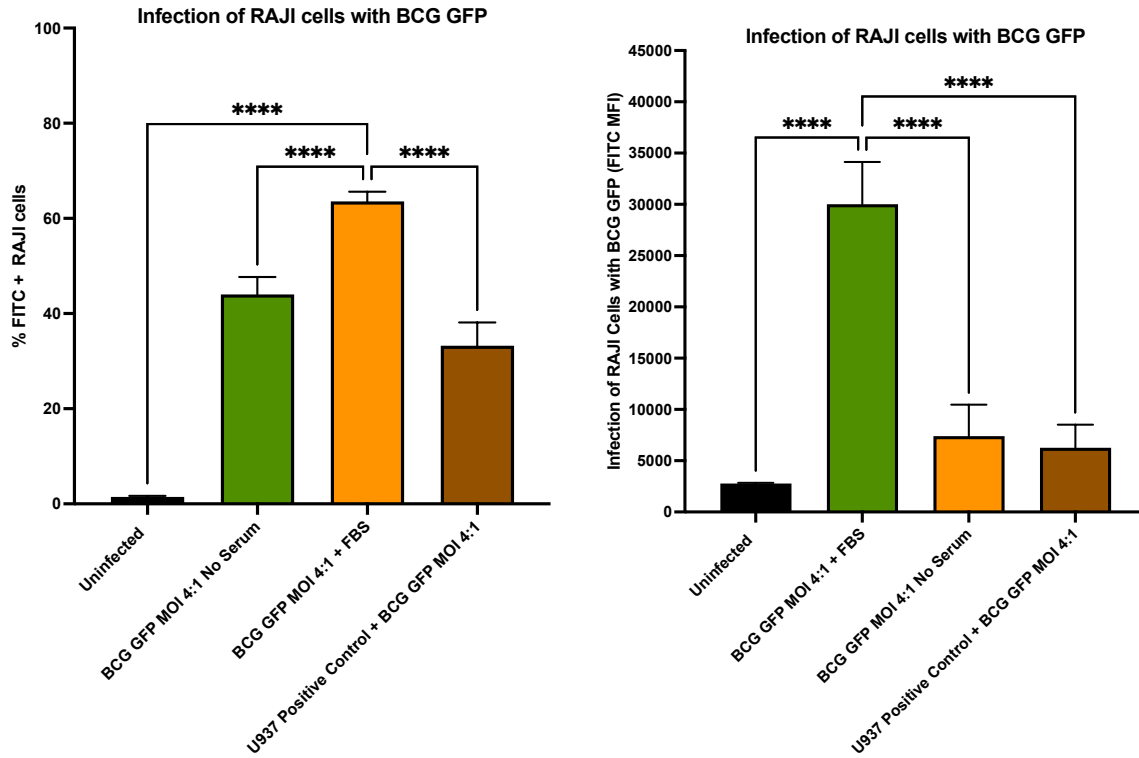


Figure 7.5.1. Effect of foetal bovine serum on the interaction of B cells and BCG. Duplicate wells containing 1×10^5 RAJI cells were infected with BCG GFP at an MOI of 4:1. Wells were prepared containing RPMI media without serum or RPMI media with 10% foetal bovine serum (A). Wells were also prepared containing 10% monkey serum or heated monkey serum. The monkey serum was sourced from BCG vaccinated animals. After 3 hours the cells were analysed by flow cytometry. The percentage of FITC + B cells was measured. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Tukey's multiple comparisons test ($n=3$).

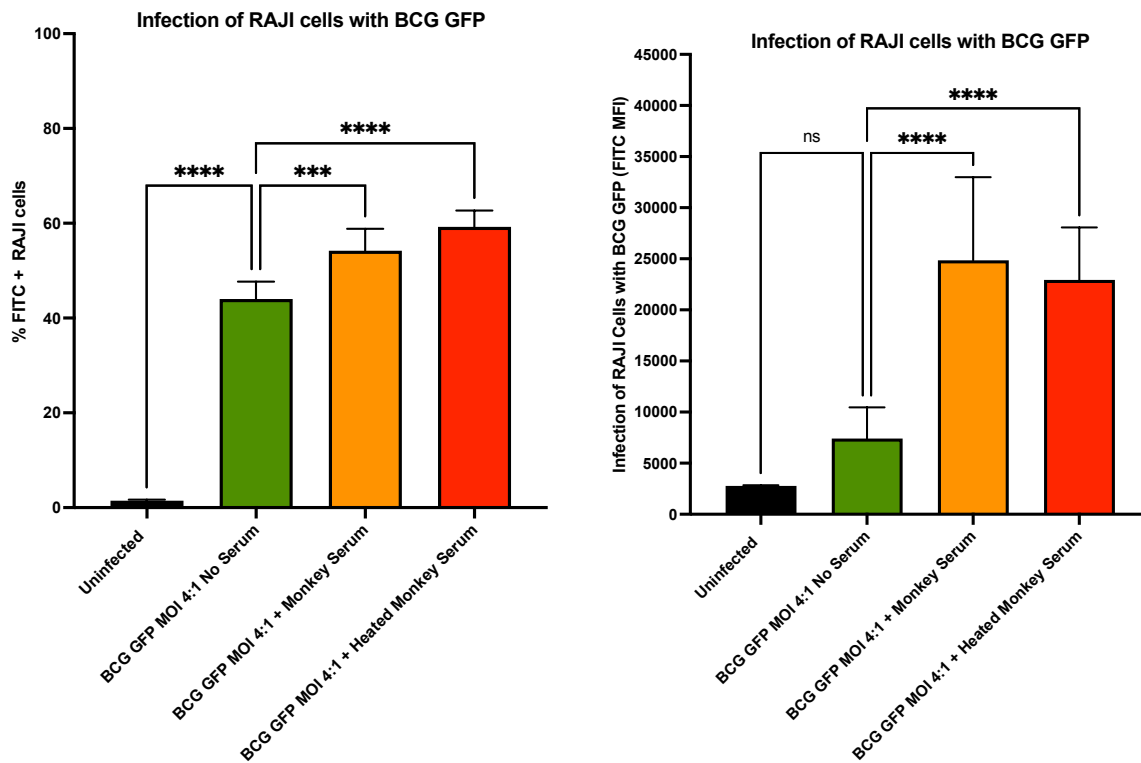


Figure 7.5.2. Effect of monkey serum on the interaction of B cells and BCG. Duplicate wells containing 1×10^5 RAJI cells were infected with BCG GFP at an MOI of 4:1. Wells were prepared containing RPMI media without serum or with 10% monkey serum or heated monkey serum. The monkey serum was sourced from BCG vaccinated animals. After 3 hours the cells were analysed by flow cytometry. The percentage of FITC + B cells was measured. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . Statistical analysis was performed using one way ANOVA and Dunnett's multiple comparisons test (n=3).

7.6 Effect of human serum and immunoglobulin on the interaction of B cells and BCG

Foetal bovine serum and monkey serum both enhanced the binding of BCG GFP to RAJI cells. To test whether human serum had the same effect, RAJI cells were infected with BCG GFP for 24 hours with or without 10% human serum. Figure 7.6.1A shows that 1% of uninfected RAJI cells were FITC+. After infection with BCG without serum, 44% of RAJI cells were FITC+ ($p < 0.0001$). When 10% human serum was added to the culture medium, 65% of RAJI cells became FITC+ during BCG GFP infection. This was a significant increase compared to infection without serum ($p < 0.0001$). This increase in FITC+ RAJI cells confirms that human serum enhances the infection of B cells by BCG.

The experiment shown in figure 7.6.1A also investigates the role of immunoglobulin in the infection of B cells. RAJI cells were infected with GFP BCG with human serum which did not contain any immunoglobulin. This was to ascertain whether human immunoglobulins were responsible for the serum dependant enhancement of B cell infection. The RAJI cells which were infected with BCG GFP with immunoglobulin depleted serum were only 31% FITC+. This was significantly lower than the 65% of cells which became FITC+ in the presence of BCG and serum ($p < 0.0001$). This demonstrates that human immunoglobulin is responsible for increasing the interaction of B cells and BCG.

Figure 7.6.1 B confirms that the human serum without immunoglobulin did not contain anti BCG IgG. The presence of human anti BCG IgG was tested by ELISA. The negative control had an absorbance of 0.25. An anti Acr monoclonal antibody TBG65 was used as a positive control. This bound to the plate and generated a positive signal with an absorbance of 0.61. This was a fourfold increase compared to the negative control ($p < 0.0001$). Human serum generated a stronger signal. The absorbance increased 13 fold, from 0.25 in the negative control to 3.47 in the cells containing human serum. The human serum without Ig did not generate a signal any higher than the negative control. The negative control had an absorbance of 0.25 whilst human serum without Ig had an absorbance of 0.13 ($p = 0.4087$). This provides confirmation that the human serum used in figure 7.6.1 A did contain anti BCG antibodies and that these were entirely absent in the human serum without Ig.

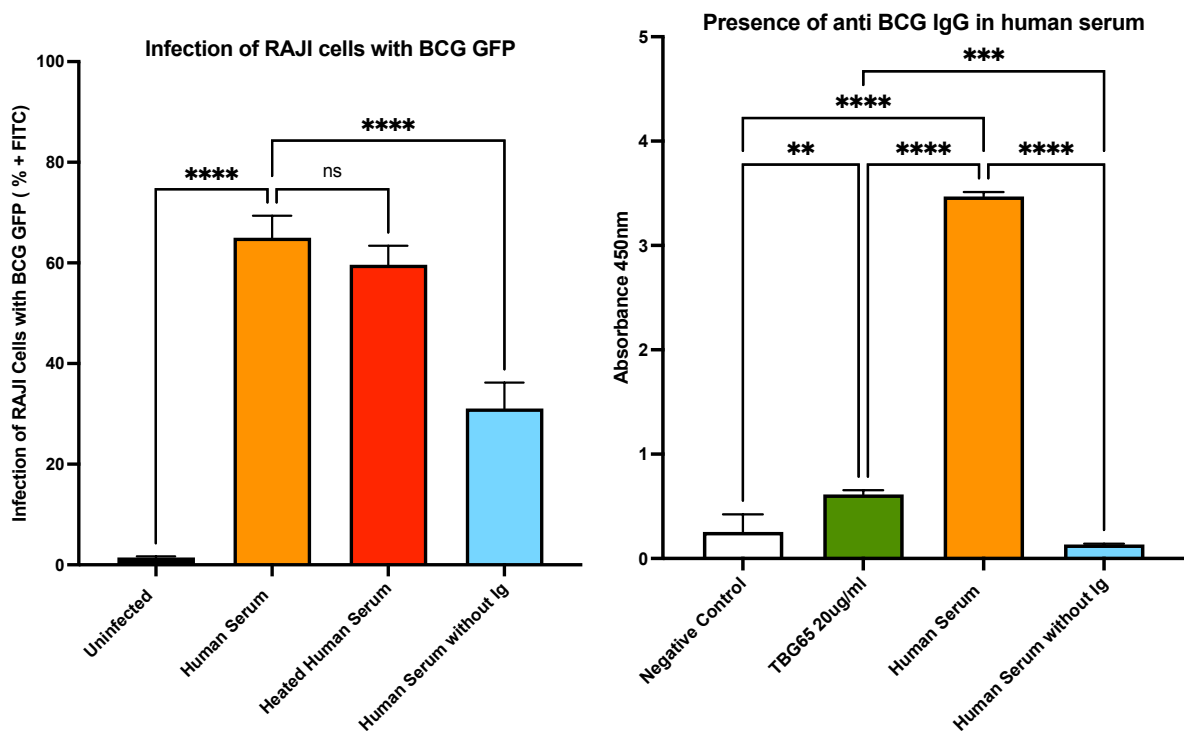


Figure 7.6.1. Effect of human serum and immunoglobulin on the interaction of B cells and BCG.

Duplicate wells containing 1×10^5 RAJI cells were infected with BCG GFP at an MOI of 4:1. Wells contained RPMI media without serum, RPMI media with 10% human serum or RPMI media with human serum which lacked immunoglobulin (A). After 3 hours the cells were analysed by flow cytometry. The percentage of FITC + B cells was measured. Statistical analysis was performed using one way ANOVA and Tukey's multiple comparisons test ($n=3$). The presence of anti BCG IgG in the human serum was tested by ELISA (B). The monoclonal antibody TBG65 was used at a concentration of 20 $\mu\text{g}/\text{ml}$ as a positive control. Triplicate wells containing human serum and human serum without Ig were also prepared. Mean values and $\pm$ standard deviations are shown. * indicates differences of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows differences of ≤ 0.0001 . The results recorded as absorbance ($n=1$). Statistical analysis was conducted using One way ANOVA and Tukeys multiple comparisons.

7.7 Imaging of B cell interactions with BCG GFP

B cells interact directly with BCG GFP. This has now been confirmed by flow cytometry and confocal microscopy. It is important to determine whether this was due to adherence of external bacteria to B cells or internalisation of BCG by B cells. To resolve this question, B cells were infected with BCG and confocal microscopy was used to generate a 3 dimensional z stack analysis. Optical slices were taken at 8 μ M intervals. These images were then combined into 3D models which could be rotated to reveal the spatial relationship between individual B cells and bacilli. These 3D models are included in the supplemental information. RAJI B cells and tonsillar B cells were used for this experiment. THP1 cells are known to phagocytose BCG GFP and were used as a positive control for internalisation.

Figure 7.7.1 shows the infection of THP1 cells with BCG. Uninfected THP1 cells are shown in 7.7.1A. THP1 cells infected with BCG GFP phagocytosed individual bacilli. This can be seen in figure 7.7.1B in overview and in figure 7.7.1 C and D in closeup. The phagosomes surrounding the bacteria were clearly visible due to phalloidin 647 staining of actin filaments. These z stack sections were combined to generate 3 dimensional models of the infected THP1 cells. Rotating these images reveals that the BCG GFP is internalised within the THP1 cells.

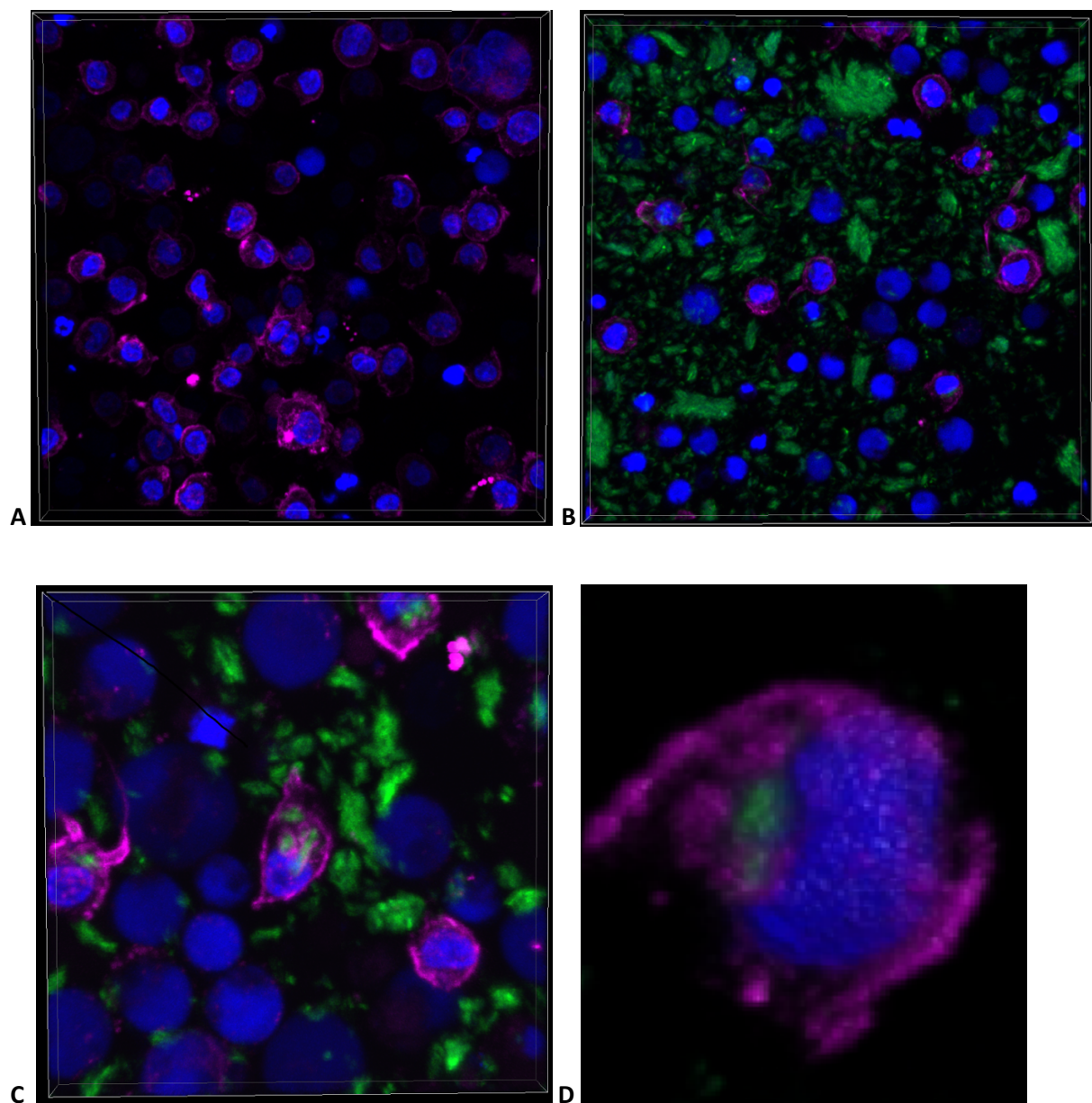


Figure 7.7.1. THP1 cell interaction with BCG GFP. 1×10^5 THP1 cells were infected in triplicate at an MOI of 4:1 with BCG GFP for 24 hours. Cells were stained with DAPI (Blue) and Phalloidin 647 (Magenta). Images were taken using a NIKON A1R confocal microscope at 200x magnification. The figure shows uninfected THP1 cells (A). THP1 cells infected with BCG GFP (Overview) (B) and closeup views of THP1 cells infected with BCG GFP (C) and (D) (n=2).

In Figure 7.7.2, RAJI B cells were infected with GFP BCG. There was a clear interaction between RAJI cells and BCG GFP. The bacteria adhered onto the surface of the RAJI cells. Once the images were combined into 3 dimensional models which could be rotated, it was evident that the RAJI cells did not internalise BCG GFP. It was notable that RAJI cells formed aggregates around the mycobacteria. This can be seen in images B, D and E. This may be related to the phenomenon of B cell aggregates forming in the lungs of TB patients.

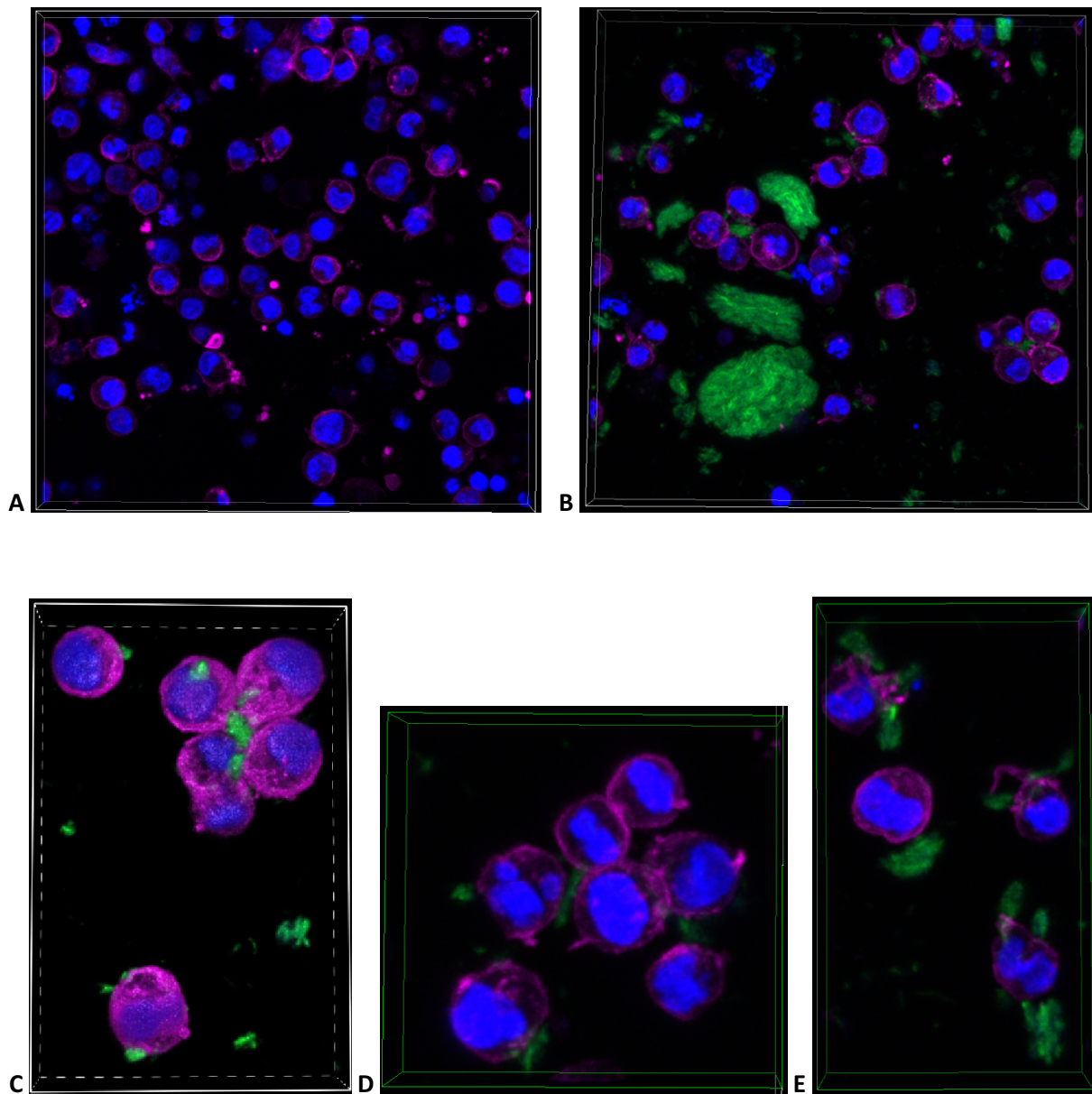


Figure 7.7.2. RAJI B cell interaction with BCG GFP. 1×10^5 RAJI cells were infected at an MOI of 4:1 with BCG GFP for 24 hours in triplicate wells. Cells were stained with DAPI (blue) and Phalloidin 647 (magenta). Images were taken using a NIKON A1R confocal microscope at 200x magnification. The photographs depict uninfected RAJI cells (A) RAJI cells infected with BCG GFP (Overview) (B) and RAJI cells infected with BCG GFP (closeup) (C), (D) and (E) (n=2).

To further investigate this phenomenon, TMCs were used as a source of primary human B cells. These results are shown in figure 7.7.3. The interaction between tonsillar B cells and BCG GFP was investigated using TMCs from 6 different patients. BCG GFP bound to the surface of tonsillar B cells. There were no examples of B cells internalising BCG GFP. In this experiment, B cells can be identified by a red colour.

In Figure 7.7.3, images C and D show B cells which have bound to the polar ends of BCG bacilli. Mycobacteria generate peptidoglycan at their poles. Mycolyltransferase activity is localised to the poles of BCG cells where new extracellular cell envelope is generated (Hodges et al., 2018). Staining with FITC labelled trehalose has shown that Ag85 has greater enzymatic activity at the poles of mycobacteria (Backus et al., 2011). Some of these tonsillar B cells may have antibodies specific for Ag85. It is possible that they are binding to the BCG via anti Ag85 antibodies. This could be tested in further experiments by incubating the TMC with His conjugated Ag85b and then probing with anti His antibodies. These results clearly demonstrate that B cells directly bind to BCG. They also demonstrate that B cells do not phagocytose BCG.

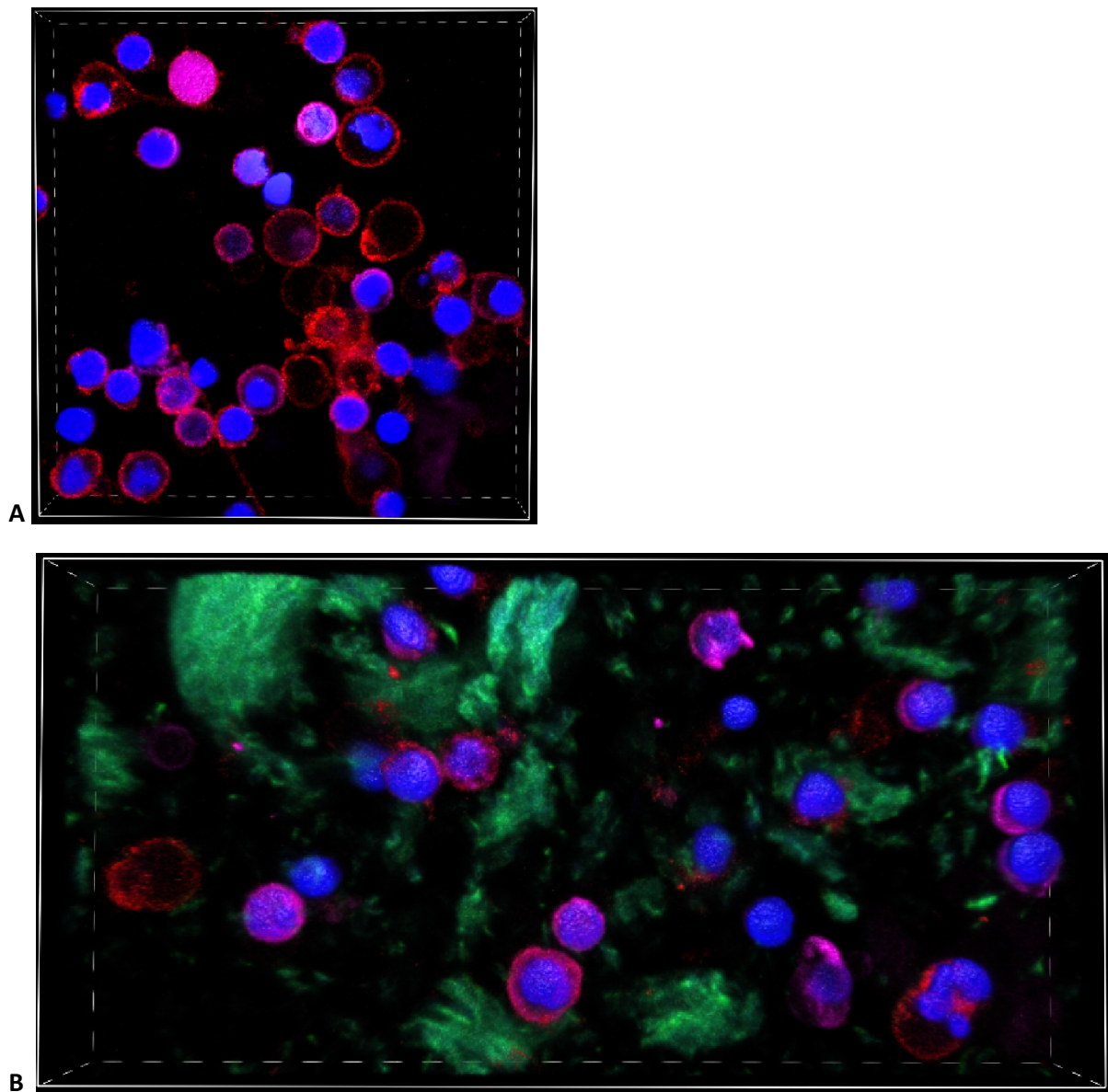


Figure 7.7.3. Tonsillar B cell interaction with BCG GFP. 1×10^5 TMCs were infected at an MOI of 4:1 with BCG GFP for 24 hours in triplicate wells. TMCs from six different patients were used. (n=2). Cells were stained with DAPI (Blue) and Phalloidin 647 (Magenta). B cells were stained with CD19 PE (Red). Images were taken using a NIKON A1R confocal microscope at 200x magnification. The photographs show uninfected tonsillar B cells (A) and tonsillar B cells infected with BCG GFP (Overview) (B). The photographs overleaf show closeup views of tonsillar B cells infected with BCG GFP (C-E).

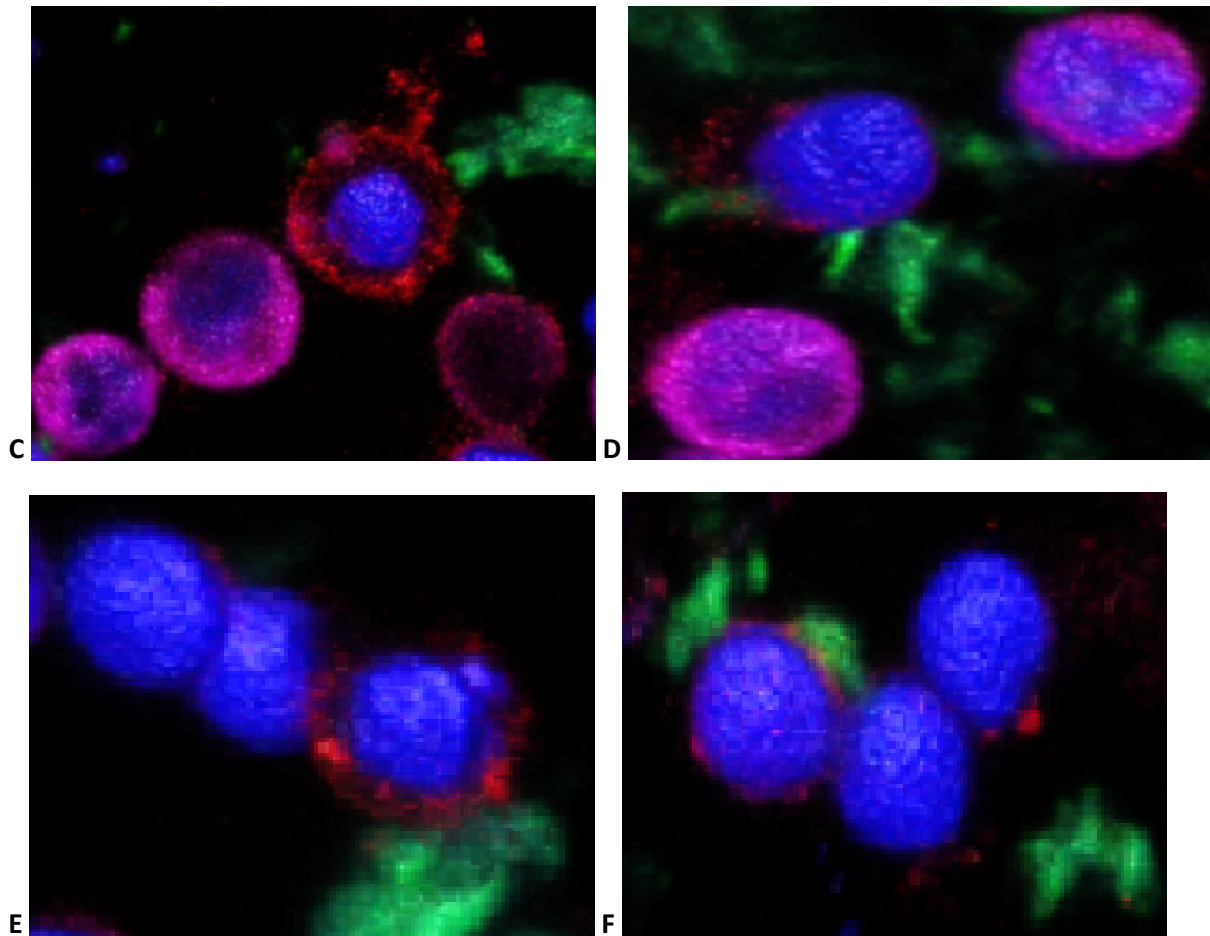


Figure 7.7.3 continued. Tonsillar B cell interaction with BCG GFP. 1×10^5 TMCs were infected at an MOI of 4:1 with BCG GFP for 24 hours in triplicate wells. TMCs from six different patients were used. (n=2). Cells were stained with DAPI (Blue) and Phalloidin 647 (Magenta). B cells were stained with CD19 PE (Red). Images were taken using a NIKON A1R confocal microscope at 200x magnification. These photographs show closeup views of tonsillar B cells infected with BCG GFP (C-E)..

7.8 Functional effects of BCG binding to B cells

Mycobacterium species are known to have profound immunomodulatory effects. *Mycobacterium tuberculosis* is able to use macrophages and dendritic cells as intracellular niches. In macrophages, it can block intracellular trafficking, induce cell necrosis and inhibit autophagy (Lerner et al., 2015). The mycobacterial enzyme nucleoside diphosphate kinase (npK) inhibits the assembly and functioning of NADPH oxidase leading to an interruption of ROx production (Sun et al., 2013). MTB is also capable of disrupting the macrophages ability to use toxic metals by upregulating the efflux of copper (Neyrolles et al., 2015). In dendritic cells, ManLAM from MTB binds to DC-SIGN blocking DC maturations and inducing IL-10 production (Geijtenbeek et al., 2003). Mycobacterial ManLAM is also known to induce IL-10 production in B cells (Yuan et al., 2019).

This study provided an opportunity to investigate the immunomodulatory effect of BCG on human B cells. Flow cytometry was used to measure the effect of BCG infection on the expression of relevant cell surface markers on B cells. Expression of CD40, CD69, CD80, HLA DR, CCR7, Ki67 and surface IgM was measured. IL-10 expression and CD32 expression were also measured. This work was first done in RAJI cells and then repeated using TMCs.

7.9 Effect of BCG infection on HLA DR, CD40 and CD80 on RAJI cells.

It was found that BCG profoundly suppressed the expression of HLA DR, CD40 and CD80 in RAJI cells. This would dysregulate antigen presentation by B cells and the development of B cell and T cell antigen specific responses. Figure 7.9.1 A shows HLA DR expression, as measured by PE Cy7 MFI. The PE Cy7 MFI was 72,280 in uninfected cells. BCG infection reduced this down to 25,716 ($p<0.0001$). HLA DR plays an important role in presenting peptide antigens to TCRs on T cells. Reducing the expression of HLA DR on B cells would assist mycobacteria in evading T cell immune responses.

Figure 7.9.1 B shows that 91% of uninfected RAJI cells were CD40+. Following infection with BCG, this fell to 52% CD40+ ($p<0.0001$). CD40 fulfils a number of roles in B cells. CD40 signalling plays a costimulatory role in the development of T cell immunity. CD40 engagement also leads to cytokine production which drives T cell development. In B cells, CD40 signalling is responsible for the development of germinal centres, isotype switching and the development of naïve B cells into plasmablasts and memory B cells (Elgueta et al., 2009). These functions of B cells will all be disrupted as CD40 expression is lost during BCG infection.

CD80 on B cells acts as a costimulatory molecule and stimulates CD4+ T cells to proliferate. It is upregulated on activated B cells and negatively regulates B cell proliferation and immunoglobulin secretion. The effect of BCG infection on RAJI cell CD80 expression is shown in Figure 7.9.1C. 99% of uninfected cells express CD80. BCG infection reduced the percentage of CD80+ RAJI cells down to 68% ($p<0.0001$).

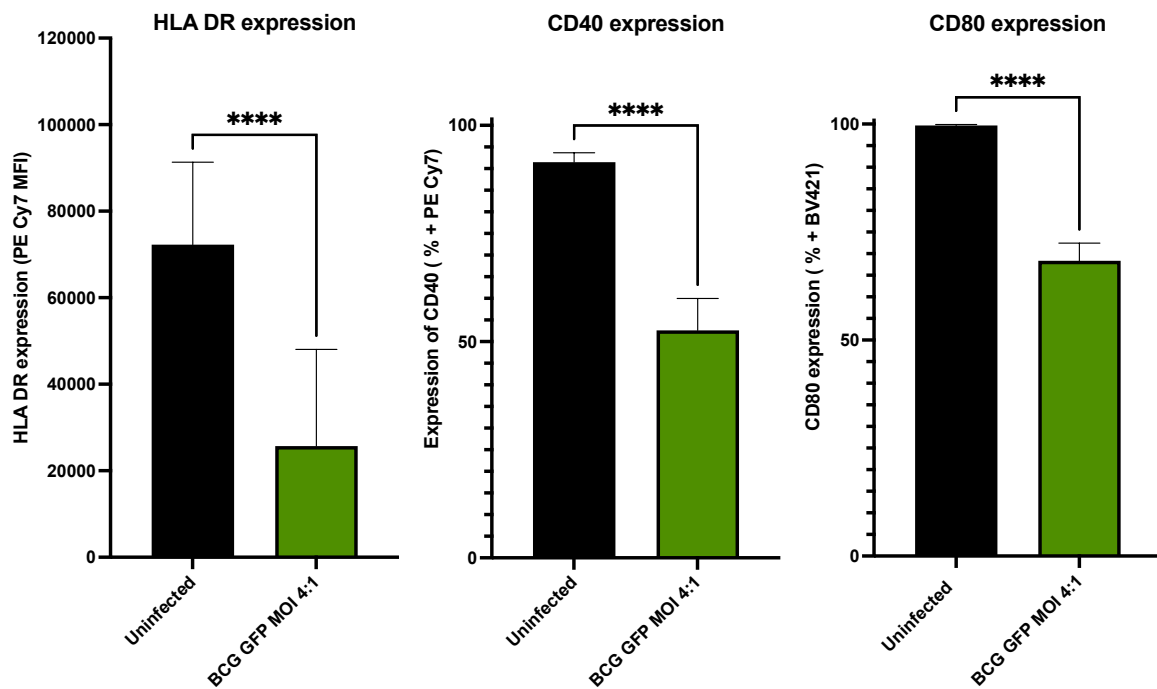


Figure 7.9.1. Effect of BCG infection on HLA DR, CD40 and CD80 on RAJI cells. 1×10^5 RAJI cells were infected with BCG GFP at an MOI of 4:1 in duplicate wells. After 24 hours the cells were analysed by flow cytometry. The expression of; A) HLA DR, B) CD40 and C) CD80, was measured. Mean fluorescence values are shown for HLA DR and percentage + cells are shown for CD40 and CD80 along with $\pm$ standard deviations. **** shows differences of ≤ 0.0001 . Statistical analysis was performed using an unpaired T test ($n=3$). Infection with BCG led to significantly decreased expression of HLA DR, CD40 and CD80 on RAJI cells.

7.10 Effect of BCG infection on CD69 and CCR7 expression by RAJI cells

B cell activation and chemotaxis of RAJI cells were both affected by BCG infection. CD69 and CCR7 were both downregulated during infection. The greatest effect was seen with infection at an MOI of 10:1. In Figure 7.10.1A, expression of CD69 fell from 11,579 in uninfected cells to 1,039 in BCG infected B cells ($p < 0.0001$). In figure 7.10.1 B, CCR7 expression also decreased from 7,726 MFI on uninfected cells to 4,362 in BCG infected RAJI cells ($p < 0.0001$). CD69 is an early activation marker for lymphocytes which is expressed once B cells internalise antigen via the BCR or after TLR4 stimulation. CCR7 expression is also upregulated in B cells following exposure to antigen. The downregulation of CD69 and CCR7 on B cells shows that the normal activation of B cells in the presence of antigen is being suppressed. Both CD69 and CCR7 play roles in the chemotaxis of activated B cells. By suppressing the expression of these receptors, BCG would disrupt the migration of B cells and subsequent B cell development.

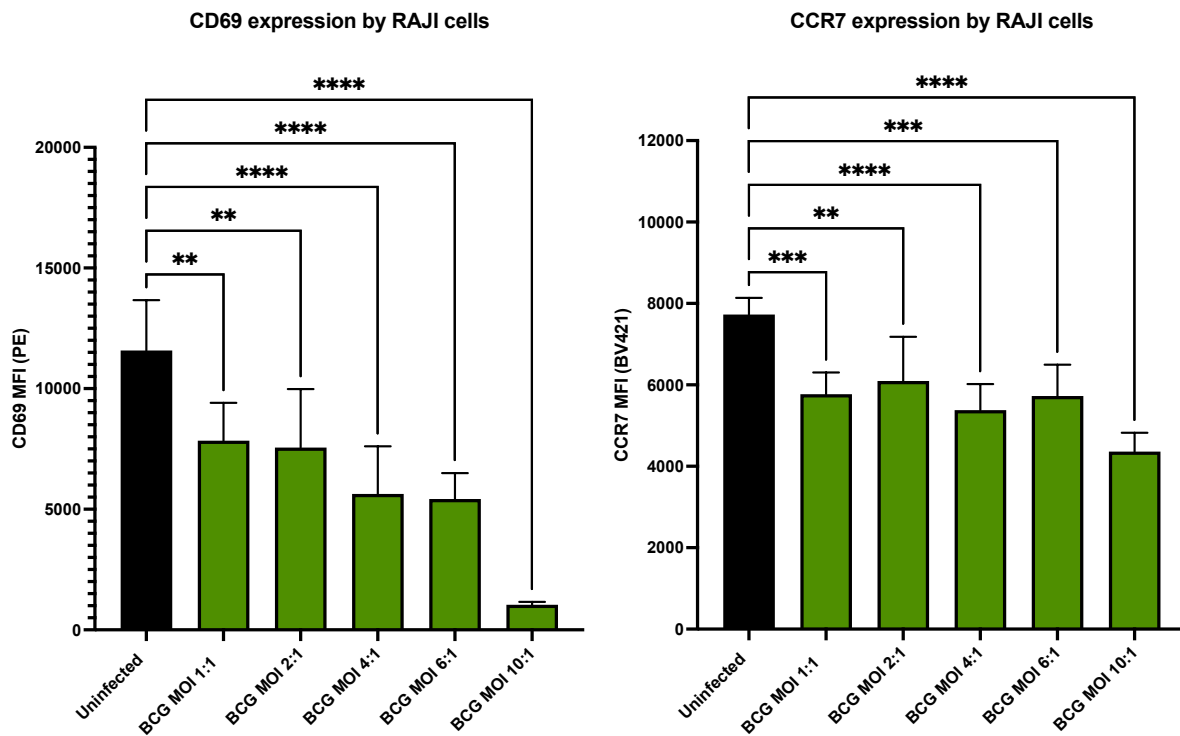


Figure 7.10.1. Effect of BCG infection on CD69 and CCR7 expression by RAJI cells. 1×10^5 RAJI cells were infected with BCG GFP at a MOIs of 10:1, 6:1, 4:1, 2:1 and 1:1 in duplicate wells. After 24 hours the cells were analysed by flow cytometry. Mean fluorescence values for; A) CD69 and B) CCR7, are shown. Statistical analysis was performed using one way ANOVA and Dunnetts multiple comparisons test. (n=3). Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Infection with BCG in RAJI cells reduced expression of the activation marker CD69 and the chemotaxis marker CCR7.

7.11 Effect of BCG infection on proliferation and IgM expression of RAJI cells

Infection of RAJI cells with BCG impaired their proliferation and their expression of IgM. This can be seen in Figure 7.11.1. BCG infection at a MOI of 10:1 had the strongest suppressive effect.

Dysregulation of B cell proliferation has previously been observed in TB patients during active disease (Joosten et al., 2016). Figure 7.11.1 A shows that BCG is capable of preventing RAJI B cells from proliferating. The proliferation marker Ki67 had an MFI of 7,357 in uninfected cells. This diminished to 1,357 in BCG infected cells ($p < 0.0001$).

Immunoglobulin production has also been shown to be suppressed in TB patients. Total immunoglobulin levels were reduced in TB patients, with decreased levels of IgA and IgM (Joosten et al., 2016). Figure 7.11.1 B shows a similar effect, with cell surface IgM on RAJI cells greatly reduced by BCG. IgM expression on the surface of B cells was reduced 16 fold following BCG infection, from 48,632 down to 2,972 ($p < 0.0001$).

These results correspond with previous understanding of the immunomodulatory effects of TB and shows that BCG prevents the development of appropriate B cell responses during infection.

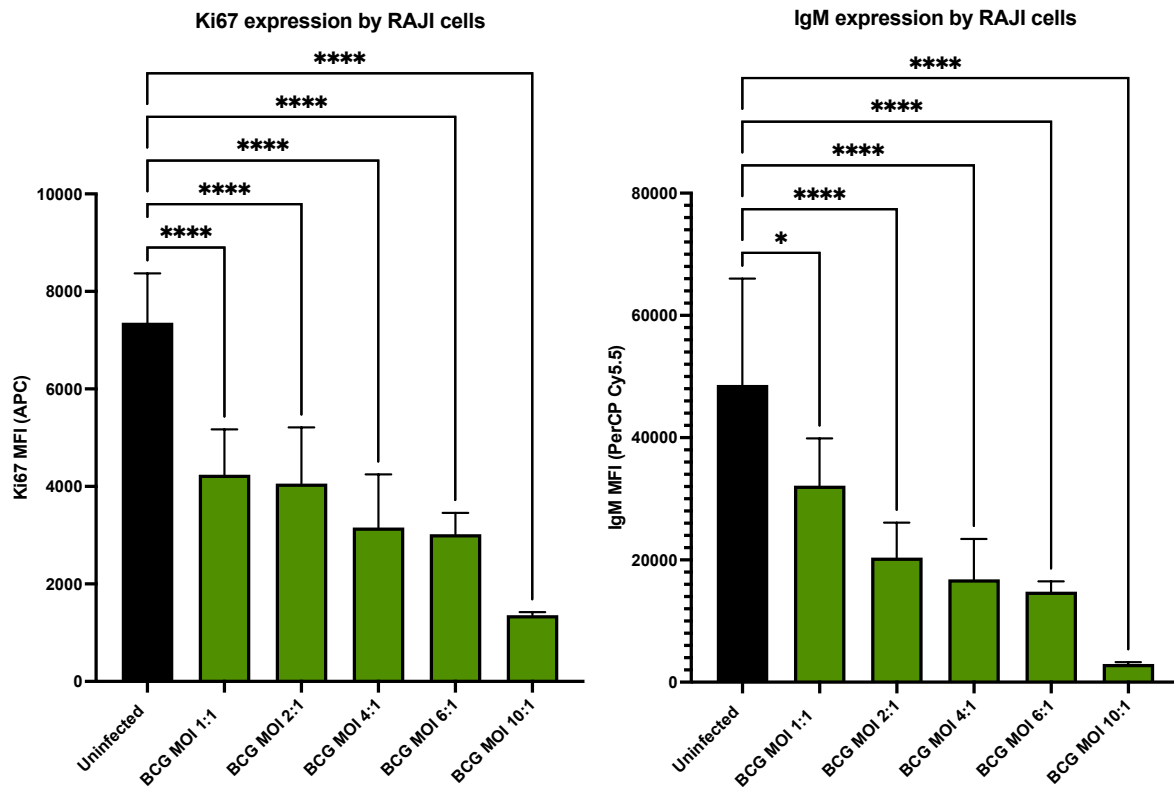


Figure 7.11.1. Effect of BCG infection on proliferation and IgM expression of RAJI cells. Duplicate wells were prepared containing 1×10^5 RAJI cells. These were infected with BCG GFP at an MOI of 10:1, 6:1, 4:1, 2:1 and 1:1. After 24 hours the cells were stained and analysed by flow cytometry. Mean fluorescence values and $\pm$ standard deviations for; A) Ki67 and B) IgM, are shown. Statistical analysis was performed using one way ANOVA and Dunnetts multiple comparisons test. (n=3). * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Infection with BCG led to significantly decreased expression of Ki67 and IgM in RAJI cells.

7.12 Effect of BCG infection on IL-10 and CD32 Fc receptor expression by RAJI cells.

The mechanism by which BCG suppresses B cells is unclear. It was hypothesised that suppression might be caused by induction of the inhibitory cytokine IL-10. To test this, intracellular levels of IL-10 were measured in RAJI B cells infected with BCG. The effect of BCG on B cell expression of the inhibitory receptor CD32 was also measured. The expression of the Fc receptor CD32 and the cytokine IL-10 were both found to be suppressed in infected RAJI cells. This can be seen in figure 7.12.1. 97% of uninfected RAJI cells expressed CD32, whereas, only 63% of cells were CD32+ after BCG infection ($p<0.0001$). Expression of IL-10 was high in uninfected RAJI cells, with an MFI of 115,062. This fell sharply to 2,232 in the infected cells ($p<0.0001$). This was a 50 fold reduction in IL-10 expression. This fits into a wider pattern of B cell dysregulation and functional suppression but does not provide an explanation for the mechanism of B cell suppression.

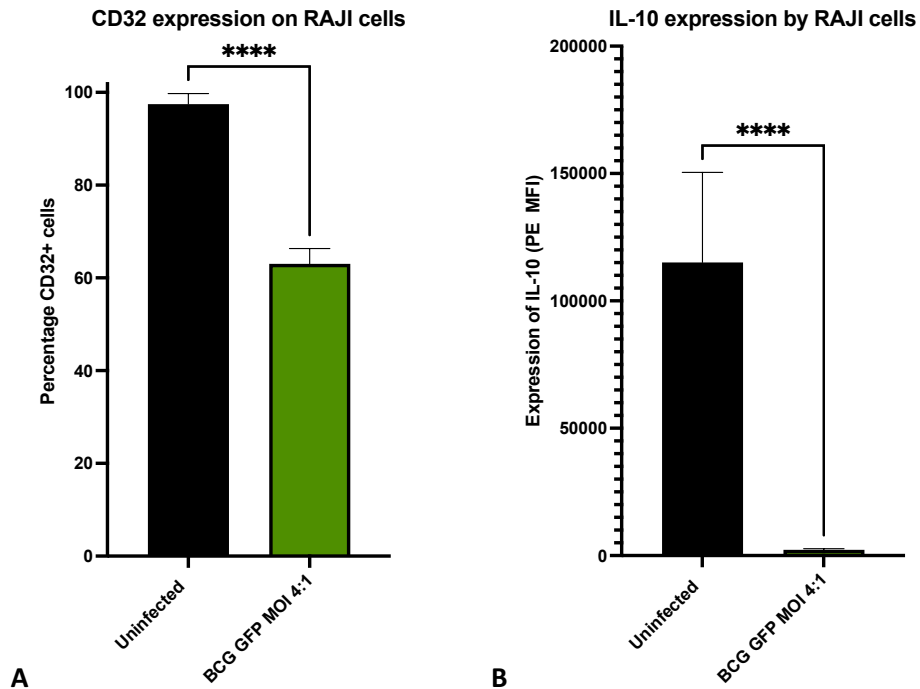


Figure 7.12.1. Effect of BCG infection on IL-10 and CD32 Fc receptor expression by RAJI cells. 1×10^5

RAJI cells were seeded in duplicate wells. These were infected with BCG GFP at an MOI of 4:1 for 24 hours ($n=3$). The cells were then stained and analysed by flow cytometry. The mean percentages of CD32+ RAJI cells is shown in (A). The mean fluorescence value for IL-10 is shown in (B). $\pm$ standard deviations are shown. **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using an unpaired T test. ($n=2$). Infection with BCG reduced expression of CD32 in RAJI cells as well as the expression of IL-10.

7.13 Effect of BCG infection on tonsillar B cells.

BCG suppresses the function of B cells from the RAJI cell line. It was important to determine whether this effect is also seen in primary human cells. To investigate this, TMCs were infected with BCG and the expression of cell surface markers on B cells. The function of B cells as antigen presenting cells was studied by measuring expression of HLA DR, CD40 and CD80. The activation and chemotaxis of B cells was examined by measuring CD69 and CCR7 expression. B cell proliferation, antibody production, IL-10 production and CD32 expression were also measured. BCG infection increased the proliferation of tonsillar B cells and their expression of IgM. The increased proliferation and immunoglobulin production of these tonsillar B cells may be due to recall responses due to prior BCG vaccination. In spite of this, BCG suppresses the expression of CD69, CCR7, the Fc receptor CD32, IL-10 and HLA DR on tonsillar B cells.

7.14 Effect of BCG infection on HLA DR, CD40 and CD80 on tonsillar B cells.

Figure 7.14.1 shows that BCG suppresses the expression of HLA DR and the costimulatory molecules CD40 and CD80 in TMCs. HLA DR expression is shown in graph A. The uninfected cells had a PE Cy7 MFI of 22,884. This fell 60 fold to 379 in BCG infected cells ($p < 0.0001$). Graph B shows that 87.85% of uninfected tonsillar B cells were CD40+. BCG infection reduced this to 71.55% CD40+ ($p < 0.0001$). Graph C shows the percentage of tonsillar B cells expressing CD80 was increasing following infection with BCG. 2.27% of uninfected cells expressed CD80. After infection with BCG, this increased to 18.98% of tonsillar B cells expressing CD80 ($p < 0.0001$). These results confirm the findings shown in figure 7.9.1 where HLA DR and CD40 were suppressed in RAJI B cells. BCG also suppresses HLA DR and CD40 on primary tonsillar B cells. This would have a profound effect on the ability of B cells to engage in antigen presentation. In RAJI cells, CD80 expression was downregulated by BCG whereas in tonsillar B cells, BCG infection led to a greater proportion of cells expressing CD80.

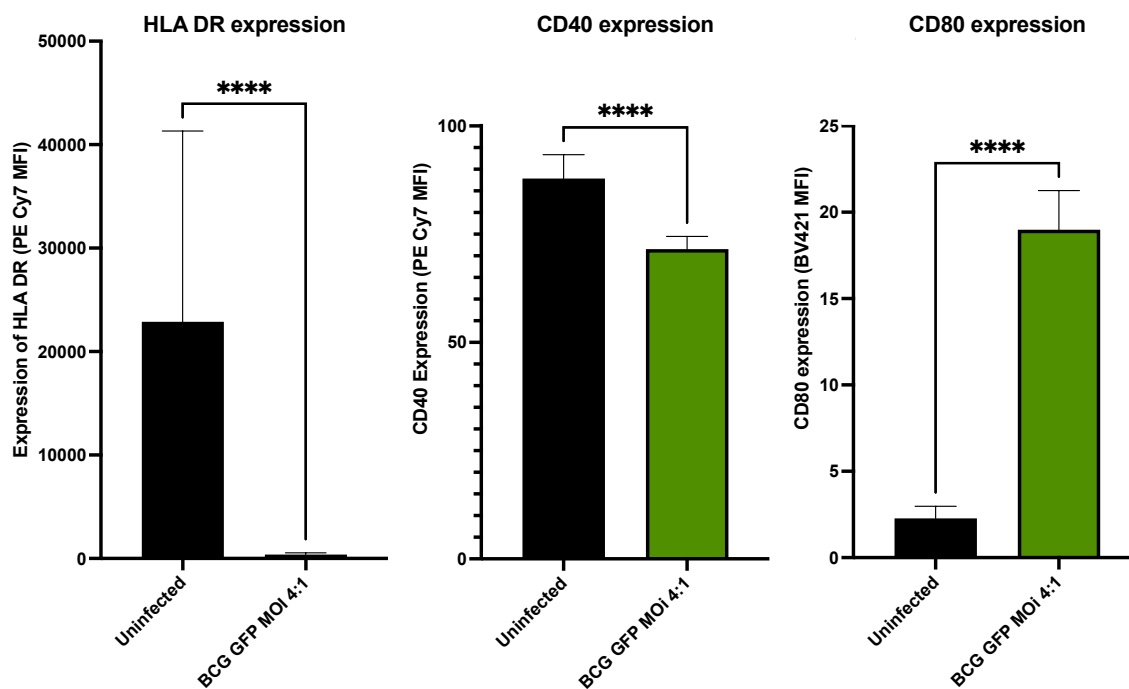


Figure 7.14.1. Effect of BCG infection on HLA DR, CD40 and CD80 on tonsillar B cells. 1×10^5 TMCs were infected for 24 hours with BCG GFP at an MOI of 4:1 in duplicate. The expression of; A) HLA DR, B) CD40 and C) CD80 by B cells is shown. Mean fluorescence values and standard deviations are shown for HLA DR (n=3). **** shows a p value of ≤ 0.0001 . The percentage of CD40+ and CD80+ B cells is also shown (n=3). Statistical analysis was performed using an unpaired T test. Infection with BCG suppressed expression of HLA DR and CD40 in tonsillar B cells. The percentage of B cells expressing CD80 was increased.

7.15 Effect of BCG infection on CD69 and CCR7 expression by tonsillar B cells

In Figure 7.10.1, expression of CD69 and CCR7 by RAJI cells was shown to be suppressed by BCG. This same effect can be seen in tonsillar B cells, which are primary cells rather than a cell line. CD69 and CCR7 were both downregulated during infection. On figure 7.15.1, expression of CD69 fell from 2,029 in uninfected cells to 746 in BCG infected B cells ($p=0.0002$). CCR7 expression was high in tonsillar B cells. It decreased from 877,250 MFI to 603,000 in BCG infected cells ($p=0.0470$). This demonstrates that BCG is capable of suppressing expression of CD69 and CCR7 on B cells. This would impact B cell development and their migration to lymph nodes.

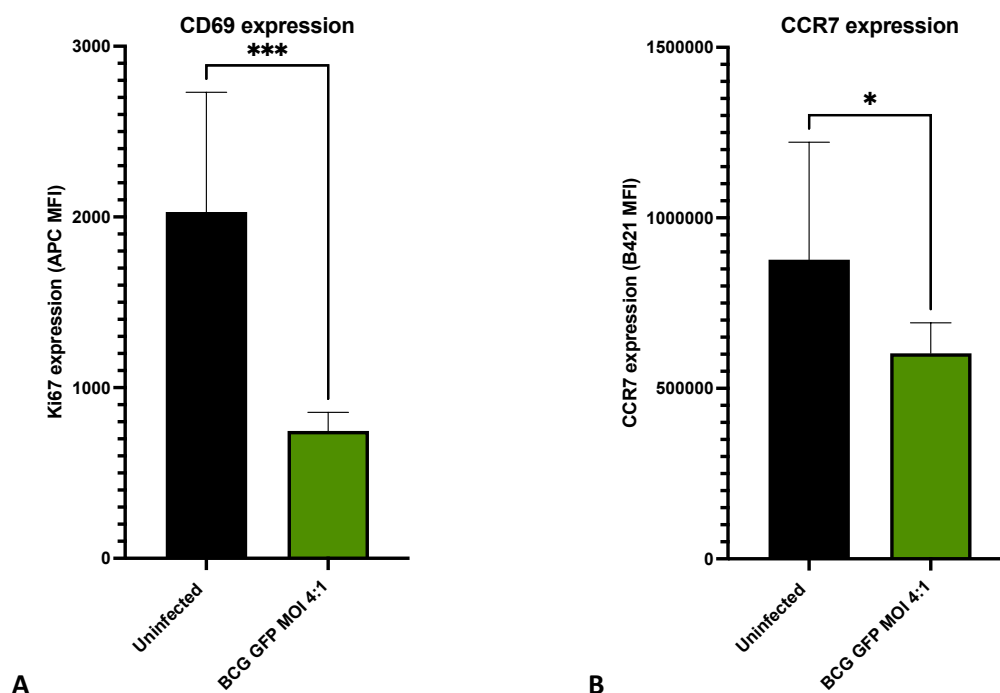


Figure 7.15.1. Effect of BCG infection on CD69 and CCR7 expression by tonsillar B cells. TMCs were infected with BCG GFP at an MOI of 10:1 in triplicate for 24 hours. B cells were identified by flow cytometry. The MFI values for A) CD69 and B) CCR7, are shown. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using an unpaired T test. (n=3).

7.16 Effect of BCG infection on proliferation and IgM expression of tonsillar B cells

Figure 7.16.1 depicts the effect of BCG infection on the proliferation of B cells and their expression of IgM. Infection of tonsillar B cells with BCG increased the proliferation of these cells and increased their surface expression of IgM. Ki67 expression increased from 887 MFI in uninfected cells up to 1,230 in BCG infected cells ($p=0.0045$). BCG infection increased IgM expression on the surface of B cells from 1418 MFI up to 1,812 ($p=0.0022$). RAJI cells and primary tonsillar B cells react differently to BCG infection. Figure 7.11.1 shows that in RAJI cells, Ki67 and IgM expression are reduced by BCG. In contrast, tonsillar B cells have increased proliferation and IgM expression following infection with BCG. This is likely due to a recall response generated by prior BCG vaccination.

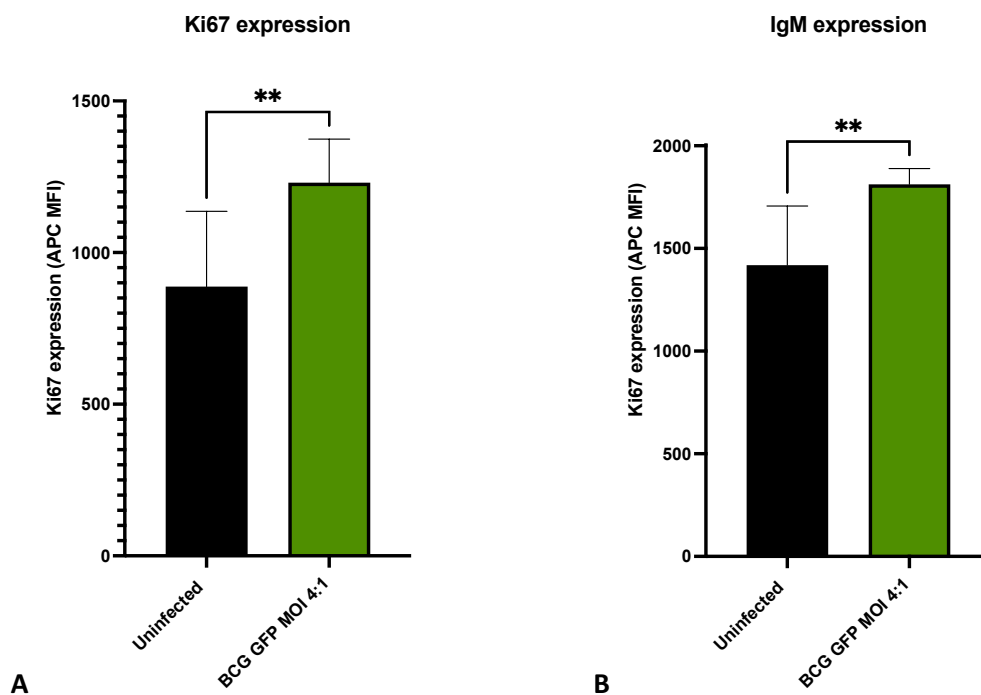


Figure 7.16.1. Effect of BCG infection on proliferation and IgM expression of tonsillar B cells. 1×10^5

TMCs were infected with BCG GFP at an MOI of 4:1 in triplicate. After 24 hours the B cells were analysed by flow cytometry. Mean fluorescence values and standard deviations for; A) Ki67 and B) IgM, are shown. Statistical analysis was performed using an unpaired T test. ($n=3$). ** indicates p values of ≤ 0.01 . Infection with BCG elicited increased expression of Ki67 and IgM in tonsillar B cells.

7.17 Effect of BCG infection on IL-10 and CD32 Fc receptor expression by tonsillar B cells.

CD32 and IL-10 were both suppressed in tonsillar B cells infected with BCG. In Figure 7.17.1, 23.8% of uninfected B cells expressed CD32, whereas, only 5% of cells were CD32+ after BCG infection ($p<0.0001$). This was a more than 4 fold reduction in CD32 expression, which limits the ability of the cells to bind IgG immune complexes and receive stimulatory signals. Expression of IL-10 was relatively low in uninfected TMCs, with an MFI of 721. BCG infection further reduced this to 380 ($p<0.0001$). This was accompanied by a drop in the percentage of B cells which were IL-10+. 35% of the uninfected cells were producing IL-10 whereas only 10% of the BCG infected B cells expressed IL-10. This data reveals that BCG suppresses IL-10 and CD32 in primary B cells from human tonsils.

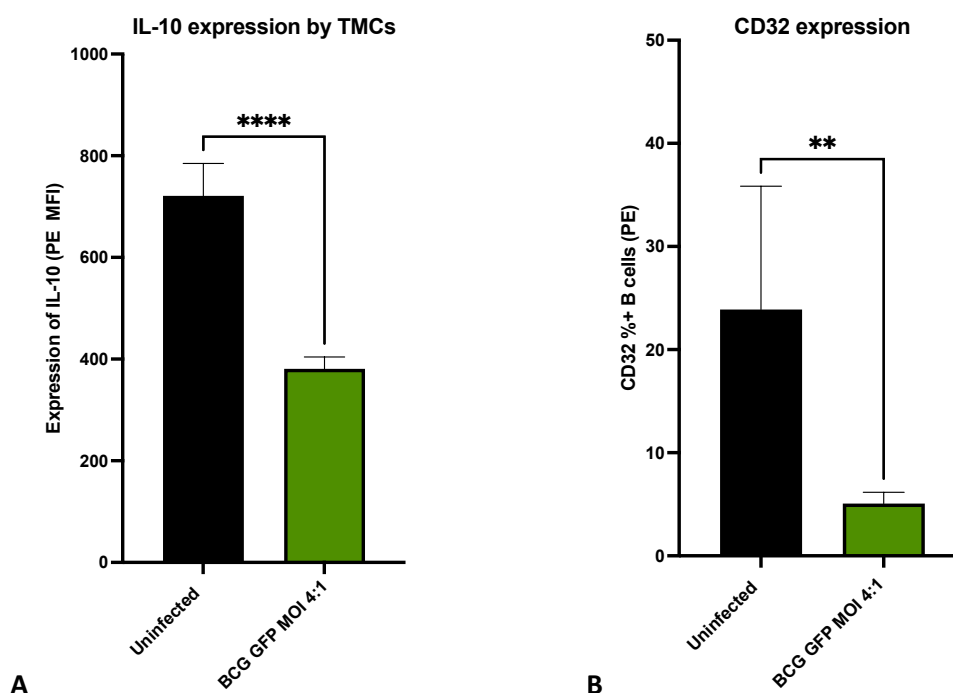


Figure 7.17.1. Effect of BCG infection on IL-10 and CD32 Fc receptor expression by tonsillar B cells.

1×10^5 TMCs were infected for 24 hours with BCG GFP at an MOI of 4:1 in duplicate. The mean percentages of CD32+ B cells are shown in A. The mean fluorescence value for IL-10 is shown in B. standard deviations are shown. Statistical analysis was performed using an unpaired T test. ($n=3$).

7.18 Effect of BCG infection on U937 cells

BCG is able to suppress the function of B cells. The mechanism by which this suppression occurs is unclear. One possibility is that BCG uses immunomodulatory molecules to suppress B cell function. Alternatively, the suppression of B cells could be due to anergy. B cells require two signals for development. The first signal can be generated by soluble antigen. Antigen specific T cells are required for the secondary signal. Without this secondary signal, B cells do not further develop, but instead are silenced and become anergic and unresponsive.

It is important to determine whether the BCG induced suppression of B cells observed in this study is caused by immune modulation or whether it is due to an anergic response to soluble antigen in the absence of secondary signals.

U937 cells are a monocyte cell line which, unlike B cells, do not undergo anergy. These cells were infected with BCG for 24 hours. Expression of HLA DR, CD40, CD80, CD69, CCR7, Ki67, IL-10 and CD32 by U937 cells following BCG infection were measured by flow cytometry. These results are shown in figures 7.18.1, 7.18.2, 7.18.3 and 7.18.4. It was found that BCG infection suppressed U937 cells and reduced the expression of HLA DR, CD69, CCR7, Ki67, IL-10 and CD32. CD40 expression was not altered by BCG infection, whilst CD80 expression increased on the infected U937 cells. The pattern of suppression seen in U937 is similar to that seen in B cells. Because U937 cells are monocytic cells which do not undergo anergy, this finding reveals that the BCG induced suppression of RAJI and tonsillar B cells is not due to an anergic response.

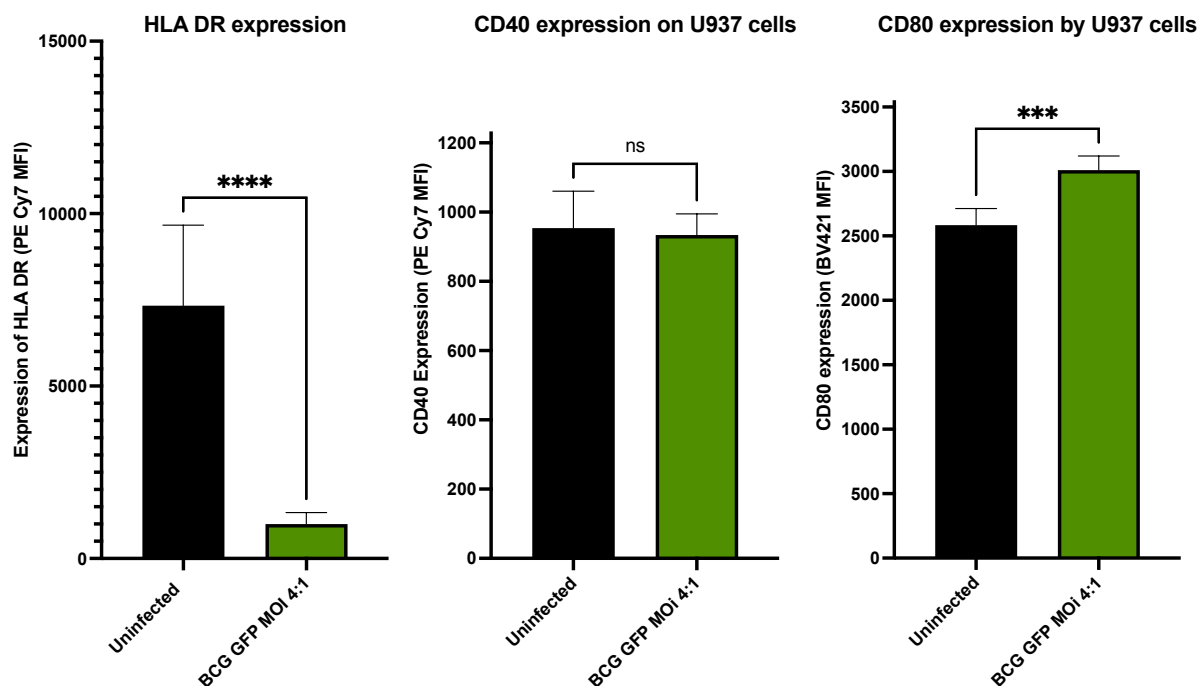


Figure 7.18.1. Effect of BCG infection on the HLA DR and costimulatory molecules on U937 cells.

1x10⁵ U937 cells were seeded in duplicate in 96 well plates. These were infected for 24 hours with BCG GFP at an MOI of 4:1. The cells were stained with anti HLA DR -PE Cy7, anti CD40- and anti CD80 BV421. The expression of; A) HLA DR B) CD40 and C) CD80 was measured by flow cytometry. Statistical analysis was performed using an unpaired T tests. (n=3). Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Infection with BCG reduced the expression of HLA DR in U937 cells. CD40 expression was unchanged and CD80 expression increased.

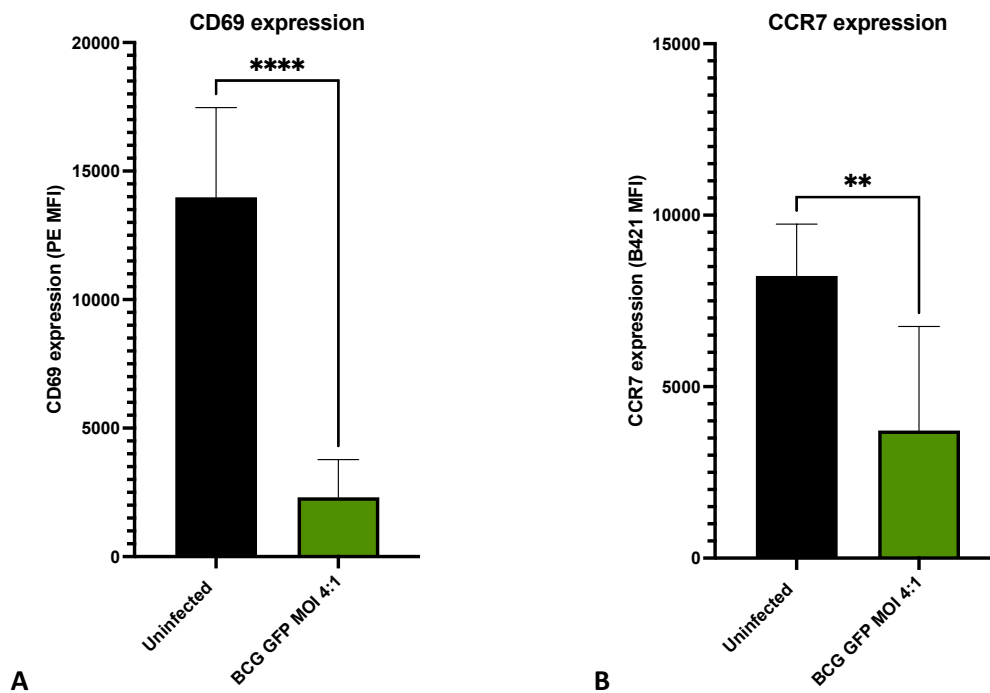


Figure 7.18.2. Effect of BCG infection on chemotaxis markers on U937 cells. 1×10^5 U937 cells were infected for 24 hours with BCG GFP at an MOI of 4:1 in duplicate wells. After 24 hours the cells were stained with anti CD69-PE and anti CCR7-BV421. The expression of; A) CD69 and B) CCR7 was determined by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using an unpaired T tests. (n=3). Infection with BCG reduced the expression of CD69 and CCR7.

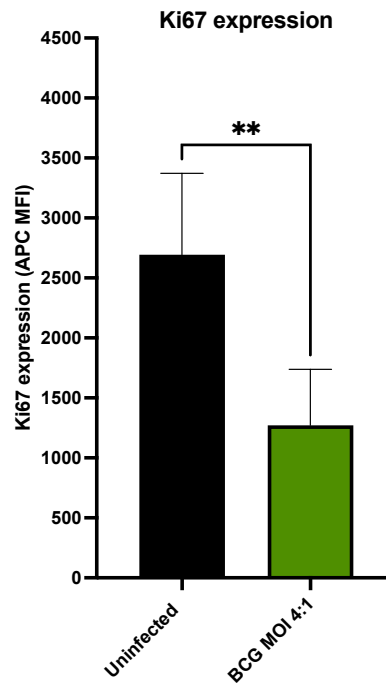


Figure 7.18.3. Effect of BCG infection on the proliferation of U937 cells. 1×10^5 U937 cells were infected for 24 hours with BCG GFP at an MOI of 4:1 in duplicate wells ($n=3$). After 24 hours of infection, the cells were stained with anti Ki67 – APC using the intracellular staining protocol. The expression of Ki67 by U937 cells was measured by flow cytometry. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using an unpaired T test. Infection with BCG reduced the expression of Ki67.

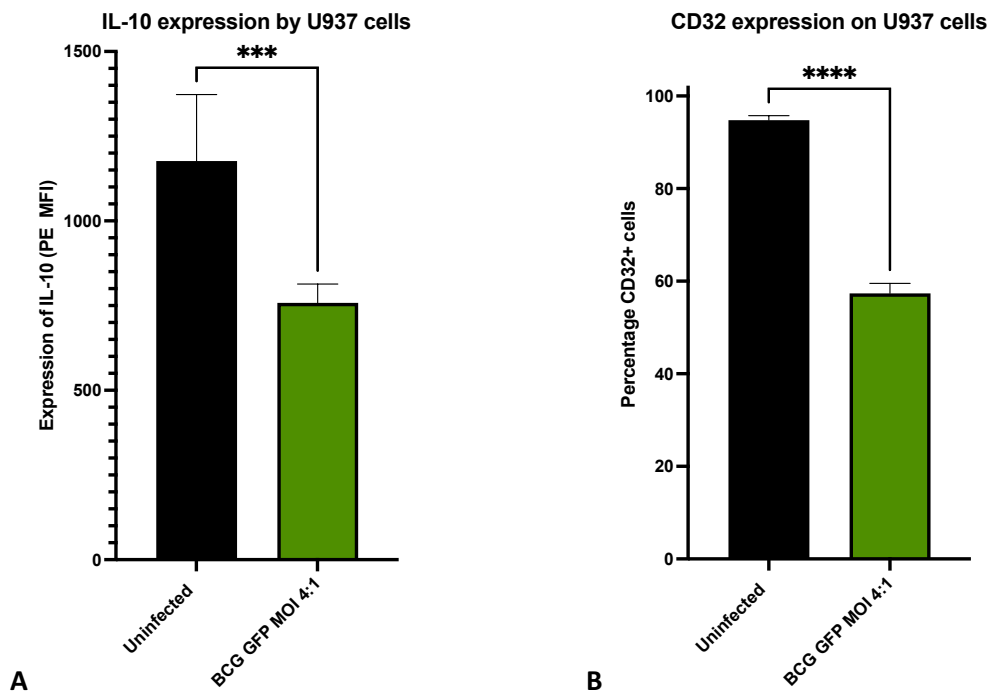


Figure 7.18.4. Effect of BCG infection on IL-10 and CD32 Fc receptor expression by U937 cells. 1×10^5

U937 cells were infected for 24 hours with BCG GFP at an MOI of 4:1 in duplicate wells. Flow cytometry was used to measure the expression of A) IL-10 and B) CD32. Mean values and $\pm$ standard deviations are shown. * indicates p values of ≤ 0.05 , ** of ≤ 0.01 , *** of ≤ 0.001 and **** shows a p value of ≤ 0.0001 . Statistical analysis was performed using an unpaired T tests. (n=3). Infection with BCG reduced the expression of both IL-10 and CD32 in U937 cells.

Key Findings

BCG binds to the external surface of tonsillar B cells

Antibodies contribute to the binding of BCG to B cells

BCG suppresses the function of B cells

BCG suppresses the expression of cytokines, activation markers and chemotaxis markers by B cells.

BCG suppresses antibody production and proliferation of RAJI B cells

Tonsillar B cells upregulate antibody production and proliferate in the presence of BCG.

8. Discussion

8.1 Murine dendritic cell models

This study used *in vitro* models based on murine DCs to test the immunogenic effects of novel vaccine candidates. Expression of activation markers and pro inflammatory cytokines by DCs was investigated. This provided information on the effects of vaccine delivery systems and the appropriate TLR agonists which could be used in combination with vaccine delivery systems. Murine DCs were used to investigate the phagocytosis of dead cells by DCs. This study also used BMDCs to model the ability of vaccine stimulated DCs to induce the proliferation of naïve T lymphocytes. This work provided mechanistic insights for these vaccine delivery systems.

8.2 Effect of Spore delivery system on murine DCs.

Bacillus subtilis spores exert a pro inflammatory effect on dendritic cells. Cells treated with spores alone show enhanced expression of IL-6, IL-12 and IL-1 β . These cells did not show enhanced expression of cell surface markers. This corresponds with previous work which shows *B. subtilis* spores activate APCs (Copland et al. 346). Stimulation with *B. subtilis* spores encouraged efferocytosis of dead cells by DCs. *B. subtilis* could be combined with TLR 3, TLR 4 and TLR 9 agonists to further enhance expression of MHC II.

This work indicates that *B. subtilis* spores may be a suitable vaccine delivery system for TB vaccines, because they activate proinflammatory cytokine expression in DCs. The published literature shows that vaccination with *B. subtilis* spores combined with the antigen FP1 is immunogenic, increasing antigen specific IFN γ and IL-17 responses and inducing TB antigen specific T cell proliferation. It also generates TB antigen specific IgG and IgA. Importantly, it confers protection against MTB in a murine

model (Copland et al. 346). Combining Poly I:C with Spore FP1 conferred a similar level of protection against TB.

Combining *B. subtilis* spores and Poly I:C further activates DCs and leads to increased expression of MHC II and the costimulatory molecules CD80 and CD86. Poly I:C has previously been used to enhance the immunogenicity of a DC targeted vaccine and found to increase protective polyfunctional T cell responses (Trumpfheller et al. 2574-2579). It was expected that using Poly I:C in combination with the spore delivery system would enhance antigen presentation by DCs of a model recombinant antigen, FP1, to naïve T cells.

The effect of this stimulation on antigen presentation by DCs to T cells was unknown. This was tested using an *in vitro* model based on co-culture of naïve splenocytes with DCs pulsed with Spore, FP1, Poly I:C. Proliferation of T cells following co-culture with pulsed DCs was used as an indirect measure of antigen presentation. The *in vitro* data presented in figure 3.10.1 unexpectedly showed Spore FP1 Poly I:C pulsed DCs exerting a tolerogenic effect, inhibiting T cell proliferation.

Following on from this, a murine *in vivo* experiment was performed to investigate the effect of Spore FP1 Poly I:C on the development of antigen specific T cell responses. Again, it was found that stimulation with Spore, FP1, Poly I:C did not induce detectable proliferative effect in T cells, as seen in Figure 3.11.1A. Spore FP1 Poly I:C induced a low level antigen specific IFN γ release from lymphocytes. This is presented in Figure 3.11.1C. Immunological correlates of protection for TB are lacking, however, IFN γ is known to be necessary for protection and antigen specific IFN γ CD4 $^{+}$ T cell responses have previously been used as an immunogenicity marker for novel TB vaccines (Rodo et al. e1007643). Antigen specific IL-2 and IFN γ CD4 $^{+}$ T cell responses also been shown to distinguish latent TB from active disease (Sester et al. e17813).

The results shown in figure 3.11.1 demonstrated that vaccines can be co-administered with vaccine pulsed DCs to generate enhanced immunogenic effects. Combining Spore, FP1, Poly I:C with syngeneic pulsed DCs induced 10 fold greater expression of IFN γ by splenocytes. It also induced the proliferation

of CD4+ T cells and CD8+ T cells. Expression of IL-17 was also increased. This is an important finding as it shows that it is possible to further enhance the immunogenicity of spore FP1 Poly I:C and that it has not reached a biological ceiling.

This study has demonstrated that Spore FP1 Poly I:C is immunogenic and generates antigen specific CD4+ T cell responses. However, it has also demonstrated that these responses can still be enhanced. The immunogenicity of Spore FP1 Poly I:C may be partially limited by the expression of the inhibitory ligand PD-L1 by DCs. Expression of PD-L1 acts as a limiting factor in the development of T cell responses. PD-L1 co-stimulation contributes to down-modulation of T cells and limits the generation of antigen specific T cell responses (Arasanz et al. 51936; Escors et al. ; Karwacz et al. 581-592).

Combining *B. subtilis* spores and Poly I:C together increased expression of PD-L1 on DCs. Figure 3.12.1 shows that both spores, and Poly I:C individually induced significant increases in PD-L1, but in combination, a further increase in PD-L1 expression was seen. Spores generate TLR 4 signalling, activating the MAPK pathway, leading to enhanced PD-L1 expression (Beswick et al. 2218-2229). Exopolysaccharide (EPS) derived from *B. subtilis* inhibits T cell activation through TGF- β and PD-L1 dependent mechanisms (Gao, Jing et al. 701-706)(Paynich, Jones-Burrage, and Knight 2689-2698). TLR3 agonists like Poly I:C are also able to induce expression of PD-L1 (De Waele et al. e1407899; Takeda et al. 1874-1887). This may explain the sub optimal antigen presentation seen in vitro in figure 23.

Coincidentally, antigen specific T cell responses are also limited during TB infection by a component of *Mycobacterium tuberculosis*, EsxH. This sub optimal antigen presentation can be counteracted with addition of syngeneic pulsed DCs (Grace and Ernst 357-364; Portal-Celhay et al. 16232). This same effect was seen in figure 3.11.1, when Spore FP1 Poly I:C was administered in combination with syngeneic pulsed DCs to mice.

Further work is needed to establish whether Poly I:C is the optimal adjuvant for the spore delivery system. It enhances the activation of dendritic cells, but it may lead to overexpression of PD-L1 by DCs

and subsequently, tolerogenic effects of T cell populations. Spores can also be combined with CPG DNA, MPL or LPS. These stimulations enhance the activation of DCs without increasing PD-L1 expression.

One confounding factor in these studies is the fact that mice and humans react differently to TLR3 signalling. In murine macrophages and DCs Poly I:C generates Nfkb signalling which leads to the production of TNF α and IL-6. This effect is not seen in human macrophages and DCs (Lundberg A.M. et al. 2007)(Shen-Ying Zhang et al. 2013).

Regardless, Spore FP1 either with or without adjuvants is still able to generate protective responses against TB (Copland et al. 346).

8.3 Effect of YC NaMa delivery system on murine DCs.

Stimulation with YC NaMa alone has no discernible effect on the expression of MHC I, MHCII, CD40, CD80, CD86, PDL1 or CCR7 by murine BMDCs. YC NaMa did not induce the production of TNF α , IL-1 β , IL-6 or IL-12. YC NaMa did not affect the ability of DCs to engage in efferocytosis, nor did it induce apoptosis in stimulated cells. This would suggest that YC NaMa only acts as an inert antigen delivery system, but this is not the case.

Co-stimulation of murine BMDCs with YC NaMa and TLR agonists led to enhanced production of cytokines and the expression of activation markers. Stimulation with YC NaMa combined with TLR 4 agonists upregulated CD80, CD86, MHC I, PD-L1, MHC II, IL-6, IL-12 and TNF α . YC NaMa and TLR 9 agonists increased BMDC expression of MHC I, CD80, CD86, IL-1 β , IL-6, IL-12 and TNF α . This activation of DCs was not accompanied by increased PD-L1 expression. TLR 4 and TLR 9 agonists are suitable adjuvants for vaccine candidates which use YC NaMa as a delivery system.

The mechanism by which YC NaMa enhances TLR signalling is not yet fully understood. YC NaMa is known to induce IRF 3 signalling, but how this occurs has not been determined (Hart et al. 822-833).

The tyrosine kinase c-src is recruited to TLR3 and induces activation of IRF-3 (Johnsen et al. 3335-3346). One of the core components of YC NaMa is myristate. Myristic acid binds to the myristic acid binding pocket of c-src and enhances its activity (Panjarian et al. 6116-6129). It is possible that IRF3 signalling is increased by YC NaMa through a c-src dependant mechanism. IRF-3 activation is necessary for memory T cell function and is therefore important for effective vaccine responses (Guinn et al. 141-149; Petro, Guinn, and Lampe).

Myristic acid also binds to the tyrosine kinase c-abl, putting it into an auto inhibitory state, in which c-abl functionality is lost (Nagar et al. 859-871). If YC NaMa blocks c-abl, this may be beneficial in the context of tuberculosis. Imatinib, a src inhibitor, which blocks c-abl, has been shown to reduce the number of granulomas and bacterial load during MTB infection (Mahadik et al. 85).

c-abl has a number of functions which may be relevant to mycobacterial infection. It is upregulated in macrophages infected with mycobacteria and promotes entry and survival of microbes/mycobacteria by preventing acidification of the phagosome. It is also required for TNF alpha induced apoptosis (Chau et al. 4438-4447). During TB infection, MTB induces TNF α and apoptosis of macrophages by expressing ESAT6 (Yang et al. 1276-1288). YC NaMa generates increased levels of phosphorylated Nf- κ B and C-Jun (Hart et al. 822-833). C-Jun acts with Nf- κ B to suppress TNF alpha induced apoptosis (Wisdom, Johnson, and Moore 188-197). YC NaMa may be able to block TNF alpha induced cell death which would be relevant in generating effective immune responses against mycobacteria.

8.4 Effect of Liposome P/S delivery system on murine DCs.

This study found that Liposome P/S does not impact the expression of CCR7, CD40, CD80, CD86, MHC I or MHC II by murine BMDCs. Alone, it has no effect on the production of TNF alpha, IL-12, IL-1 β or IL-6. The existing literature suggested that phosphatidylserine is immunosuppressive and limits the expression of pro inflammatory cytokines (Huynh, Fadok, and Henson 41-50; Voll et al. 350). In this study, there was no evidence in this study of P/S inhibiting TLR signalling. Indeed, liposome P/S combines with a range of TLR agonists to upregulate MHC I expression. TLR3, TLR4 and TLR9 agonists

combine with Liposomes to PD-L1 expression which may exert an inhibitory effect. This study found that liposome P/S blocks efferocytosis (phagocytosis of dead cells) by DCs. It is possible that efferocytosis is blocked by liposome competitively binding to P/S receptors on the surface of live phagocytes, limiting DC binding to apoptotic target cells.

Unexpectedly, liposome P/S combines with the TLR 5 agonist, Flagellin, to strongly activate DCs. Flagellin in combination with liposome P/S increases DC expression of CD40, CD80, MHC I, MHC II and PD-L1, compared to flagellin or liposomes alone. Flagellin, or another TLR 5 agonist could be an appropriate adjuvant for liposome P/S as they act together to activate DCs. Combining Liposomes with Flagellin increased PD-L1 expression on DCs so it will be important to avoid overstimulating the DCs when combining liposomes with a TLR5 agonist.

TRIF promotes the degradation of TLR 5 by activating Caspase 8. Blocking TRIF prevents TLR 5 degradation and enhances the effects of TLR 5 agonists such as Flagellin. This study found that Liposome P/S enhanced TLR 5 signalling by Flagellin. Activation of caspase 8 is mediated by the C-terminus of TRIF (Choi, Y. J., Im, Pothoulakis, and Rhee 21382-21390). It is possible to hypothesise that liposome P/S blocks the activity of the c terminal of TRIF, whilst enhancing the activity of the N terminus or the TIR domain. This study did not provide any evidence that Liposome P/S inhibits TRIF mediated TLR signalling. Based on this finding, it is unlikely that the entire TRIF molecule is blocked by liposome P/S. It is more likely that the TIR domain and the N terminal domain retain their functionality.

Based on the data from the *in vitro* DC models, it is possible to hypothesise that liposome P/S binds to TIR-domain-containing adapter-inducing interferon- β (TRIF). Liposome P/S may phosphorylate the N terminal of TRIF, releasing the molecule from its normal auto-inhibitory state. Phosphorylation is known to be essential for IRF3 signalling generated by TRIF (Liu, S. et al. 2006). This model would explain why liposome P/S does not generate TRIF signalling by itself, but instead enhances TRIF signalling by TLR agonists. TRIF plays a number of roles in cell biology. It mediates MyD88 independent

signalling by TLR4 agonists and it acts as the sole adaptor molecule for TLR 3 signalling. It enhances Nf- κ B signalling and activates IRF-3 and IRF-7 (Mahita and Sowdhamini 9). TRIF upregulates MHC II expression on DCs through a GEFH1/RhoB pathway(Kamon et al. 4108-4119).

Further experiments will be needed to test the theory that phosphatidylserine liposomes exert an immunogenic effect via the TRIF molecule.

8.5 BCG PD-L1 Study

T cell responses are regulated by costimulatory molecules on APCs. The ligands PD-L1 and PD-L2, expressed on APCs bind to PD-1 on T cells acting as negative regulators of T cell activation and proliferation. The costimulatory ligands CD80 and CD86 are part of the same B7 family but generate an activatory and inflammatory effect by binding to CD28.

The *in vitro* experiments performed here, show that BCG upregulates expression of B7 family cell surface markers on macrophages and DCs. BCG infection led to enhanced expression of PD-L1, PD-L2, CD80 and CD86 in macrophages and DCs. In macrophages, BCG infection led to expression of PD-L1 and CD86 increasing by around 3 fold. Infected dendritic cells showed 3.5 fold greater expression of PD-L2 and CD86 than the unstimulated cells. These results showed that BCG infection is capable of subverting the immune responses of macrophages and dendritic cells by inducing the expression of the tolerogenic molecules PD-L1 and PD-L2.

Enhancing PD-L1 expression in macrophages may represent a mechanism of mycobacterial immune evasion. Increased PD-L1 expression is known to be important in tolerogenesis, malignancy and chronic disease (Shen, T. et al. 1152-1159). MTB and BCG are both known to upregulate PD-L1. In the context of bladder cancer, blocking PD-L1 using anti PD-L1 therapy has been shown to enhance BCG induced immune responses (Wang et al. 2891).

PD-L2 on dendritic cells serves as a negative regulator of T cells. PD-L2 knockout mice have been studied previously. PD-L2 knockout APCs were more effective at activating T cells *in vitro* and *in vivo*.

PD-L2 was also shown to be necessary for tolerogenesis (Zhang, Y., Chung, Bishop, Daugherty, Chute, Holst, Kurahara, Lott, Sun, Welcher, and Dong 11695-11700). PD-L2 expression on DCs is associated with diminished responses to TLR agonists and subsequent Th2 responses (Mendoza-Coronel et al. 36-46). *Mycobacterium avium* induces PD-L2 expression in BMDCs via the MAPK pathway. This PD-L2 expression was linked to reduced antigen specific T cell responses to BCG infection (Mendoza-Coronel et al. 36-46). This study has shown for the first time that BCG induces PD-L2 expression on DCs.

Filtered cell culture media from infected and uninfected macrophages cells was added to cultures of uninfected J774 cells. PD-L1 expression was then measured by flow cytometry. PD-L1 expression was comparable between BCG infected cells and cells which had received filtered supernatant from infected cells. This work reveals that BCG is able to indirectly enhance PD-L1 expression. Secreted factors in culture media are sufficient to generate BCG induced PD-L1 upregulation. These secreted factors are either cytokines generated during infection, or secreted mycobacterial antigen, or a combination of the two. It may be possible to enhance the efficacy of BCG vaccination against TB by limiting the expression of PD-L1 and PD-L2 by BCG infected DCs.

8.6 Immunogenicity of novel TB vaccines

This study has used TMCs to develop an *in vitro* model to investigate the immune responses generated by novel TB vaccine candidates. The model has focused on IFN γ responses and IL-17 responses in CD4 $^{+}$ and CD8 $^{+}$ T cells. These proinflammatory responses are associated with protection against TB. Vaccine candidates which generate IFN γ and IL-17 responses may be promising TB vaccine candidates. The TMC model has also investigated T cell proliferation and IL-2 production. These have been used as indicators that a particular vaccine may be capable of generating a sustained T cell response.

The BCG vaccine is the only widely used vaccine against TB. It generates IFN γ and IL-17 responses in both CD4 $^{+}$ T cells and CD8 $^{+}$ T cells and is protective against disseminated TB and meningitis. When

BCG was tested in the TMC immunogenicity model, it was found that BCG elicited IFN γ and IL-17 responses. It also induced proliferation and the production of IL-2. These findings conformed to the known immunogenic characteristics of BCG. This was significant because it demonstrated that the tonsil immunogenicity model was able to detect the key elements which are thought to be involved in protection against TB.

The tonsil immunogenicity model has been used to investigate the immunogenicity of some vaccine adjuvants which could be useful for future TB vaccines. Four adjuvants were tested; Alum, Peptidoglycan, Poly I:C and Quil A.

8.7 Immunogenicity of Alum

Alum was introduced as a vaccine adjuvant in the 1920s. It has previously been used in vaccines against a range of diseases, including hepatitis B (Burakova et al., 2018), diphtheria, tetanus and pertussis and human papilloma virus (CDC, 2023). Alum has recently been included in two novel COVID vaccines BBIBP-CorV and Coronavac (Xia et al., 2021; Zhang, Y. et al., 2021). Alum engages the NLRP3 inflammasome (Eisenbarth et al., 2008; Elgueta et al., 2009; Kool et al., 2008), leading to the development of Th2 and Th1 responses (Coffman et al., 2010a) (Brewer et al., 1996; Burny et al., 2017; Elgueta et al., 2009; Nardin et al., 2004; SALMON-CÉRON et al., 1995). Alum also induces production of IgG1 and IgE (HogenEsch, 2002; Ulanova et al., 2001).

In TMCs, Alum had no impact on the production of cytokines by CD4 $^{+}$ T cells. It also had no impact on the expression of activation markers on CD4 $^{+}$ T cells. Stimulation with Alum upregulated CD80 expression on CD8 $^{+}$ T cells. It had no effect on cytokine production by B cells or their expression of activation markers. This immunogenic profile did not match the anticipated immunogenicity of Alum. We did not observe any increased production of IFN γ or IL-4 in TMCs stimulated with Alum. The fact that Alum generates a different immunogenic profile in the mucosal tissue of TMCs may have implications for the design of mucosal vaccines.

The TMC immunogenicity data also showed that Alum plays a role in the activation of CD8+ T cells.. Alum upregulated CD80 expression on CD8+ T cells. The effect of alum on antibody production in the tonsil model was not examined, but it would be useful to determine whether it is capable of generating IgG1 and IgE immunoglobulins in a tonsil model.

8.8 Immunogenicity of Peptidoglycan

TLR2 signalling is important for several vaccines. The BCG vaccine signals through TLR2, TLR4, TLR9 and DC SIGN (Moliva et al., 2017; Tsuji et al., 2000). TLR signalling is essential for the live attenuated yellow fever vaccine YF-17D (Querec, Troy, Bennouna, Alkan, Laouar, Gorden, Flavell, Akira, Ahmed, & Pulendran, 2006; Querec, Troy D. et al., 2009). YF-17D is capable of activating TLR 2 as well as TLR 3, TLR7 and TLR9 receptors (Querec, Troy et al., 2006). The Classical Swine Fever (CSF) subunit vaccine used in livestock contains Oprl lipoprotein from *Pseudomonas aeruginosa*. Oprl lipoprotein generates TLR2 signals which activates APCs leading to enhanced T cell responses and antibody responses (Rau et al., 2006).

Peptidoglycan is a TLR2 agonist which is also recognised by the NOD1 receptor. TLR2 signalling suppresses the function of Treg cell populations. NOD1 signalling contributes to the development of antigen specific responses. NOD1 is also required for Th1, Th2 and Th17 responses (Fritz et al., 2007). Peptidoglycan has previously been used as a component of Complete Freund's adjuvant (CFA). CFA signals through the NLR, the inflammasome and TLR2. CFA induces humoral responses as well as Th1 and Th17 CD4+ T cell responses (Coffman et al., 2010b; Eisenbarth et al., 2008; Gavin et al., 2006).

It was expected that peptidoglycan would induce T cell responses in the tonsil model. Peptidoglycan did upregulate the expression of CD80 and CD86 on CD8+ T cells but it had no effects on CD4+ T cells or B cells. Peptidoglycan did not upregulate the production of IFN γ , IL-17 or IL-2 in the TMCs. When peptidoglycan has been used previously in vaccines, it has been combined with other TLR agonists. It may be that peptidoglycan alone is unsuitable as an adjuvant as it does not elicit a suitable response in the TMC model. Overstimulation with TLR agonists can cause septic shock (Kanzler et al., 2007). There was no evidence that the concentration of peptidoglycan used in this study impacted the viability of the TMCs.

8.9 Immunogenicity of Poly I:C

Poly I:C is a synthetic derivative of dsRNA. It is a TLR3 agonist which has been used widely as an adjuvant for novel vaccine candidates (Coffman et al., 2010c). It has been shown to enhance antibody production, Th1 responses and CD8+ T cell responses (Salem et al., 2005; Tewari et al., 2010).

Poly I:C induced IL-4 production by tonsillar B cells. It did not induce cytokine responses in CD4+ or CD8+ T cells. It also had no impact on the expression of CD40, CD69 or CD80 activation markers on these cells.

8.10 Immunogenicity of Quil A

Quil-A is a saponin adjuvant derived from bark of the *Quillaja saponaria* tree. Quil A has a high affinity for Cholesterol. It binds to membrane cholesterol, opening pores in cells (Kersten & Crommelin, 2003). It is known to be immunogenic it enhances antibody production and induces mixed Th1/ Th2 responses as well as CTL responses (Hackett & Harn Jr, 2007; Kensil, C. Read, 1996; Kensil, Charlotte R. et al., 1991; Newman et al., 1992). Quil A has been used as an adjuvant for the Inactivated Polio vaccine (IPV). Quil A adjuvant significantly enhanced the serum levels of specific IgG, IgG1 and IgG2. Antigen specific Th1 responses were also elevated with the inclusion of a Quil A adjuvant (de Costa et al., 2014).

One of the components of Quil A, QS-21, has been combined with MPL as an adjuvant in an influenza vaccine in mice. This vaccination resulted in lower viral loads, increased Th1 responses and increased titres of IgG and haemagglutinin inhibiting antibody (Bhatnagar et al., 2022).

QS-21 is a core component of the AS01b adjuvant. AS01b is composed of QS-21 and MPLA within liposomes. AS01b has been approved for use as an adjuvant in the Shingrex and Mosquirix vaccines for herpes zoster and malaria (James et al., 2018; Lacaille-Dubois, 2019).

Quil A was the most immunogenic adjuvant examined in this study. It elicited IFN γ , IL-17, IL-4 and IL-10 in CD4 $^{+}$ T cells. This conformed with the previous literature which indicated that Quil A is capable of generating Th1 and Th2 responses. CD86 expression in CD4 $^{+}$ T cells was also increased by Quil A. It has previously been established that Quil A is capable of generating IgG responses. The effect of Quil A on B cell cytokine production was not well studied. This study showed that Quil A induced IL-4, IL-10 and IL-2 cytokines in tonsillar B cells. Quil A increased CD40 expression on CD8 $^{+}$ T cells.

8.11 Immunogenicity of Resiquimod

Resiquimod is a TLR 7/8 agonist which has known antiviral and anti tumour properties. It has been investigated as a potential vaccine adjuvant (Tomai et al., 2007). Previously, Resiquimod has been shown to upregulate CD40, CD8, CD86 and CCR7 on DCs (Gibson et al., 2002). It is also known to activate B cells and enhance IFN γ and CTL responses in T cells (Bishop et al., 2000; Gorski et al., 2006). In this study, Resiquimod was tested as a potential adjuvant for novel TB vaccines. TLR8 is non functional in mice, making a human tonsil model useful for assessing Resiquimods immunogenicity. Resiquimod was found to be immunogenic in TMCs. Resiquimod generated IFN γ responses in CD4 $^{+}$ T cells. It activated T lymphocytes, with upregulated expression of CD69 on CD4 $^{+}$ T cells and CD8 $^{+}$ T cells. It also upregulated CD40 on CD8 $^{+}$ T cells. In B cells, Resiquimod induces a Be-1 phenotype, with increased percentages of IFN γ and IL-2 $^{+}$ B cells. Expression of CD40, CD69 and CD86 on B cells was also upregulated in the presence of Resiquimod. These characteristics make Resiquimod an appealing adjuvant for novel TB vaccines.

8.12 Immunogenicity of novel TB vaccine candidates

Six novel vaccine candidates against TB were tested; Spore FP1, YC NaMa FP1, Liposome FP1, Liposome A/E, IgA ICM and IgG ICM. Each of these vaccines produced distinct immune responses. From these results it is possible to predict the most promising vaccine candidates and the appropriate adjuvant combinations.

TMCs which had been treated with Spore FP1, generated IFN γ + CD4+ T cells responses. There was no corresponding increase in IL-17 production. Nor was there any observed increase in IL-2 production or proliferation of T cells. IFN γ + T cell responses are known to be protective against tuberculosis and this may form the basis of the enhanced protective immunity previously observed in mice vaccinated with BCG and Spore FP1 as a prime boost strategy (Copland et al. 346).

YC NaMa FP1 elicited a strong proinflammatory immune response. TMCs stimulated with YC NaMa FP1 developed CD4+ T cell responses with increased expression of IFN γ and IL-17. This is expected to assist the development of protective immunity against TB (Sakai, Mayer-Barber, and Barber 137-142; Torrado and Cooper 455-462). In addition to this, YC NaMa FP1 also induced proliferation of CD4+ T cells and the production of IL-2. This gives it an immunogenic profile similar to the BCG vaccine.

The cytokine responses observed in the TMC study were not always correlated with proliferation of T cells. This is consistent with the published literature. Tonsillar DCs typically suppress T-cell proliferation and can decouple proliferation from cytokine responses (Hallsley, Heyderman, and Williams 1847-1856). YC NaMa FP1 overcame this suppressive effect and induced pro-inflammatory cytokine production and proliferation in T cells.

Poly I:C was tested as an adjuvant for YC NaMa FP1 and Spore FP1. The results of this study did not find any enhancement of the immune responses generated by Spore FP1 or YC NaMa FP1 vaccines following the addition of a Poly I:C adjuvant. YC NaMa FP1 and Spore FP1 without Poly I:C both enhanced the development of Th1 T cell responses in the TMCs. YC NaMa FP1 also enhanced Th17

responses. These immunogenic effects were lost with the addition of Poly I:C. This corresponds with the findings of the murine *in vitro* studies shown in chapter 1. Poly I:C has previously been used as an adjuvant for YC NaMa FP1 in a mouse model (Hart et al., 2018a). This study has found that Poly I:C is not an optimal adjuvant for either Spore FP1 or YC NaMa FP1 as it abrogates the immunogenicity of these vaccines.

Liposome FP1 was immunogenic in the TMC model. It enhanced IFN γ and IL-17 production by CD4 $^{+}$ T cells. Liposome FP1 did not induce the proliferation of CD4 $^{+}$ or CD8 $^{+}$ T cells or IL-2 expression. This immunogenic profile indicates that Liposome FP1 may be a suitable for further development as a TB vaccine candidate. One caveat, is that Liposome FP1 did not generate any proliferative effects indicating that it might not produce a sustained effect. Combining Liposome FP1 with exogenous IL-2 or some other adjuvant may improve the long term protection conferred by Liposome FP1.

Liposome A/E had a different immunogenic profile to liposome FP1. It did not generate significant IFN γ production by CD4 $^{+}$ T cells, nor did it induce proliferative responses. Instead, it caused CD4 $^{+}$ and CD8 $^{+}$ T cells in TMC cultures to express IL-17. IFN γ + CD4 $^{+}$ T cell responses are thought to be important for TB vaccines, without these, Liposome A/E may not be a suitable TB vaccine candidate by itself. Previously Liposome A/E has been combined with BCG vaccination in a mouse model (Diogo et al., 2019b). Boosting BCG with Liposome A/E led to IFN γ and IL-17 responses and reduced bacterial loads. This prime boost strategy would have generated IFN γ responses from BCG which would then have been complemented by Th17 responses generated by Liposome A/E.

IgG Immune Complex Mimic (ICM) was not an immunogenic vaccine formulation. TMCs stimulated with it did not show increased percentages of CD4 $^{+}$ or CD8 $^{+}$ T cells expressing IFN γ , IL-2 or IL-17. It also failed to induce proliferation of T cells. There was no evidence to suggest that IgG ICM would be a suitable vaccine. Combining IgG ICM with Quil A induced IFN γ expression by CD4 $^{+}$ T cells and encouraged them to proliferate. This was only due to the addition of Quil A, the IgG ICM complex did not measurably activate the TMCs. IgG ICM was designed to bind to Fc γ receptors. Immune complexes

binding to FcγRIIA receptors induce immune activation. In contrast monomeric Ig binding to FcγRIIA results in inhibition. Monomeric Ig is also capable of co-ligating to FcγRIIB and the BCR leading to inhibition (Ben Mkaddem et al., 2019). The inability of the IgG ICM complex to induce activatory signals in this study may indicate that it was binding to the TMCs in a monomeric form.

IgA ICM alone enhanced IL-17 expression by CD4⁺ T cells. It was not capable of inducing IFNγ production or proliferative responses by itself. Combining IgA ICM with Quil A produced a promising immunogenic profile. A greater proportion of CD4⁺ T cells produced IFNγ and IL-2 following treatment with IgA ICM Quil A. It also induced the proliferation of CD4⁺ T cells. Quil A suppressed the IL-17 inducing effect of IgA ICM. Based on this study, IgA ICM, with a Quil A adjuvant may be a suitable candidate for further development. Monoclonal IgA therapy has been shown to reduce MTB burden (Balu et al. 3113-3119). Further work is needed to determine whether IgA ICM combined with Quil A would also reduce TB burden.

IgG ICM and IgA ICM are both based on the anti Acr Immunoglobulin 2E9. Anti Acr IgA 2E9 has been shown to have a protective effect against tuberculosis (Balu et al., 2011). Interestingly, the IgG version of 2E9 does not confer any protection against TB (Tran et al., 2020). This corresponds well with the TMC immunogenicity data gathered in this study, with IgA ICM being immunogenic and IgG ICM having no immunogenic effect on TMCs.

The number of reported Dengue cases has increased over the past twenty years. In 2019, there were more than 5.2 million recorded cases (World Health Organisation, 2023). The only existing vaccine against Dengue virus, CYT TDV (Dengvaxia) is unsuitable for widespread use. As such, it is important to develop new vaccines against Dengue virus. This study investigated four novel dengue vaccine candidates; D-PIGS, DERICS, My42J and Mod D-PIGS.

Firstly, it was important to determine whether the dengue vaccine candidates are capable of interacting with human cells. This study showed that there was detectable binding of DERICS and D-PIGS vaccines to tonsillar monocytes. There was no detectable binding of My42J or Mod D-PIGS to

tonsillar monocytes. This may indicate that the addition of a J chain to this recombinant construct has disrupted the interaction of the vaccine with a human FcγR receptor.

These experiments have developed a tonsillar model which can be used to assess the T cell responses generated by novel Dengue vaccines. Four novel dengue vaccine candidates D-PIGS, My42J, Mod D-PIGS and DERICS were examined and their effect on T cell responses was examined. At a 12 day timepoint, three of the vaccines, D-PIGS, Mod D-PIGS and My42J generated TNFα, IFNγ and IL-4 responses in CD4+ T cells. D-PIGS, Mod D-PIGS and My42J also generated IL-4 responses in CD8+ T cells. The DERICS vaccine generated IFNγ and IL-4 responses in CD4+ T cells. But there were no detectable CD8+ T cell responses to DERICS. These experiments provide compelling evidence that these vaccine candidates are immunogenic in a human model.

T cell responses are known to be relevant to Dengue infection. In murine models, CD4+ T cells and CD8+ T cells both confer protection against Dengue infection (Ngono et al., 2016; Yauch et al., 2009c; Yauch et al., 2010b; Zellweger et al., 2015). CD4+ T cell responses are redundant in the development of CD8+ T cell responses and the creation of antibodies against Dengue virus (Yauch et al., 2010b). In spite of this, CD4+ T cell responses are still relevant for dengue vaccine design. Inducing CD4+ T cells by immunization enhances viral clearance (Yauch et al., 2010b). Dengue infection induces DENV specific CD4+ T cells which express IFNγ, TNFα and IL-2 (Lindow et al., 2012). These IFNγ, TNFα and IL-2 dengue responses in CD4+ T cells are protective and have been associated with subsequent subclinical infection (Hatch et al., 2011b). D-PIGS, Mod D-PIGS and My42J each elicited proinflammatory responses in CD4+ T cells which are comparable to these protective proinflammatory responses. The proinflammatory CD4+ T cell responses created by these vaccines may contribute to protection against Dengue virus. The DERICS vaccine had a different immunogenic profile. It generated IFNγ and IL-4 CD4+ T cell responses which differed from the known protective proinflammatory profile.

Dengue specific CD4+ T cells also have cytotoxic activity (Tian et al., 2016b). These cytotoxic CD4+ T cells are more abundant in patients with less severe Dengue disease (Duangchinda et al., 2010). This study did not investigate the cytotoxic function of tonsillar CD4+ T cells stimulated with these vaccines. Further studies would be needed to assess whether these vaccines were able to promote the expansion of a DENV specific cytotoxic CD4+ T cell population

Polyfunctional CD8+ T cell responses are correlated with reduced severity of dengue disease (de Alwis et al., 2016; Weiskopf et al., 2013b). Dengue specific CD8+ T cells are more abundant in children with subclinical Dengue secondary infection rather than active disease (Hatch et al., 2011b). These CD8+ T cell responses are thought to be significant for vaccine design. It has been hypothesised that the Dengvaxia vaccine did not generate adequate T cell responses (Halstead, 2018). TV003 generates strong CD8+ T cells responses and confers substantial protection with one dose (Chandele et al., 2016; Kirkpatrick et al., 2016).

The IL-4 responses generated by D-PIGS, Mod D-PIGS and My42J in CD8+ T cells were different to the protective polyfunctional CD8+ T cell responses previously described and due to limitations of the tonsil model, it was not possible to demonstrate that they were DENV specific. This makes it difficult to relate the observed immunogenicity to any potential protective effect. The DERICS vaccine did not elicit any detectable tonsillar CD8+ T cell responses at all. Given the importance of CD8+ T cell responses for Dengue vaccine, this may be an indication that D-PIGS, Mod D-PIGS, My42J and DERICS would produce suboptimal immune responses.

This immunogenicity model examined expression levels of IFN γ , IL-4, IL-10 and TNF α and Ki67. The Dengue vaccines tested in this study generated IFN γ , TNF α and IL-4 responses. The vaccines may have generated other responses which were not recorded. DNA microarray analysis could be used in future to monitor the effects of these vaccines on all gene expression without bias. IL-17 responses have been associated with increased severity of dengue disease, whilst Macrophage inflammatory protein-1 β (MIP1 β) is associated with milder forms of dengue disease (Jain et al. 613-618; Bozza et al. 86).

Additionally, as soluble CD27 and CD38 are associated with the severity of dengue fever, it may be appropriate to measure them in future *in vitro* studies of dengue vaccine immunogenicity (Castañeda, Salgado, and Narváez 136-145).

This work has revealed limitations of the tonsil model. It was not possible to detect any recall responses to CEDIII in tonsillar T cells stimulated with the vaccine candidates. The vaccine candidates used in this study are known to be immunogenic and have previously generated recall responses in mouse models. In spite of this no CEDIII recall responses were observed. From this, it is possible to conclude that the tonsil model is unsuitable for generating antigen specific responses *de novo*.

Neutralising antibody responses are highly associated with protection against Dengue infection. In screening novel dengue vaccine candidates, it is important to ascertain whether they elicit antibody responses and whether those antibody responses are neutralising. It was not possible to generate antibody responses at all using the tonsillar model. D-PIGS is known to elicit neutralising antibodies in mice. D-PIGS generated anti CEDIII antibodies in a mouse model which used transgenic mice with human CD64 (Kim, Mi Young, Copland, Nayak, Chandele, Ahmed, Zhang, Diogo, Paul, Hofmann, & Yang, 2018c). The DERICS vaccine candidate is also known to generate neutralising antibodies in mice (Kim, Mi-Young et al., 2015). By contrast, it was not possible to detect any anti CEDIII antibodies from tonsil cells stimulated with D-PIGS or DERICS.

Antigen specific responses require naïve T cells and B cells which are specific for the selected epitope. The precursor frequency, the number of naïve cells specific for a specific antigen, is generally low. For T cells, the precursor frequency is between 1 in 10^4 and 1 in 10^5 . For B cells, the precursor frequency is significantly lower. It is estimated that the precursor frequency for B cells is between 1 in 10^8 and 1 in 10^{12} . In these immunogenicity experiments, cells were seeded with 1×10^5 TMCs. The phenotype analysis shown in chapter 2, figure 4.2.2 and 4.3.9 makes it possible to estimate the number of naïve lymphocytes which would be present in these wells. In a well containing 1×10^5 TMCs, there would be approximately, 14,000 naïve B cells, 2,400 naïve CD4+ T cells and 1,200 naïve CD8+ T cells. Given the

rarity of naïve antigen specific lymphocytes, it is not practical to generate new antigen specific responses *in vitro* with this number of cells. From this, it is possible to conclude that this tonsil model is unsuitable for generating antibody responses or antigen specific responses *de novo*.

Tonsil cultures have previously been used to generate antibodies *in vitro* (Mohn et al., 2016). Patients were vaccinated with Live attenuated Influenza vaccine (LAIV) 2 weeks prior to tonsillectomy. This induced the development of mucosal B cell responses in tonsil tissue. Antibodies against Influenza, including IgA, were detected. Vaccination with LAIV prior to tonsillectomy has also been shown to induce the activation of follicles and tonsillar follicular T helper cells (Lartey et al., 2020). Both of these studies vaccinated patients with LAIV prior to tonsillectomy.

More recently, LAIV has been added to tonsil cells after tonsillectomy (Wagar et al., 2021a). This paper was significant because it showed several key functions of lymphoid tissue could be recapitulated *in vitro*. Influenza specific antibodies were generated. There was also formation of germinal centres. B cell isotype class switching was observed. Plasmacytoid dendritic cells were found to be important for antibody production by tonsil cells. Antigen specific IgG levels were significantly reduced when these cells were depleted. Type I interferon added exogenously restored the production of antigen specific IgG. Type I interferon may therefore be useful as an additive to the cell culture media of tonsil cells as it plays a role in promoting antibody production.

It is significant that the tonsillar cells in this paper generated antibodies against influenza. The antibody responses were most likely recall responses from patients who have previously been exposed to influenza. There is very little evidence that tonsil cultures can be used *in vitro* to generate new antibody responses against a previously unknown antigen.

Tonsils have been stimulated with malaria antigens and used to generate antibodies *in vitro* against *P. Falciparum* (Garraud et al., 2001) but these were memory responses which already existed in the patients prior to the tonsillectomy. 65% of TMCs from Senegalese villagers generated *P. falciparum* specific IgG. These villagers lived in areas with endemic malaria. None of the TMCs from malaria naïve

European generated *P. falciparum* specific IgG *in vitro*. In addition, the tonsillar B lymphocytes responding to *P. falciparum* antigens belonged to the surface γ^+ subset and had therefore already undergone isotype switching. This confirmed that these were pre-existing antibody responses.

Further work could be done with TMCs to develop Dengue vaccines and investigate the development of new memory responses.

1. Increase the scale of the experiments to maximise the chance of finding rare antigen specific naïve B cells.
2. Incorporate type I interferon into the cell culture media to boost antibody production.
3. Include IL-2 in the culture media. IL-2 signalling promotes the survival of CD4⁺ and CD8⁺ T cells during primary memory response (Goldrath et al., 2002).
4. Examine the expression of OX40 and PD-L1 on tonsillar T cells. These are markers for TCR activation of tonsillar follicular helper cells (Dan et al., 2016; Havenar-Daughton et al., 2016).
5. Examine the expression of CD45RA and CCR7 on CD4⁺ T cells following stimulation with Dengue vaccines. CD4⁺ T cells with a CD45RA⁺CCR7⁻ phenotype are associated with protection against severe Dengue disease (Weiskopf et al., 2015).
6. Examine the development of cytotoxic anti Dengue CD4⁺ T cells and CD8⁺ T cells.

8.13 TMC infection model for TB

Tonsil infection models have previously been developed for several diseases. This is the first study to use tonsil cells to model mycobacterial infection *in vitro*. We have also developed an *in vitro* model which can be used to measure the immunogenicity of novel TB vaccines. Tonsils are an easily accessible source of primary human tissue. They contain B cells helper and cytotoxic T cells and macrophages. These cells are suitable for immunogenicity studies and infection models. Using tonsillar *in vitro* models, we show that the novel vaccines Spore FP1, YC NaMa FP1 and Liposome FP1 are all immunogenic and aid in clearance of mycobacterial infection. In addition we have characterised the interaction between BCG GFP and human lymphocytes; showing that mycobacteria adhere to the surface of T lymphocytes and tonsillar B lymphocytes.

There are currently 14 TB vaccines in human clinical trials and numerous others in development. Progress on TB vaccine development is hindered by a lack of known correlates of protection and *in vitro* models which predict vaccine efficacy.

Murine models have previously been used to characterise the immunogenicity of the FP1 vaccines. YC Nama FP1 and Spore FP1 both provide protection against infection with TB. Vaccinated animals infected with either Spore FP1 or YC NaMa FP1 had reduced bacterial loads in lungs and spleens. High titres of antigen specific IgA were detected in the lungs and IgG in the serum. YC NaMa FP1 and Spore FP1 both induce Ag85B recall cytokine responses in T cells, with IFN γ , IL-17 and IL-10 responses as well as proliferation.

In murine TB infection models Spore FP1, YC NaMa FP1 and Liposome FP1 all reduced mycobacterial load in lungs and spleens of infected animals. In the tonsillar infection model, prior stimulation with YC NaMa FP1, Spore FP1 or Liposome FP1 led to reduced mycobacterial burden. Stimulation with BCG or IFN γ in the tonsillar model also reduced mycobacterial load. The short time frame of this experiment rules out an adaptive immune response. Instead, it is most likely that these stimulations

activate monocytes allowing them to successfully phagocytose mycobacteria. A similar phenomenon has been observed in THP1 derived macrophages which have been stimulated with LPS. These stimulated cells demonstrate increased phagocytosis and elimination of MTB along with increased ROS production. This effect was dependant on TLR4 and NOX2 (Lv J. et al. 2017).

The findings of the mycobacterial challenge assay were therefore comparable to those seen in murine models. The tonsillar model generated useful information about the immunogenicity of the FP1 vaccine candidates. The FP1 vaccines generated IL-17 and IFN γ T cell responses in tonsil cells, just as they did in mice. These IL-17 and IFN γ responses are thought to play a protective role against TB infection.

These results demonstrate the usefulness of the tonsil model and show that it can be used to screen novel TB vaccine candidates. A further challenge will be to compare the outcomes of the mycobacteria clearance assay with the results of human clinical trials.

This model has value for screening novel vaccine candidates, it can identify vaccines which elicit clearance of mycobacteria. However it has the limitation that it is unable to quantify the efficacy of novel vaccine candidates. This model showed mycobacterial clearance with IFN γ treatment and BCG vaccination. The novel FP1 vaccines showed a similar level of mycobacterial clearance. Both BCG vaccination and IFN γ are only partially protective against active TB but it is not possible to determine whether the novel FP1 vaccines would be more or less effective than BCG or IFN γ treatment. For this, *in vivo* studies and human clinical trials would still be necessary.

Animal models will also be necessary to model dissemination of mycobacteria to other organs, as this cannot be assessed using the tonsil model.

The BCG vaccine is known to confer protection against TB and it also has the ability to induce mycobacterial clearance. In a murine model, BCG vaccination leads to reduced mycobacterial burden in the lungs (Bickett, T.E. et al 2020). Stimulation with live BCG reduces mycobacterial lung burden

whilst stimulation with irradiated BCG does not. This mycobacterial clearance does not require CD4+ T cells or CD8+ T cells. IFN γ and TNF α responses are also unnecessary for mycobacterial clearance by BCG. Instead, the mycobacterial clearance is accompanied by an influx of monocytes into the lungs. Neutrophils have also been identified as being crucial to BCG induced mycobacterial clearance (Bickett, T.E. et al 2020).

The mechanism by which the neutrophils and monocytes eliminate mycobacterial infection has not been elucidated. Lysozyme is not required for mycobacterial elimination. (Bickett, T.E. et al 2020). Both Elastase and IL-8 are upregulated, but there is no evidence of enhanced ROS production or phagocytosis (Moorlag S.J. et al 2020).

It is reasonable to think that mycobacterial clearance is an important characteristic for a novel TB vaccine. The BCG vaccine is known to be protective and it induces mycobacterial clearance. By contrast, the MVA85A vaccine was not protective against TB and does not induce mycobacterial elimination (Tameris M.D. et al 2013) (Tanner R. et al 2019). In this context, it is promising that Spore FP1, Liposome FP1 and YC NaMa all induce mycobacterial clearance.

8.14 Interaction of BCG with B cells

This study demonstrates that BCG binds to the surface of B lymphocytes. It also binds to the cell surface of CD4⁺ and CD8⁺ T cells. The tonsil model has been used here to investigate the interaction of BCG with human B cells. Tonsillar cells are a good model for B cell biology because approximately 60% of all tonsil cells are B cells. The tonsil model can also be used to investigate the interaction of T lymphocytes with BCG.

The mechanism by which BCG bound to B cells was unclear. B cells are known to be capable of engaging in phagocytosis. Phagocytosis is controlled by actin filaments and can be disrupted by the addition of cytochalasin D. Pre-treatment with cytochalasin D did not reduce the FITC MFI of B cells during infection with BCG GFP. This indicates that interaction of B cells with BCG is not due to phagocytosis or any process which involves the development of actin filaments.

B cells are also known to engage in macropinocytosis (Castaneda- Sanchez J.I. et al 2017). Amiloride was used to block macropinocytosis. This blockade had no impact on the interaction between B cell and BCG, indicating that some process other than macropinocytosis was responsible for the interaction.

The complement receptors 1, 2 and 3 (Schlesinger L.S. et al 1990) have been shown to bind to mycobacteria. These are present on B cells and we investigated whether the complement component C1q was opsonising BCG leading to binding to B cells. Additional C1q did not enhance the interaction between B cells and BCG GFP.

There was substantial evidence that the interaction between B cells and BCG was due to immunoglobulin binding to the surface of the bacteria. The interaction of BCG GFP with B cells did not involve an actin filaments. Disrupting actin filaments with cytochalasin D did not impact binding. This is consistent with binding to the immunoglobulins of BCRs.

Trypsin treatment is known to degrade IgM and generate multiple monomeric IgM components. When B cells were infected with BCG and then treated with trypsin, there was an increase in the percentage of B cells adhering to GFP BCG. We theorise that individual molecules of immunoglobulin were degraded into multiple monomeric IgM components. These monomeric fragments of IgM were able to bind more BCG and thus enhanced binding of BCG GFP to the B cells.

Trypsin treatment followed by washing steps and then BCG infection led to significant reductions in adherence. This is consistent with IgM being degraded to monomeric IgM components which were then washed away, limiting binding of BCG to B cells.

The clearest evidence that B cells bind to BCG via antibodies is shown in figure 7.6.1. Cells infected in the presence of serum showed higher levels of infection than those without serum. Human serum enhanced binding of BCG to B cells whilst human serum without immunoglobulin did not increase binding at all.

Monocytes and macrophages play a critical role during mycobacterial infection. They act as an important part of the innate immune response to infection. Myeloid cells can also become infected with mycobacteria, serving as an intracellular niche in which the bacteria are able to replicate. We sought to establish whether B cells also become infected with BCG and act as an intracellular reservoir for mycobacteria. Using confocal microscopy, it was determined that BCG GFP was adhering to the cell surface of B cells but not being internalised. In a murine model, B cells have been implicated in the dissemination of tuberculosis out of the lungs (Catherine M Bosio et al. 2000). Attachment of mycobacteria to B cells which then migrate elsewhere may provide an explanation for this finding.

This interaction has a profound effect on B cells. Binding to BCG suppresses the expression of numerous markers on B cells. BCG suppresses the expression of CCR7 and CD69 on tonsillar B cells and RAJI cells. This suppression would have a significant impact on the development of B cell responses. CCR7 is an important chemotaxis marker which drives the migration of activated B cells into germinal follicles where they can interact with antigen specific T cells. Without CCR7, activated B

cells linger at the outskirts of germinal follicles and do not appropriately interact with T cells. It has recently been found that antigen specific B cells play an important role in the maturation of T follicular helper cells (T_{FH}) during TB infection. The interaction of antigen specific B cells with T_{FH} cells drives their localisation to the TB granuloma leading to effective control of MTB infection (Swanson R.V. et al 2023). Disruption of B cell function by mycobacteria may be a survival strategy to evade immune control.

CD69 is an activation marker, upregulated following engagement of the BCR. It serves as a chemotaxis receptor. CD69 controls cell migration following B cell activation. It inhibits the movement of activated B cells out of lymphoid tissue. CD69 also plays an important role in cytokine regulation, in T cells, CD69 signalling controls IFN γ and Th17 cytokine production, with CD69 deficiency leading to increased Th1 and Th17 responses. BCG induced suppression of CD69 in B cells may impact the development of antimycobacterial B cell responses and dysregulation of B cell cytokine production.

B cells also play an important role as antigen presenting cells. This function is suppressed by BCG. Expression of HLA DR and CD40 was reduced in the presence of BCG.

B cells internalise, process and present antigenic peptides to CD4 $^{+}$ T cells. This interaction is dependent on histocompatibility complex II molecules like HLA DR. As BCG suppresses the expression of HLA DR by B cells, the presentation of antigenic peptide by B cells to CD4 $^{+}$ T cells will be curtailed. This will impact the development of CD4 $^{+}$ T cells responses which are essential for control of mycobacterial infection. This subversion of B cell antigen presentation represents a form of immune evasion which allows BCG to establish persistent infections. It has previously been established that MTB is capable of suppressing of antigen presentation and MHC II expression in macrophages (Harding, C. V. & Boom, 2010). This study presents for the first time evidence that BCG is capable of suppressing antigen presentation by human B cells.

BCG inhibited HLA DR expression by monocytes from the U937 cell line, as shown in figure 7.18.1. This mirrors a previous finding that *Mycobacterium tuberculosis* suppressed HLA DR expression in

macrophages of the THP1 cell line (Wang, Yue et al., 2005). MTB infection led to increased expression of the corepressor Sin3A, which represses HLA DR expression. This prevented the THP1 macrophages from upregulating HLA DR in response to IFN γ stimulation. It is likely that this same mechanism is responsible for the BCG induced suppression of HLA DR in U937 cells. The tonsil model can be used for further experiments to determine whether BCG suppression of HLA DR in B cells is also caused by upregulation of Sin3A.

In this study it was found that BCG infection decreased CD40 expression on RAJI B cells and tonsillar B cells. Antigen presentation to CD4 $^{+}$ T cells requires CD40 binding to CD40L. This interaction drives the differentiation of germinal centre B cells into centroblasts and their subsequent development (Victora & Nussenzweig, 2012). CD40 / CD40L interactions play an important role in the regulating peripheral B cell tolerance. Deficiencies in CD40 signalling prevent autoreactive B cells from being removed (Hervé et al., 2007).

In a murine model, engaging CD40 with an agonist led to reduced mycobacterial lung burdens in an aerosol challenge experiment (Sia, Bizzell et al., 2017). In addition, the development of protective antigen specific IL-17 responses in TB is dependent on CD40L binding (Sia, Madan-Lala, & Rengarajan, 2017). Suppression of CD40 will therefore profoundly impact the development of B cell responses and T cell responses against mycobacterial infection. MTB infection is known to impair CD40 expression on human dendritic cells (Sia et al., 2017). This study shows that the same suppressive effect is exerted by BCG on human B cells.

The costimulatory molecule CD80 was downregulated on RAJI B cells infected with BCG but upregulated in the U937 monocyte cell line and in tonsillar B cells. CD80 regulates the interactions between T cells and B cells by binding to CD28 or CTLAT-4 on T cells. It is upregulated on activated B cells and binding to CD28 provides a costimulatory signal which activates CD4 $^{+}$ T cells. Binding to CTLAT 4 generates inhibitory signals. Dendritic cells stimulated with BCG upregulate CD80 (Copland et al., 2019) U937 cells and tonsillar B cells stimulated with BCG also upregulate CD80, as seen in

figures 7.14.1 and 7.18.1. Upregulation of CD80 may lead to increased inhibitory signalling via CTLA4 binding. Upregulating CD80 expression on B cells may therefore limit their ability to engage in appropriate antigen presentation and dysregulate the adaptive immune response. This may assist mycobacteria in evading the immune response. Further work will be needed to resolve this question. In RAJI B cells, expression of CD80 was suppressed by BCG. This would deprive B cells of the ability to engage in costimulatory or coinhibitory regulation of T cells.

BCG infection suppressed the proliferation of RAJI B cells and reduced their surface expression of IgM. This indicates that BCG is capable of dysregulating the development of antibody responses. Passive transfer studies in mice have shown that antibodies against mycobacterial antigens confer protection against tuberculosis. High titres of antimycobacterial antibodies reduce susceptibility to infection. Deficits in antimycobacterial antigens are associated with weakened protection against tuberculosis (Achkar et al., 2015).

The fact that BCG inhibited the proliferation of RAJI cells may be clinically significant. RAJI cells are a cancer cell line derived from Burkitts lymphoma. BCG is used as an immunotherapeutic for several forms of cancer (Redelman-Sidi et al., 2014). BCG has been tested as a therapeutic for Burkitts lymphoma, though it did not improve outcomes (Magrath & Ziegler, 1976). An improved understanding of the mechanism by which BCG suppresses proliferation of B cells might be useful for the development of novel anti-cancer treatments.

BCG infection of tonsillar B cells did not suppress IgM expression or proliferation. Instead, surface IgM was upregulated on BCG infected tonsillar B cells. The proliferation marker Ki67 was upregulated as well on these cells. This may represent a recall response in patients already vaccinated with BCG. Although these B cells upregulated IgM and were proliferating, their expression of HLA DR, CD40, CD69 and CCR7 was suppressed.

The finding that BCG suppresses B cell function matches with the known effect of *Mycobacterium tuberculosis* on human B cells. Patients with active tuberculosis have a dysfunctional B cell population.

There are fewer B cells in TB patients than in healthy controls. There is a greater proportion of abnormal and atypical B cells in B cells from TB patients. The B cells from TB patients show reduced proliferation, impaired immunoglobulin production and reduced cytokine production. It was also observed that HLA DR expression was lower on B cells from TB patients (Joosten et al., 2016). These characteristics closely match the suppression of B cells caused by BCG observed in this study.

The role of B cells in TB infection and immunity has not been fully elucidated. B cell deficient mice have been shown to be more susceptible to TB. This increase in tuberculosis infection was not associated with a loss of specific T cell responses (Vordermeier et al., 1996). B cell deficient mice have increased IL-10 expression in their lungs, greater numbers of neutrophils in their lungs and more inflammation. This coincides with increased mycobacterial burdens and mortality (Maglione et al., 2007). This would suggest a protective role for B cells in TB. Conversely, B cells may also play a role in the dissemination of TB and the development of lung pathology. B cell deficient mice have less severe pulmonary granulomas and decreased dissemination to peripheral organs compared with controls.

In humans, active pulmonary TB is associated with B cell deficiency. These patients have fewer peripheral B cells than uninfected or latently infected individuals (Corominas et al., 2004; Hernandez et al., 2010). Antibodies play an important role opsonising and neutralising mycobacteria. IgA responses confer protections against TB, reducing mycobacterial burden. B cells also contribute to the development of T cell responses which are crucial for immune protection against TB. B cells play a role in TB immunity by generating cytokines which co-ordinate the immune response (Loxton A.G. 2019 & Stewart P. et al. 2023). B cells production of IFN γ and IL-12 drives the development of Th1 responses. Regulatory B cells use IL-10 to control autoimmunity and induce Th2 responses (Moulin et al., 2000). IL-10 also drives the polarization of macrophages towards a M2 phenotype (Wong et al., 2010). In the context of TB, regulatory B cells which produce IL-10 are important in preventing immunopathology and they also induce apoptosis of infected cells (Loxton A.G. 2019). This study has shown that BCG suppresses expression of IL-10 by RAJI B cells and tonsillar B cells.

Disrupting B cell development and function enables TB to evade the immune response and promote its own survival (Achkar et al., 2015) (Kozakiewicz et al., 2013). This study reveals that the same phenomenon occurs with BCG and reveals that BCG binds to the surface of B cells via immunoglobulins. The tonsillar model provides a good means to investigate the interactions between mycobacteria and B cells in primary human cells.

The Fc receptor CD32 promotes maturation of APCs and increases antigen presentation (Liu & Janeway Jr, 1992; Medzhitov & Janeway Jr, 2000). This drives the growth of Th1 or Th2 responses (Bandukwala et al., 2007). The expression of CD32 (FcγRII) was reduced in U937 cell line monocytes following infection with BCG. CD32 expression was also reduced on RAJI B cells and tonsillar B cells. Disruption of stimulatory CD32 signalling is associated with suboptimal responses to influenza, leishmania and *S. enterica* (Huber et al., 2001; Kima et al., 2000; Menager et al., 2007; Uppington et al., 2006). In the absence of activatory FcγR signalling, mice have a greater susceptibility to TB and immunopathology driven by excessive production of IL-10 (Maglione et al., 2008).

A previous study from our group used the human monoclonal antibody 2E9 as a treatment for TB. It was found that IgA isotypes of the antibody 2E9 conferred protection against TB. The IgG isotype of the same antibody did not confer protection (Tran et al., 2020). CD32 binds to IgG immune complexes. Downregulation of CD32 by mycobacteria may explain the relative inefficacy of antimycobacterial IgG antibodies compared to IgA antibodies.

Previous studies in humans and in non human primates have noted aggregates of B cells forming in the lungs of TB infected individuals (Tsai, M. C., Chakravarty, Zhu, Xu, Tanaka, Koch, Tufariello, Flynn, & Chan, 2006; Ulrichs et al., 2004). These B cells aggregates are a characteristic part of granulomas (Gonzalez-Juarrero et al., 2001; Tsai, M. C. et al., 2006; Turner et al., 2001). When analysing the confocal images generated in this study, it was noted that similar aggregates of B cells had formed around BCG GFP. In these instances, it was seen that multiple B cells formed a cluster around a central

bacteria. These B cells were adhered to the surface of the bacteria. This observation may provide an insight into the phenomenon of B cell aggregates in the lungs of TB patients.

The mechanism by which BCG suppresses B cell function has not been determined. The mycobacterial antigen PPE18 is known to downregulate proinflammatory responses and inhibit NF- κ B signalling. PPE18 induces the inhibitory cytokine IL-10 and is known to suppress B cell function and downregulate MHC II antigen presentation in mice (Dolasia et al., 2021). PPE18 contributes to increased mycobacterial burden during infection (Bhat et al., 2012). The immunomodulatory effect of PPE18 is driven by IL-10. If PPE18 was responsible for the suppression of B cell function, IL-10 expression would be increased in the presence of BCG. This study instead found that IL-10 expression was suppressed in RAJI cells, tonsillar B cells and U937 cells infected with BCG GFP. It is therefore unlikely that PPE18 is the cause of the observed suppression in B cells. Further studies could be performed using a BCG strain without the *PPE18* gene to determine whether this molecule is responsible for the suppression of B cells.

B cell activation requires two activatory signals. The first signal can be provided by soluble antigen binding to the BCR. The second activatory signal is provided by antigen specific T helper cells. Without this secondary signal, B cells do not develop correctly, but instead enter into a state of anergy. These anergic cells are quiescent and do not proliferate or respond to antigen. In this study, RAJI cells infected with BCG showed suppressed function and proliferation. Tonsillar B cells, which were cultured alongside other tonsillar cells, including T helper cells showed a different pattern of suppression following BCG infection. The tonsillar B cells showed decreased expression of CCR7, CD69, HLA DR, CD40 and CD80 but increased proliferation and IgM expression.

These results might seem to fit a model of B cell anergy induced by soluble antigen – RAJI cells encounter soluble antigen but do not have T helper cell support and so become fully anergic. Tonsillar B cells encounter soluble antigen and have additional signal from a small population of T helper cells, some of them become anergic but cells which receive both signals proliferate and generate IgM.

However, this model cannot explain the suppression of BCG infected U937 cells. These are a monocytic cell line and do not undergo anergy. It was found that BCG infection suppressed U937 cells, reducing CCR7, CD69, HLA DR expression and proliferation. The fact that a similar suppressive effect is seen in U937 and RAJI cells and tonsillar B cells strongly indicates that the suppression of B cells by BCG is not due to an anergic response. Further work will be needed to uncover the mechanism by which BCG and TB suppress and dysregulate B cells.

9. Conclusions

- Based on DC *in vitro* models, Spore FP1, YC NaMa FP1 and Liposome FP1 are all suitable for further development as TB vaccine candidates.
- Spores are immunogenic and can be used in combination with FP1 without further adjuvants.
- It is possible to enhance the antigen specific IFN γ response generated by Spore Poly I:C FP1 with a syngeneic transfer of pulsed dendritic cells.
- Liposome can be coupled with a TLR 5 adjuvant to activate DCs. Hence, TLR 5 agonists may be appropriate adjuvants for a vaccine which uses a liposome delivery system.
- BCG infection induces upregulation of B-7 cell surface markers, PD-L1 and PD-L2 in macrophages and DCs.
- A tonsil binding study showed that D-PIGS and DERICS both bind to human monocytes.
- IgG formulations which contained the protein component J chain did not show any demonstrable binding to TMCs and may require further development.
- The three vaccine candidates, Spore FP1, YC NaMa FP1 and Liposome FP1 have different immunological properties but all generate IFN γ + CD4+ T cell responses in a TMC model.
- Tonsil *in vitro* models showed that IgA ICM in combination with Quil A was immunogenic and associated with IFN γ + CD4+ T cell responses and proliferative responses from CD4+ T cells.
- This study has developed a tonsillar infection model can be used to assess the anti mycobacterial effects of novel vaccines.
- BCG Pasteur and IFN γ were both effective at reducing mycobacterial infection in this model.
- The FP1 vaccine formulations Spore FP1, YC NaMa FP1 and Liposome FP1 also showed anti mycobacterial effects. Nitric oxide production was not responsible for this effect.
- Lymphocyte populations directly interact with BCG
- BCG is able to bind to the surface of B cells and suppress their function.

10. Supplemental tables.

Table 10.1. Antibody panel used for flow cytometry analysis of BMDCs.

Cell Marker	Fluorochrome	Clone	Species	Supplier
CCR7	PerCP Cy5	4B12	Rat	Biolegend
MHC II (I-A/I-E)	BV510	M5/114.15.2	Rat	Biolegend
MHC I	FITC	M1/42	Rat	Biolegend
Viability	APC Cy7		Rat	Biolegend
CD86	PE/ Cy7	GL-1	Rat	Biolegend
CD80	APC	16-10A1	Rat	Biolegend
CD40	PE	1C10	Rat	Biolegend
PD-L1	BV421	10F.9G2	Rat	Biolegend

Table 10.2. TLR agonists tested in combination with vaccine delivery systems

Ligand	Target protein	Concentration
Lipopolysaccharide (LPS)	TLR 4	100 ng/ml
Monophosphoryl lipid A (MPL) (<i>S. enterica</i>)	TLR 4	100 ng/ml
polyinosinic-polycytidylic acid (Poly I:C)	TLR 3 + RIG I	10 µg /ml
Peptidoglycan (<i>B. subtilis</i>)	TLR 1 + TLR 2 + NOD 2	1 µg /ml
CPG DNA	TLR 9	1 µM
Flagellin	TLR 5	1 µg /ml

Table 10.3. Antibody panel used for antigen presentation and T cell proliferation assay

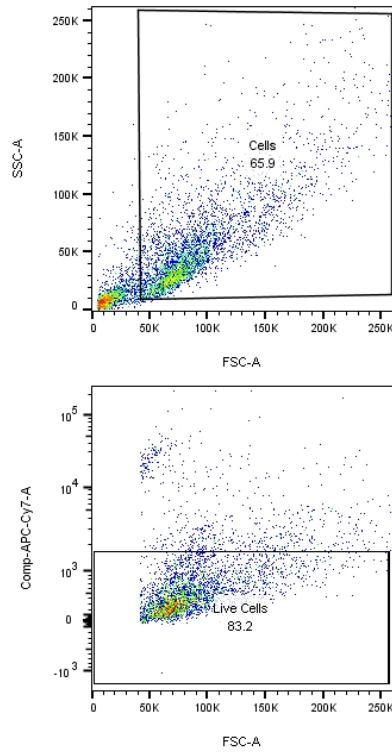
Cell Marker	Fluorochrome	Clone	Species	Supplier
Dead Cells	APC – Cy7	N/A	N/A	ThermoFisher
Ki67	APC	16A8	Rat	Biolegend
CD8	BV510	53.6.7	Rat	Biolegend
CD90.2	BV421	30H12	Rat	Biolegend
CD4	PerCP Cy5	GK 1.5	N/A	Biolegend

Table 10.4. Antibody panel used for BCG B7 family study

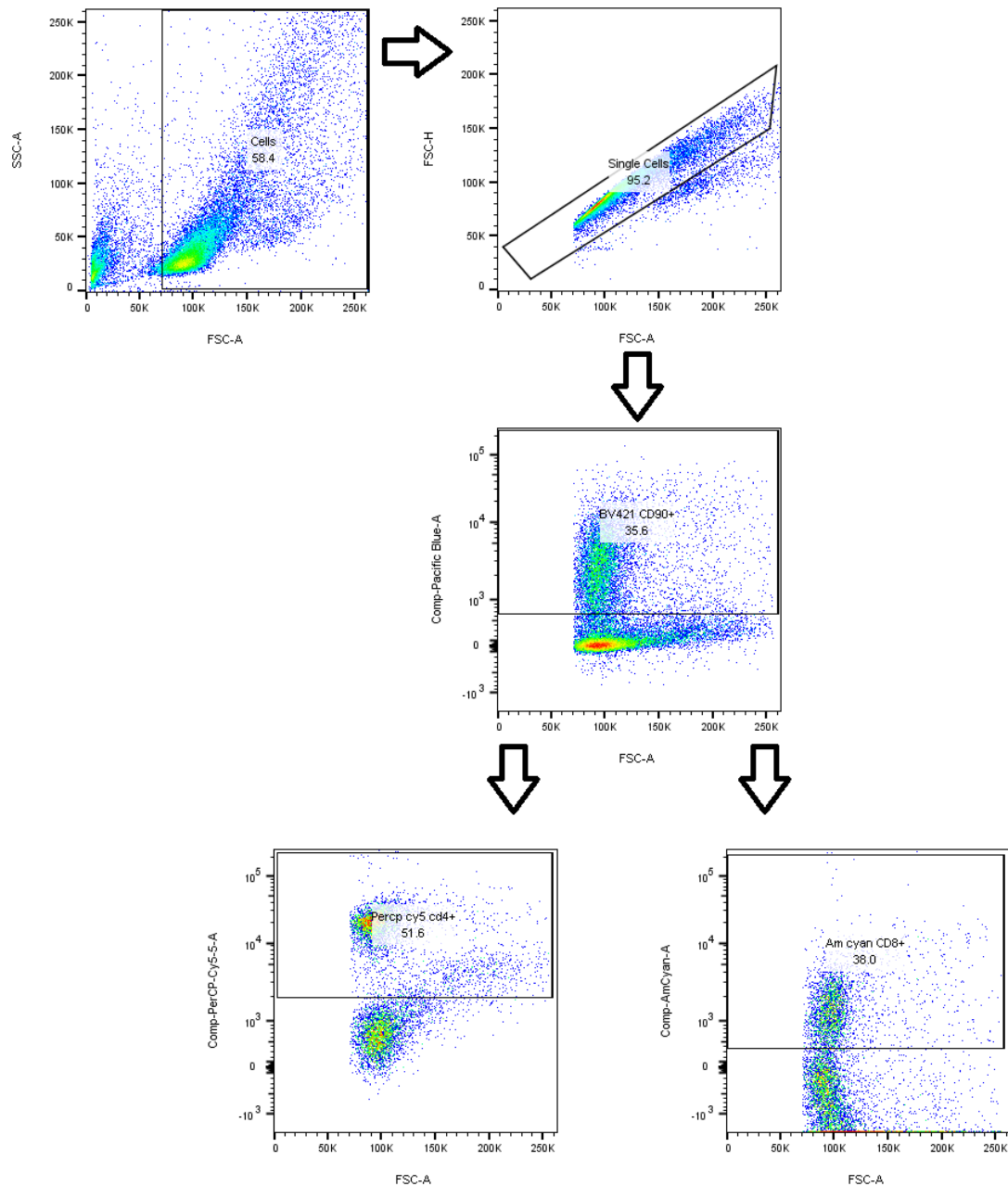
Cell Marker	Fluorochrome	Clone	Species	Supplier
Dead Cells	APC – Cy7	N/A	N/A	ThermoFisher
CD80	APC	16-10A1	Hamster	Biolegend
PD-L2	PE	TY25	Rat	Biolegend
PD-L1	BV421	10F.9G2	Rat	Biolegend
CD86	PE Cy7	GL-1	Rat	Biolegend

Table 10.5. Human T Cell Proliferation and Cytokine production panel

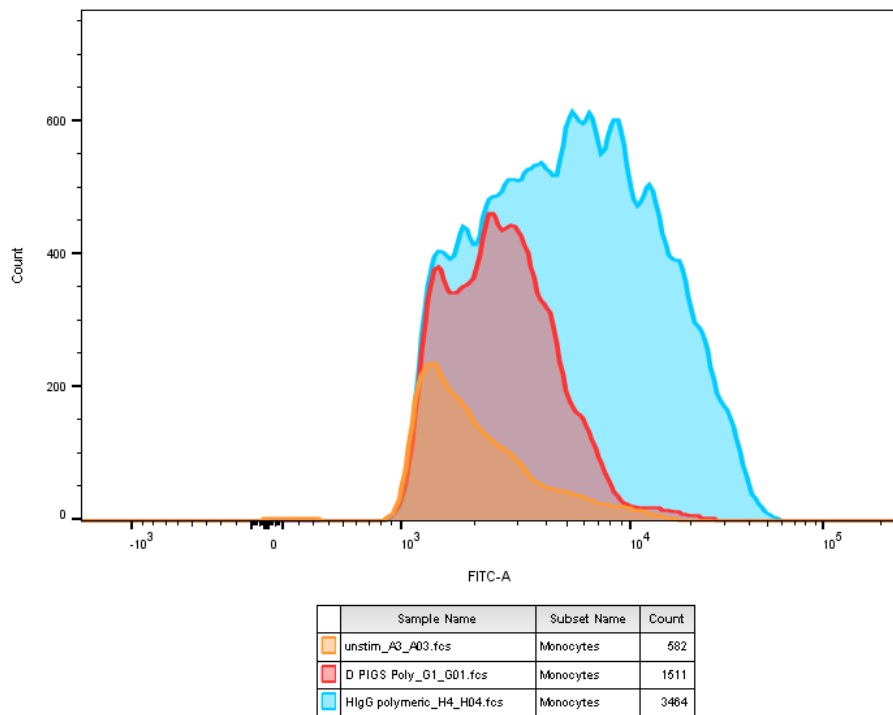
T cell marker	Clone	Fluorochrome	Biological relevance
CD3	SP 34.2	FITC	Pan T cell marker
CD4	OKT4	PerCP Cy5.5	T helper cell marker
CD8	SK1	BV510	Cytotoxic T cell marker
Ki67	B56	APC	Proliferation
IL-2	N/A	PE	Proliferative cytokine
IFN γ	B27	BV421	Th1 cytokine
IL-17	BL-168	PE Cy7	Inflammatory cytokine
Viable cells	E fluor 780	APC Cy7	Viable cells



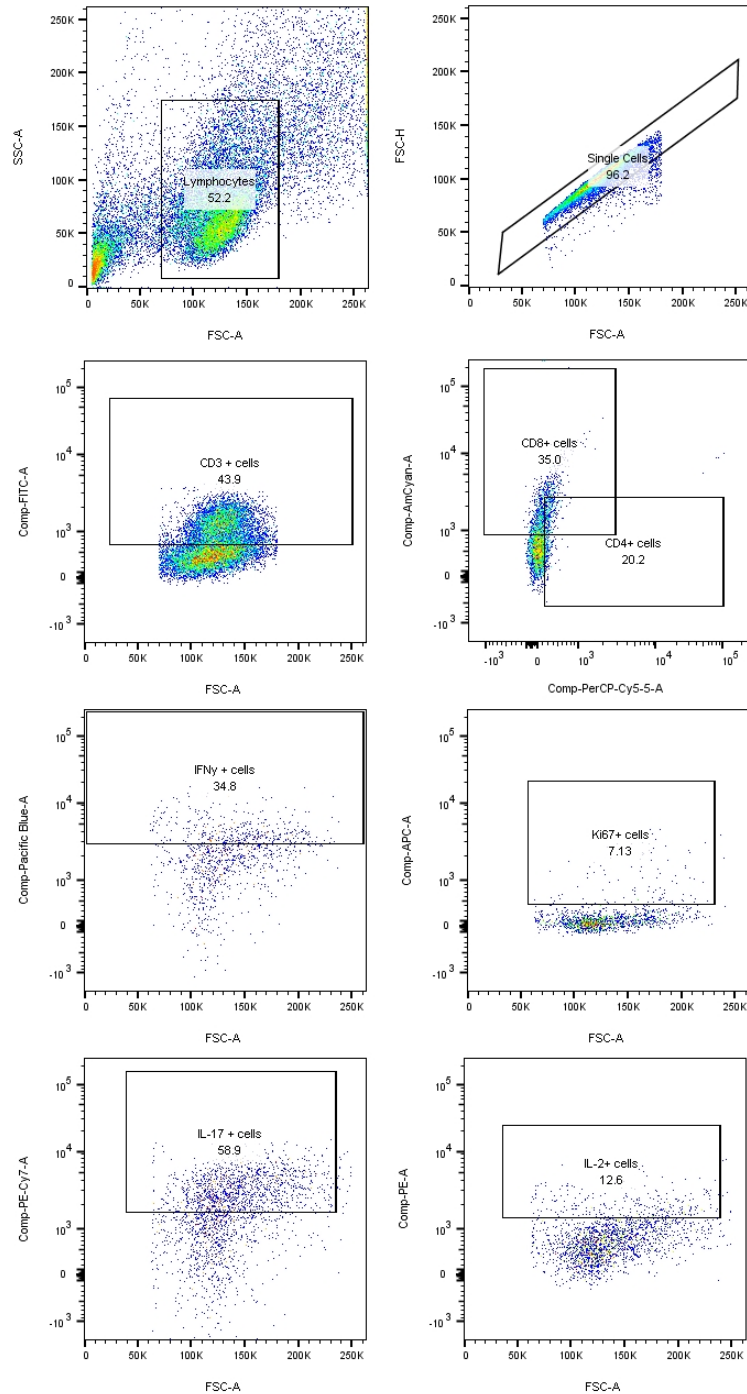
Supplemental figure 10.1. DC gating plan. DC cultures were gated by selecting populations of cells using forward scatter and side scatter. APC-Cy7 negative cells were viable. MFI values were used to measure cell surface marker expression on these viable DCs. Graphs show representative gating plan.



Supplemental figure 10.2. Murine splenocyte gating plan. Populations of single T cells were identified by forward and side scatter. Fluorescence minus one (FMO) controls were used for gating CD90+ T cell populations and CD4+ and CD8+ T cell subpopulations. Pacific Blue (BV421) positive cells were CD90+ T cells. Of these, PerCPCy5.5 positive cells were CD4+ T cells and Amcyan (BV510) positive cells were CD8+ T cells. Proliferation of these cells was measured by mean fluorescence of APC, which was bound to an anti Ki67 antibody.



Supplemental figure 10.3. Representative histogram of binding study data. Binding of polymeric IgG vaccine formulations to human monocytes was determined using a FITC conjugated anti human IgG antibody. Flow cytometry was performed and FITC fluorescence measured. This histogram shows the fluorescence of cells incubated with heat aggregated human IgG or D-PIGs in comparison with unstimulated control cells.



Supplemental figure 10.4. TMC gating plan. Populations of single lymphocytes were gated using forward scatter and side scatter. Fluorescence minus one (FMO) controls were used to set subsequent gates. CD3+ cells were selected by gating for FITC positive cells. CD4+ and CD8+ sub-populations were gated using PerCPCy5.5 and Amcyan gates. These were then gated for cells which were positive for IFN γ (Pacific Blue), Ki67 (APC), IL-17 (PE-Cy7) and IL-2 (PE). This gating plan is depicted here.

11. References

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