EXPLOITING THE POTENTIAL OF INKT CELLS TO IMPROVE VACCINATION

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ABSTRACT

Invariant (i)NKT cells are a subset of innate-like T cells that recognize glycolipids (such as α-galactosylceramide; α-GalCer) presented by the non-polymorphic MHC I-like molecule CD1d. The known specificity of iNKT cells combined with their capacity to secrete vast amounts of cytokine following activation makes them a candidate for manipulation to potentiate immune responses following vaccination. FliC is the fundamental building block of the flagella of *Salmonella Typhimurium* (STm) which has been shown to have potential as a vaccine due to it acting as its own adjuvant following recognition by TLR5. Indeed, FliC vaccination has been shown to generate both T and B cell responses to itself and confer a degree of protection from subsequent ST infection in mouse models.

We therefore sought to examine whether the coincident activation of iNKT cells would boost the immune response seen on FliC administration. To this end, mice were co-immunised with FliC and α-GalCer and the dynamics of the iNKT cell response, the conventional T cell response and the B cell/antibody response to FliC was compared to those in mice that received FliC immunisation only. we found that iNKT cell activation coincident with FliC vaccination boosts immunity following FliC vaccination by enhancing Ab production, increasing the number of Ab-secreting cells and boosting the GC reaction.

Strikingly, for optimal immunity following immunisation with FliC both TLR5 signalling and iNKT cells are required. However, the activation of iNKT cells could partially rescue Ab responses to FliC in the absence of TLR5 and the loss of NKT cells dramatically impacts the success of FliC immunisation. The use of an IFNγ-inducing glycolipid such as C-glycoside resulted in an even more robust adjuvant effect with a further increased the titre and longevity of serum IgG2C and IgA Ab over and above that seen with α-GalCer. Taken together the concomitant activation of iNKT cells can improve the efficacy of vaccines.

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ABBREVIATIONS

α-GalCer	α-Galactosylceramide
β2M	Beta 2 microglobulin
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
AMPs	Antimicrobial peptides
AP-1	Activator protein 1
APC	Antigen-presenting cells
B6	C57BL/6
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra large
BCR	B cell receptors
BFA	Brefeldin A
BLIMP-1	B-lymphocyte-induced maturation protein 1
BM	Bone marrow
BMSU	Biomedical services unit
BSA	Bovine serum albumin
CCR	Chemokine receptor
CD	Cluster of differentiation
CDRs	Complementarity regions
CGG	Complexes of NP-chicken gamma globulin
CLIP	Class II-associated invariant chain peptide
CLPs	Common lymphoid progenitors
CLRs	C-type lectin-like receptors
CMV	Cytomegalovirus
CO_2	Carbon dioxide
cRPMI	Complete Roswell Park Memorial Institute Media
cTECs	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
CXCL	C-X-C motif ligand

DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
DZ	Dark zone
EDTA	Ethylenediaminetetraacetic acid
EILPs	Early innate lymphoid progenitors
ELISAs	enzyme-linked immunosorbent assays
Erg	ETS-related gene
ETPs	early T lymphocytes progenitors
ER	endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FAS-L	FAS-Ligand
FCS	Fetal calf serum
FL	Fetal liver
FMO	Fluorescence minus one
FOXP3	forkhead fox P3
GATA3	GATA Binding Protein 3
GC	germinal centre
GF	Germ-free
GFP	green fluorescent protein
GI	gastrointestinal
HBSS	Hanks' Balanced Salt Solution
HBV	hepatitis B virus
HEB	E protein transcription factor
HIV	Human immune deficiency virus
HLA	Human leukocytes antigen
Hour	h
HSCs	Hematopoietic stem cells
i.p.	intraperitoneal
i.v.	Intravenous

IC	intracellular
Ii	invariant chain
Ig	immunoglobulin
IFN	Interferon
IL	Interleukin
IL-12r	IL-12 receptor
IM	inner membrane
iNTS	invasive NTS
iNKT cells	invariant Natural Killer T cells
ISP	immature single positive
KO	knock-out
LAG-3	Lymphocyte activation gene 3
LEF1	Lymphoid Enhancer Factor 1
LNs	lymph nodes
LPS	lipopolysaccharide
LZ	light zone
mAbs	Monoclonal antibodies
MFI Median	Fluorescence intensity
MHC	major histocompatibility complex
min	minutes
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
mTEC	medullary thymic epithelial cells
MZ	marginal zone
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKR	Natural killer receptor
NKT cells	Natural killer T cells
NKTFH	Follicular helper Natural Killer T
NLRs	Node like receptors
NO	nitric oxide
NOD	non-obese diabetic

NTS	Non-typhoidal salmonellosis
OCH	2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-N-tetracosanoyl-2-amino-1,3,4-nonanetriol
OM	outer membrane
OMPs	outer membrane proteins
PAMPs	pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PD1	Programmed cell death protein 1
PDL-1	Programmed cell death protein ligand 1
PLZF	promyelocytic leukaemia zinc finger
PRRs	pattern recognition receptors
RLRs	RIG-I-like receptors
RNI	reactive nitrogen intermediates
RORγt	retinoic acid receptor-related orphan receptor gamma thymus
ROS	reactive oxygen intermediates
RPMI	Roswell park memorial institute
RT	Room temperature
SAP	signalling lymphocytic-activation molecule -associated protein
SCVs	*Salmonella*-containing vacuoles
sec	seconds
SFUs	spot-forming units
SHM	somatic hypermutation
SI	Small intestine
SI-LP	Small intestine – lamina propria
SLAM	signalling lymphocytic-activation molecule
SLTs	Secondary lymphoid tissues
SPF	Specific pathogen free
SPI-1	*Salmonella* pathogenicity island 1
SEn	*Salmonella* Enteritidis
ST	*Salmonella* enterica serovar Typhi
STm	Salmonella enterica serovar Typhimurium

sFliC	Soluble recombinant Flagellin
STAT	signal transducer of activation
T3SS	type-3 secretory system
TAP	transport associated with antigen processing
T-bet	T-box expressed in T cells
TBM	tingible body macrophages
TCF1	T Cell Factor 1
Tcon	conventional T cells
TCR	T Cell Receptor
T Cell	Thymus Cell
TD	Thymus-Dependent
TGFβ	Transforming Growth Factor β
Th	T-Helper Cell
Th 1	T-Helper Cell 1
Th 2	T-Helper Cell 2
Th 17	T-Helper Cell 17
Th-POK	Th Poxviruses and zinc finger and Kruppel
TI	Thymus-Independent
TLR	Toll Like Receptor
TLR5	Toll Like Receptor 5
TNF	Tumour necrosis factor
Treg	Regulatory T cells
VDJ	variable, diverse and joining
WT	wild type

CHAPTER 1: GERERAL INTRODUCTION

1.1. The Immune System

The immune system is well known to be a host defence that can protect many biological organisms including animals, plants and mammals against infectious diseases. The immune system recognises and fights harmful agents known as pathogens such as viruses, bacteria, fungi and parasites and also serves to maintain homeostasis. The immune system is divided in two general arms known as innate and adaptive immune system.

1.2. The Innate Immune System

The innate immune system consists of cellular or soluble factors. The cellular arm comprises cell types such as eosinophils, neutrophils, macrophages, natural killer cells and dendritic cells (DC). These cells express pattern recognition receptors (PRRs) on their surface and recognize conserved molecular motifs shared amongst many potential pathogens called pathogen associated molecular patterns (PAMPs) (1). PAMP recognition by PRRs can trigger a cascade of cell responses to aid the clearance of any pathogenic products that enter the host.

1.2.1. Soluble Pattern Recognition Receptors

PRRs can either be soluble or cellular. Soluble PRRs circulate in plasma and recognize PAMPs on the surface of microbes and aid in their elimination by coating their surface in a process called opsonisation. This process involves the recognition of PRRs bound to pathogens by innate immune cells that express complementary receptors to the PRRs. Subsequently, innate immune cells such as neutrophils and macrophages initiate a process of phagocytosis whereby the microbe is taken into the cells in endosomes called phagolysosomes where it is destroyed (2). Soluble PRRs are composed of different molecular families such as collectins (Mannose Binding Lectin), pentraxins (C-reactive protein) and plasma complement (C3b) (1).

1.2.2. Cellular Pattern Recognition Receptors

The best characterised families of cellular PRRs are Toll-like receptors (TLR), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin-like receptors (CLRs). TLRs

consist of nine receptors: TLR1 to TLR9 that recognise different common structures found on families of pathogens (3). For example, the endotoxin Lipopolysaccharide (LPS) is recognised by TLR4 and TLR5 has an affinity for flagellin (4). Other TLRs (TLR3, TLR7 and TLR9) are expressed internally within endosomes, mainly recognising non-host nucleic acid such as double stranded RNA and CpG DNA (5).

In addition to cell surface and endosome associated TLR molecules, NOD-like receptors locate to the cytosol and detect bacterial PAMPs such as cell wall motifs, along with RIG-I like receptors that recognise RNA PAMPs (6, 7). Upon activation, these receptors initiate cell signalling and subsequent pro-inflammatory immune responses that eradicate and eliminate pathogens (Figure 1.1).

PRR triggering leads to downstream activation signals associated with the induction of inflammation resulting in the release of chemokines and cytokines which can recruit and activated innate cell that have homed to the infected area (8). During acute inflammation the phagocytosis of pathogens mediated by neutrophils plays a critical role in this process. This is possible as FC receptor or complement receptors on the surface of neutrophils can bind to an antibody or complement opsonised bacteria in which neutrophils can ingest and kill invading microbes (9, 10). Thereafter, macrophages enter the infected site where they remove dead cells and foreign bodies through phagocytosis (11). However, the other main role of the innate immune system is to activate the adaptive immunity via cytokine or chemokine release.

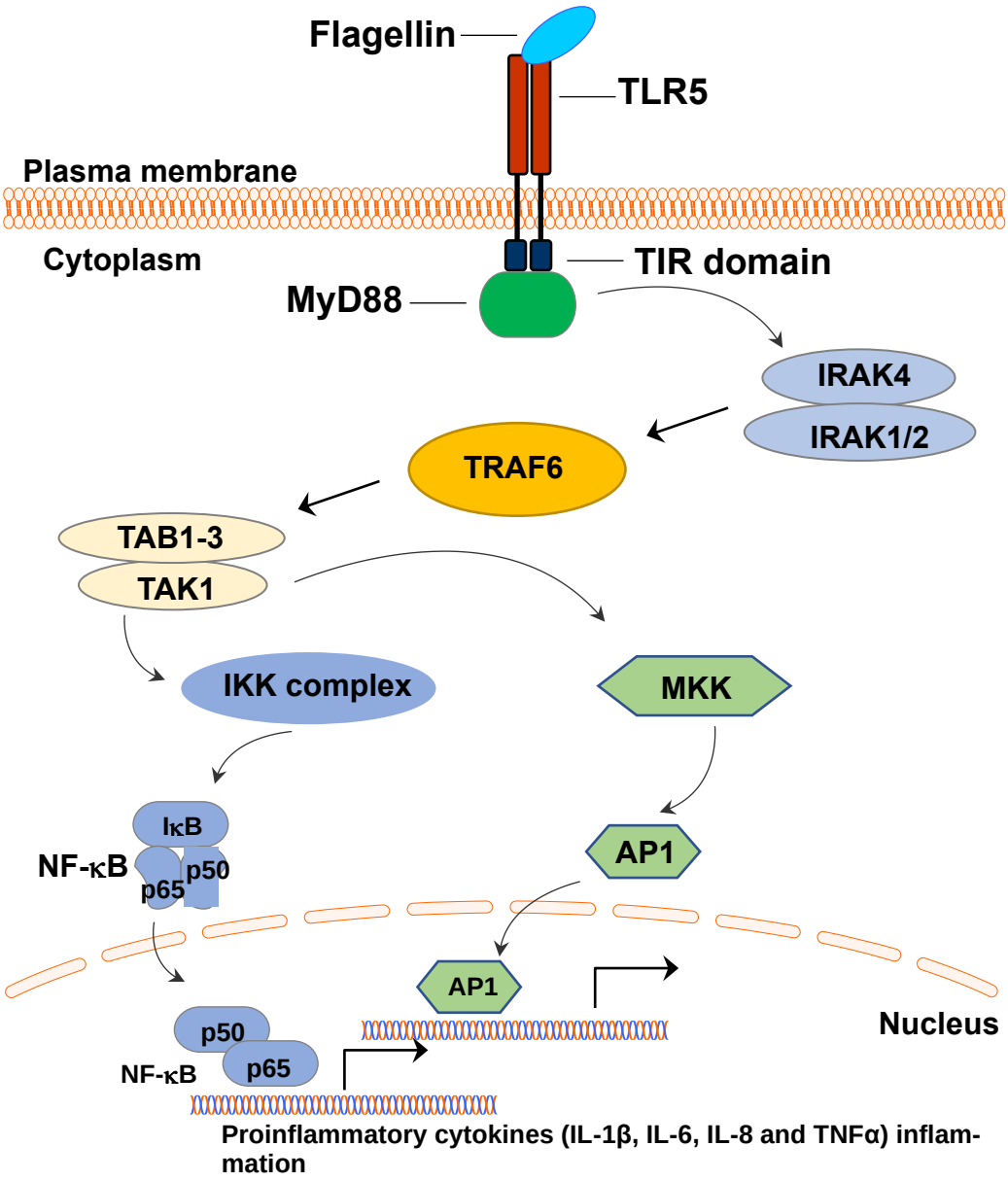

Figure 1.1 TLR5 signalling pathway.
Since TLR5 binds to Flagellin, uses MyD88-dependent pathway to start signals. MyD88 initiates signals and interacts with interleukin 1 receptor associated kinase 4 (IRAK-4). Then IRAK-4 phosphorylates to IRAK-1 and IRAK-2, results in activation of TNF receptor associated factor 6 (TRAF6). Once TRAF6 get activated lead to activate TGF-β-activated kinase 1 (TAK1) by TAK1-binding proteins (TAB1-3). TAK1 via MKK and IKK pathways can activate both AP1 and NF-κB. Together NF-κB and AP1 requires to induce the transcription of proinflammatory cytokines, results in IL-1β, IL-8, IL-6 and TNFα production.

1.2.3. Dendritic Cells (DCs) as link between the innate and adaptive response

All nucleated cells express MHC class I and present peptides derived from the proteins they synthesised. These cells are known as non-professional antigen presenting cells. However, B cells, macrophages and DCs are known as professional antigen-presenting cells (APC). In particular, DC are specialised in processing and presenting exogenously acquired antigen in the context of MHC class II (Table 1.1). Naïve $CD4^+$ T cells are activated predominantly following encounter with antigen on DCs therefore DCs are the most important APCs in generating primary immune responses (12). Immature DCs reside in non-lymphoid tissues and become activated by cytokines produced by innate immune cells and directly through triggering of their PRRs by PAMPs. This results in the processing and presentation of pathogen-derived peptides on MHCII complexes on the cell surface together with the upregulation of co-stimulatory molecules CD80/CD86 (13-15). Activated DCs also upregulate the expression of CCR7 to migrate to T-zone in spleen or lymph nodes via the binding of CCL21 or CCL19, in order to prime T cells and initiate the cell-mediated immune response (16, 17).

Table 1.1 Conventional dendritic cell subsets

DC subset	Location	Surface Marker
cDC1	Lamina propria small intestine/ Mesenteric lymph nodes	$CD11c^+$ $MHCII^+$ $CD103^+$ $CD11b^-$
cDC2	Lamina propria small intestine/ Mesenteric lymph nodes	$CD11c^+$ $MHCII^+$ $CD103^+$ $CD11b^+$
$CD103^-$ DC	Lamina propria small intestine/ Mesenteric lymph nodes	$CD11c^+$ $MHCII^+$ $CD103^-$ $CD11b^+$
Splenic DC1	Spleen	$CD11c^+$ $MHCII^+$ $CD4^-$ $CD8^+$
Splenic DC2	Spleen	$CD11c^+$ $MHCII^+$ $CD4^+$ $CD8^-$

1.3. The Adaptive Immune System

In addition to the innate system, the other arm of immunity is adaptive immunity that is dependent on highly specific antigen recognition. Adaptive immunity is diverse in terms of pathogen specificity due to its receptors (T cell receptors (TCR) and B cell receptors (BCR)) being generated by somatic genetic rearrangement of numerous V, D and J gene segments to form the complete α and β chains of the TCR and the heavy and light chains of antibodies/BCR. In addition to specificity, T and B cell responses also differ from innate immune responses as they have the capacity to develop memory for the encountered pathogen-derives antigen and are thus able to mediate a more aggressive response on re-encounter with the antigen.

1.3.1. Major histocompatibility complex

Unlike B cells/antibodies, T cells only recognise foreign antigen in the form of short linear peptides presented by major histocompatibility complex (MHC) class I or class II molecules. Human MHC antigens are also known as human leukocytes antigen (HLA) and the loci consists of more than 200 genes located on chromosome 6 (18, 19). HLA class I is composed of three genes HLA-A, HLA-B and HLA-C and HLA class II consists of HLA-DR, HLA-DQ and HLA-DP (20).

In mice the MHC locus is known as H-2 and is located on chromosome 17 (21). The H-2 complex is classified as being either class I or class II genes depending on their structure and both class I or class II subdivided to classical and non-classical genes (22).

MHC class I is expressed by all nucleated cells and is comprised of one membrane-spanning α-chain (heavy chain) and β-microglobulin (light chain). The α-chain has three subunits called α1, α2 and α3 where α2 and α1 together shape the peptide-binding groove and α3 harbours the CD8 co- stimulatory binding groove. MHC class II, the expression of which is largely restricted to APCs, consists of membrane-spanning α- and β- chains. The α- and β- chains each have two subunits called α1 and α2 and β1 and β2 respectively. The subunits α1 and β1 shape the peptide-

binding groove where the subunit β2 contains the CD4 co-stimulatory binding site (Figure 1.2) (23, 24). MHC molecules play a pivotal role in presenting processed peptide antigens to T cells.

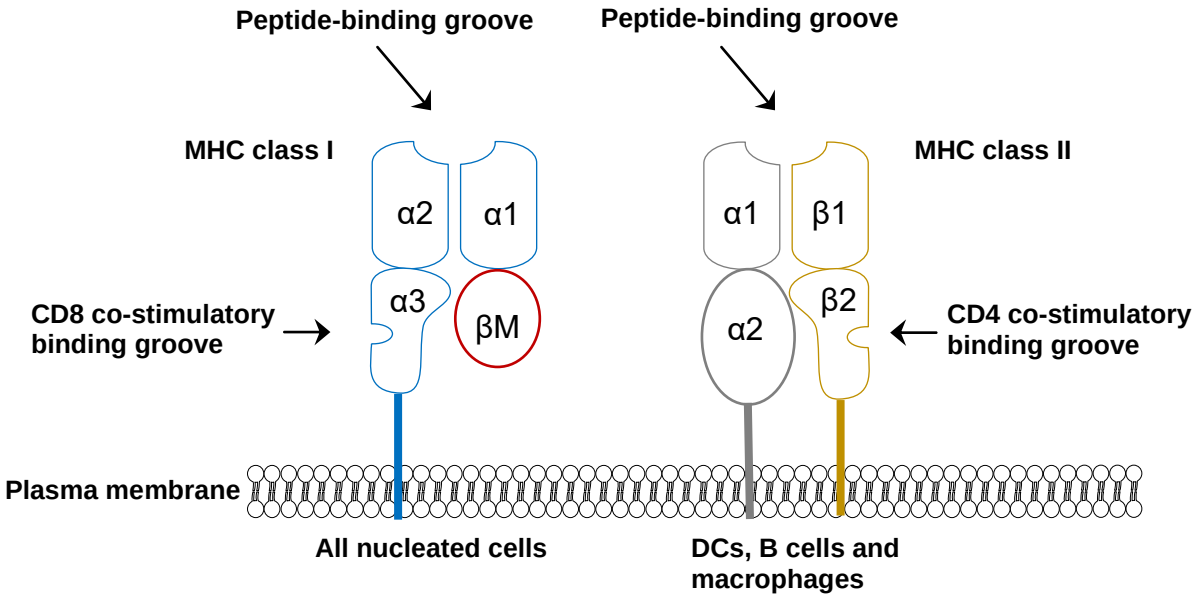

Figure 1.2 MHC Class I and MHC Class II structures.
MHC class I is expressed by all nucleated cells and MHC class II is largely restricted to APCs. In MHCI, α2 and α1 together shapes the peptide-binding groove and α3 shapes CD8 co-stimulatory binding groove. However, in MHCII the subunits α1 and β1 shape the peptide-binding groove where the subunit β2 shape the CD4 co-stimulatory binding groove.

1.3.2. T cell development and maturation

T cells initially originate in the bone marrow as early T lymphocytes progenitors (ETPs) before they migrate to the thymus and differentiate to commit to the T cell lineage. Once they enter the thymus, these progenitor cells called thymocytes, undergo a series of maturation steps which can be delineated according to the expression of different surface markers (25, 26).

The earliest developing ETPs, which lack the expression of CD25 and co-receptors CD4 and CD8, but express CD44, are known as double negative; (DN)-ETPs (27). Then the DN-ETP population undergoes four stages of differentiation known as DN1, DN2, DN3 and DN4. DN2 thymocytes are $CD4^{-}CD8^{-}CD25^{+}CD44^{+}$ cells that undergo several proliferative steps to develop into DN3 ($CD4^{-}CD8^{-}CD25^{+}CD44^{-}$) (28). At the DN3 stage proliferation is halted and cells undergo β-chain selection where cells that have rearranged a TCRβ chain successfully further differentiate and rearrange the TCRα chain and expresses both CD4 and CD8, so-called double-positive (DP) cells. Cells that don't successfully rearrange their TCR β-chain die through apoptosis. It is at this stage that DP thymocytes undergo positive selection where the TCR is tested for reactivity to self-peptide/MHC complexed (26-28).

The process of positive selection takes place in the cortex where immature DP thymocytes interact with self-antigens presented by cortical thymic epithelial cells (cTECs) in the context of MHC class I or MHC class II molecules. Cells that receive a very strong signal die by apoptosis while those that bear a TCR with too low an affinity for peptide/MHC die via neglect. Thymocytes that interact with self-antigen/MHC class I retain CD8 expression and down-regulate CD4 whilst thymocytes bearing a TCR with affinity for self-antigen presented by MHC class II retain CD4 expression and downregulate CD8 (29, 30). Post positive selection, thymocytes then upregulate the expression of CCR7 allowing them to enter the medulla to be exposed to the process of negative selection (29-31).

During negative selection, autoreactive T cells are eliminated in the medulla before they migrate to the periphery. This process takes place when medullary thymic epithelial cells (mTEC) express and present tissue specific antigens to thymocytes via the transcription factor Aire (autoimmune regulator) and the more recently identified, Fezf2 (32, 33). Perihperally-derived self-antigen is also presented to thymocytes by circulating DC n the medulla. Thymocytes with TCR that show high affinity for self-antigen/MHC are instructed to die by apoptosis whereas low affinity engagement of TCR by self-peptide/MHC allows cells survival and maturation before thymic export to the periphery (34-36). FOXP3 Treg also differentiate in the thymus in response to medium affinity TCR engagement to self-antigen presented by mTEC (37, 38).

1.3.3. Antigen Presentation for T Cell recognition

As mentioned earlier, CD4 and CD8 T cells recognise peptide antigen presented in MHC class II or MHC class I molecules. However, the mechanisms of processing and deriving the peptides for presentation differ between MHC class I and II as peptides derived from the extra-cellular compartment are preferentially loaded onto MHC class II whereas endogenous peptides are typically loaded onto MHC class I.

MHC class II is produced and assembled in the endoplasmic reticulum (ER) where it's peptide binding groove is blocked by a non-polymorphic protein known as the invariant chain (Ii). This led to prevent inappropriate activation and direct the complex through antigenic motifs towards the endosomal–lysosomal antigen processing compartments (39, 40). In this process, Ii–MHC class II complexes are transported to endosomes where the invariant chain is degraded leaving class II associated invariant chain (CLIP) peptide. This peptide remains in the binding and enables loading of lysosomally produced peptides upon disaggregation by HLA-DM (41). In this way peptides that have been acquired from extracellular sources via endocytosis and phagocytosis are loaded onto MHC class II (42, 43). Peptide-MHC class II complexes are then transported to the cell surface for presentation to CD4 T cells (Figure 1.3 A).

MHC class I presents endogenous peptides when host cells are infected with intracellular pathogens such as bacteria or virus. This mechanism of endogenous peptide presentation identifies infected or cancerous cells to be destroyed by CD8 cytotoxic T cells (44). Proteins derived from endogenous pathogens are degraded by proteasomes into peptides which are transported into the endoplasmic reticulum via the molecule transport associated with antigen processing (TAP).

TAP is also required to load such cytosolic peptides onto MHC class I molecules also generated in the ER (45). This intracellular translocation requires the formation of a peptide complex containing tapasin and another chaperone complex which is transferred peptides from ER to the MHC class I molecule, resulting in MHC class I molecule expression on the surface of infected cells (Figure 1.3 B) (39).

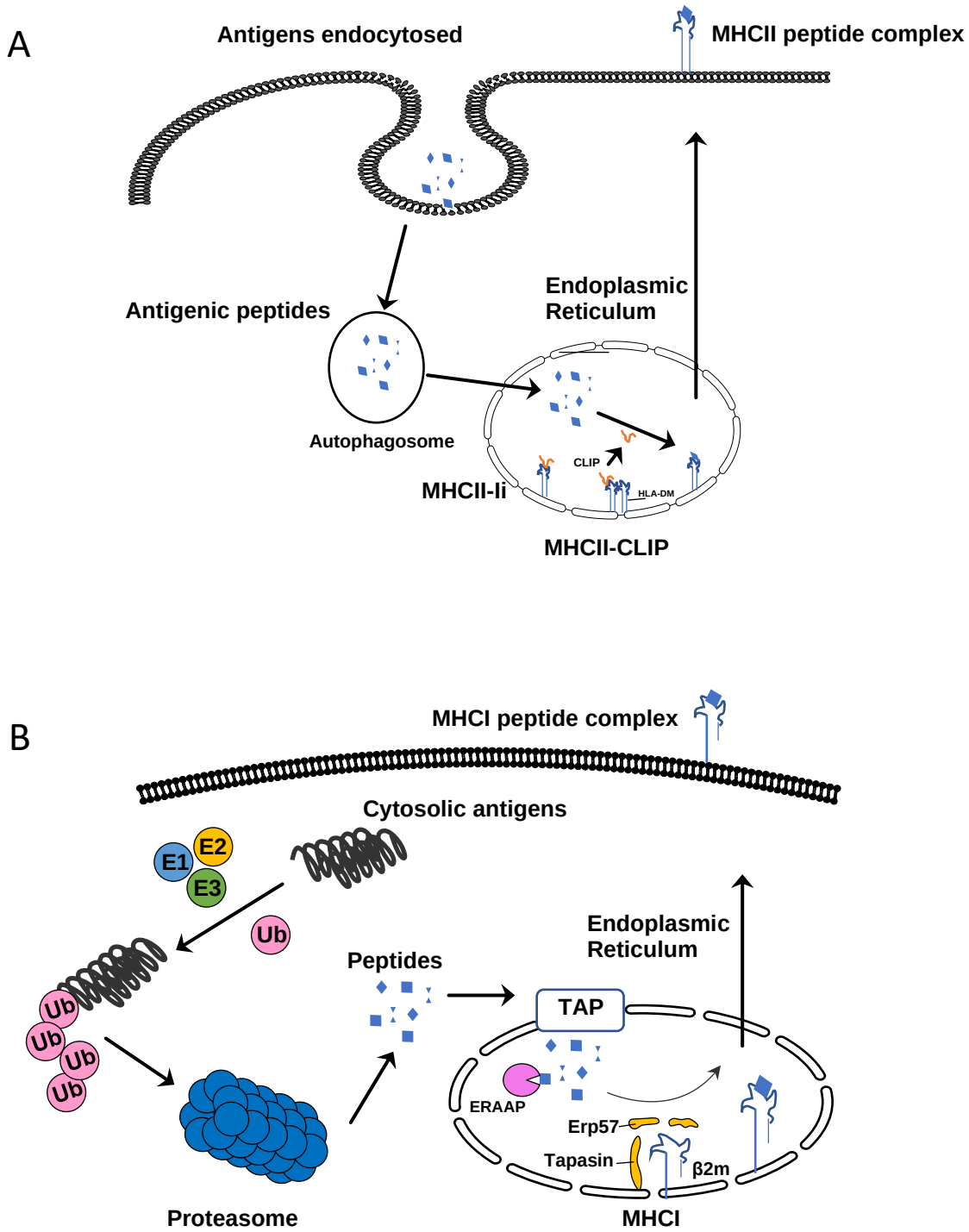

Figure 1.3 The process of production peptides for MHC class I and MHC class II presentation. A) MHC class II molecules present peptides derived from antigens that have been taken up from extracellular sources through APCs receptors mediated via the processes of endocytosis. Antigens can be taken to the endoplasmic reticulum where the production of newly synthesised MHC II molecules take place through antigenic peptides process. In endoplasmic reticulum, MHC II molecules can be then loaded with peptides via support of CLIP after degradation of invariant chain that is bound to MHCII. Then newly peptide-MHC class II complex are loaded and transported on the surface of the APCs for presentation to naïve CD4 T cells. B) MHC class I molecules present peptides derived from antigens endogenously for presentation. Endogenous antigens degrade via proteasomes into peptides and transports for MHCI loading in the endoplasmic reticulum via the molecule TAP. Then newly peptide-MHC class I complex are loaded and transported on the surface of the nucleated cells for presentation.

1.3.4. T Cell Activation

Naïve T cells require three distinct signals to be optimally activated. The first signal is generated when T cells recognise peptide presented in the context of MHC molecules. The second signal occurs on accessory/costimulatory molecule binding such as ligation of CD28 by it's ligands CD80/86 expressed by activated APCs. Indeed, CD28 expressed by T cells is the best characterised and arguably the most important co-stimulatory molecule for T cell activation (46-49). Signal 3 is provided by cytokines that direct the differentiation of naïve T cells into effector cell subsets with different functionality such as Th1 or Th17.

1.3.5. T Cell Subsets

The main T cell populations that are generated in the thymus are composed of either αβ T cells or γδ T cells. In the periphery, these two populations are known to function and localise differently. αβ T cells are located mainly in SLO such as lymph nodes or spleen whereas γδ T cells are localised in the epithelial layers of non-lymphoid tissues such as skin and small intestine (50). γδ T cell receptors appear to recognize products of cellular stress associated with infection, tumorigenesis or disease, while αβ T cells are dependent on APCs to be activated (51). There is some evidence that γδ T cells can produce cytotoxic granzymes through via TLR triggering, which can aid the clearance of pathogens (52, 53) and γδ T cells are phenotypically similar to innate immune cells as they participate in early immune response (54).

In the case αβ T cells, these cells are formed of 2 major subsets divided according to their expression of $CD4^+$ or $CD8^+$.

1.3.5.1. $CD4^+$ T Cell Subsets

As mentioned earlier, following the recognition of cognate peptides presented by APCs and the interaction between CD80/CD86 on APCs and CD28 on T-cells, providing a supportive level of co-stimulatory signals results in T cells activation. $CD4^+$ T cells differentiate into effector cell subsets such as T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17). The phenotype

of these effector $CD4^+$ T cells are usually characterised based on the expression of signature transcription factors and cytokines.

Th1 cells that express the transcription factor T-bet preferentially differentiate in microenvironments rich in IL-12, IL-28 and IFN-γ (55). Th1 cells produce proinflammatory cytokines such as IFN-γ and TNFα (in addition to IL-2) which facilitates the destruction of intracellular pathogens (56). In mice, IFN-γ secretion promote and induce B cells via T-dependant immunoglobulin to class switch toward IgG2c/IgG2a (57). In addition, IFN-γ production by Th1 cells can facilitate the clearance of protozoan infections and resolution of viral. Indeed, the production of IFN-γ can activate M1 macrophage activation, antimicrobial factor and other professional phagocytes. This leads to promote bactericidal activity of Salmonella-infected macrophages, resulting in enhanced secretion of ROI and induction of phagosome-lysosome fusion (58). Definitely, lack of IFN-γ signalling pathway leads to inability of clear infection, resulting in non-Typhi Salmonella and pathogenic mycobacteria (59).

Th2 cells are defined by the expression of the GATA-3 transcription factor. Th2 cells mainly secrete cytokines such as IL-4, IL-5, IL-10 and IL-13. Naïve T cells exposed to IL-4 during activation preferentially express GATA-3 which in turn leads to the expression of IL-4 and IL-5 to support the B cell class switching antibody and promote immunity against helminths and extracellular parasites (60, 61). B cell class switching antibody associated with Th2 responses are IgG1 and IgE (57). Th2 responses are characterized by induction of activation of mast cells, alternative activated macrophages in addition to tissue infiltration through the presence of eosinophils and basophils (62). Therefore, these responses regulate physiological changes as a consequence of asthma such as airway mucus hypersecretion, airway hyper-responsiveness and inflammation (63).

Th17 cells express the retinoic acid receptor-related orphan receptor gamma (RORγt) transcription factor and develop following T cell activation in the presence of Transforming Growth Factor β (TGFβ) and IL-6 leading to the production of IL-17, IL-22 and IL-6. Th17 cells protect against extracellular bacteria at mucosal surfaces and fungi destruction (64-66). Th17 cells are also associated specifically against *Salmonella* via producing IL-17 and IL-22 in gut post-infection, however IL-17A deficient mice showed increases of bacterial dissemination after infected with *Salmonella* (67, 68).

Finally, Treg can be generated in the periphery in addition to the thymus where they play an important role in maintaining tolerance to food antigens and microbiota (69, 70). Naïve T cells activated in the presence of TGFβ express Foxp3 and high level constitutive expression of CD25 and following activation typically produce inhibitory cytokines such as IL-10, TGFβ and IL-35 (71). These cells have been exhibited potent immunoregulatory activity in suppressing immune responses and maintain tolerance.

In certain contexts, these effector T cell subsets can become dysregulated and they have been implicated in autoimmunity (Th1 and Th17) and asthma and allergic responses (Th2) (56). In contrast peripheral Treg, particularly those that are thymically-derived, are key to the control of immune responses that may lead to autoimmunity if left unchecked. For example, $Foxp3^{-/-}$ mice develop multiple organ cellular infiltration and lymphoproliferation, dying at an early age (72).

1.3.5.2. CD8 Cytotoxic T cells

CD8 T cells like CD4 T cells require multiple signals to be activated in the periphery. These signals are TCR triggering via antigen, co-stimulatory signals and stimulation by cytokine receptors (73). Following CD8 T cells activation, these cells produce perforin and granzyme molecules that induce apoptosis or cell death via enzyme cascade activation (74). This makes

activated CD8 T cells a significant target in antiviral immunity as they have the ability to remove viruses and tumours.

1.3.6. B cell development and maturation

In the fetal liver, the process of B-lymphopoiesis initially starts from a pool of pluripotent haematopoietic stem cells, where it continues in the bone marrow where it is maintained during adult life. Upon differentiation, B cells are required to express both the endonucleases recombinase activating genes 1 and 2 (RAG1/RAG2), resulting in rearrangement of variable, diverse and joining (VDJ) segments of the surface-expressed immunoglobulin (Ig) genes (75). This means that the expression of both Rag1 and Rag2 is required to support the initial activation of VDJ recombinase activity; transfection of only Rag1 cDNA clonal to non-lymphoid cells shows an obvious failure of the ability to confer VDJ recombinase activation, suggesting that this only happens upon co-transfer of both Rag1 and Rag2 (76).

During the formation of a functional Ig heavy chain, the μ chain is rearranged, resulting in the formation of a pre-B cell receptor, where the heavy chain is expressed on the cell surface with surrogate light chains (77, 78). As a result of the rearranged light chains, variable regions combine and covalently bind with heavy chains to form an IgM molecule that is subsequently inserted into the cell membrane. This enables the exposure of the unique specificity antigen-binding domain (79). Cells that fail to successfully rearrange VDJ genes die, as they do not receive survival signals. Screening against self-antigens occurs during the rearrangement of the BCR whereby autoreactive B cells undergo clonal deletion, a process in which editing receptors to be non-autoreactive variants or clonal anergy which results in BCR being incapable of being triggered peripherally by cognate antigens (80, 81). Therefore, deletion is appeared when high-affinity interactions with membrane-bound antigen, while low affinity contact with antigen results in editing or anergy. It has been considered that a very low-affinity interactions showed a negative impact on B cells development.

Once B cells express IgM, they migrate to secondary lymphoid organs where the final maturation takes place and the B cells mature and produce IgD via alternative mRNA splicing (82). B cells can then become activated following interaction with cognate pathogenic antigens. This directs the B cells to become T-dependent; the process by which B cells undergo clonal expansion results in further maturation into memory B cells or antibody-secreting plasma cells (83). During T–B interactions, B cells are induced to proliferate and produce switch germ-line transcripts under the influence of cytokines produced by T cells, and activated B cells either proliferate within follicles, resulting in germinal centre (GC) formation or differentiate into plasmablasts in the extrafollicular areas. The most significant functional feature of B cells is that the induction of GCs leads to the production of memory cells and plasma cells that secrete antibodies that have improved/high affinity for the stimulating antigen and that such antibodies have an altered functionality due to class-switching (84). The improved affinity for antigen via the GC reaction is attributable to affinity maturation through somatic hypermutation (84).

1.3.7. B cell Subsets and Their Antigenic Targets

The process of a thymus-dependent (TD) antibody response requires cognate interaction between T and B cells whereas thymus-independent (TI) responses do not require cognate interaction with T cells (85, 86). TI antigens are divided into two general types: Type I (TI-1 antigens) and Type II (TI-2 antigens). These types have their own mechanisms for activating B cells (87, 88). Mature B cells have been sub-classified into distinct subsets: follicular (FO) B cells, B1a B cells, B1b B cells and marginal zone (MZ) B cells (Figure 1.4). Each subset can be distinguished by the surface markers they express or their anatomical locations. All subsets have their own distinct functions and immune responses.

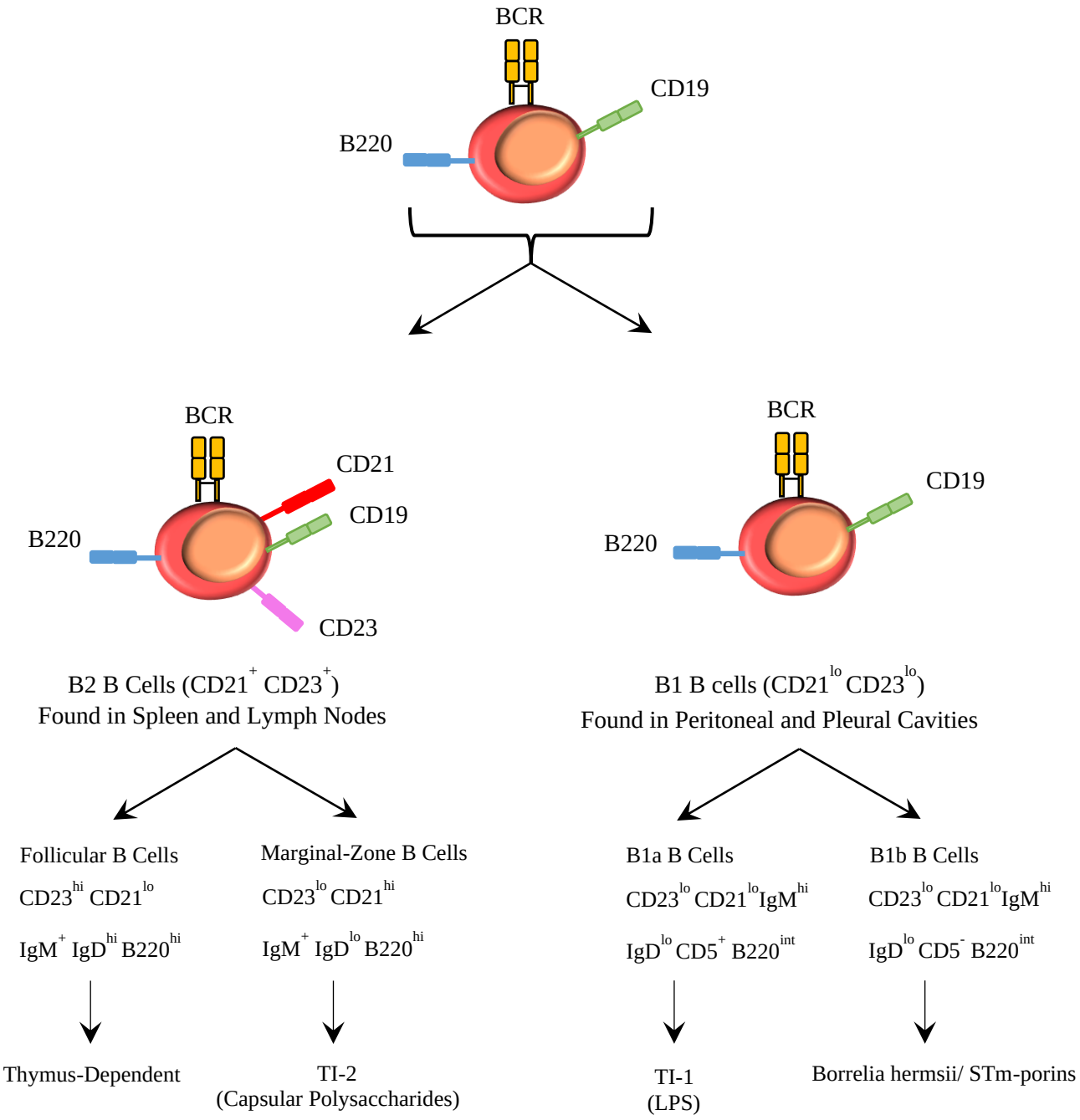

Figure 1.4 Different B cell lineages interact with TD and TI antigens
B cells subsets are subdivided into B2 B cells and B1 B cells. These subsets can be distinguished by the surface markers they express, their anatomical locations and their response towards TD or TI antigens. B2 B cells are found in lymph nodes and spleen. B2 B cells are differentiated into two distinct subsets: follicular and marginal zone B cells. These subsets can produce antibodies via TD and TI-2 responses. B1 B cells are found in peritoneal and pleural cavities and further differentiate into B1a and B1b B cells. B1a cells respond to TI-1 antigens such as LPS while B1b seems to be important in the TI response to Borrelia hermsii and STm-porins (85-88).

1.3.8. T-Dependent (TD) Antibody Responses

The majority of the adult B cell population are naïve FO B2 cells. Most of these cells are located in the follicles of secondary lymphoid organs but they constantly migrate between spleen, blood, lymph and lymph nodes (89). FO B cells can be activated through two essential signals, the first of which is provided by antigen recognition by BCR.

Following recognition of the antigen, the antigen is internalised, processed and presented on MHCII, where it is recognised by $CD4^+$ T cells (90). Subsequently, the activated T cells provide co-stimulatory signals, such as OX40 and CD40-ligand, providing an essential signal to B cells during TD antibody responses (91). The T cells involved in this process have already been primed by the DC in the T-zone whereupon they migrate to the T–B border and interact with antigen-triggered B cells. As a result of the cognate interaction with T cells the B cells proliferate and class-switch germ-line transcripts under the influence of cytokines produced by T cells. The activated FO B2 cells either proliferate within follicles, resulting in germinal centre formation or differentiate into plasmablasts in the extrafollicular areas of secondary lymphoid organs (89).

The induction of the germinal centre is required, as it leads to the production of memory cells and plasma cells that secrete high-affinity antibodies. Therefore, class switch recombination leads to changes in the immunoglobulin isotype, which gives rise to different subclasses of B cells. Somatic hypermutation of the BCR where the mutated BCR is of higher affinity than the original leads to the preferential survival signals being delivered by Tfh.

High affinity B cells then differentiate into plasma cells or return to the dark zone to undergo further rounds of proliferation and somatic hypermutation. Long-lived plasma cells migrate to the bone marrow (BM), while short-lived plasma cells move to the extrafollicular regions of the lymphoid tissue (92, 93).

1.3.9. Extrafollicular Antibody Response

Marginal zone or follicular B cell blasts migrate from the inter-follicular zone to the site of extrafollicular growth in the medullary chords or red pulp in the lymph nodes or spleen respectively. At this site, they can form extrafollicular foci, where they upregulate Blimp-1 and inhibit PAX-5 (89). This results in the differentiation and expansion of moderate- or low-affinity antibody-secreting plasmablasts (89).

Plasmablasts are generated as short-lived plasma cells and produce IgM antibody 3–5 days after antigen recognition, however, plasmablasts may undergo class-switch recombination to produce other isotypes (89). This results in plasma cells that have a low affinity antibody in compare to plasma cells derived from GC. Additionally, this suggests that the type of isotype secreted may vary based on the polarity of T cells, if they are Th1 or Th2. Indeed, it has recently been postulated that class-switching may take place prior to germinal centre formation (94). The best example of this is seen in STm infection, where a strong Th1 response is generated. This promotes the production of IgG2a and IgG2b isotype antibodies 5–7 days after infection (95). $CD11c^{hi}$ DCs can support some plasmablasts to differentiation into plasma cells, this is associated with increased of XBP-1 and Blimp-1 expression (89). Moreover, a very small number of these plasma cells continue to become long-lived plasma cells or memory B cells, most of which undergo apoptosis (89).

1.3.10. Germinal Centre Reaction

Germinal centres (GC) are microstructures located in follicles in secondary lymphoid tissues. GCs specifically act to produce memory B cells and long-lived plasma cells that can secrete high-affinity antibodies to provide long-term protection (96). B cells that are characterised by upregulation of B cell lymphoma 6 (BCL-6) and downregulation of EBI-2, are able to enter the GCs because they suppress the differentiation of plasmablasts and allow the migration of B cells to the primary follicle (96-98).

Following the proliferation of B cells, for approximately 7–10 days, GCs are formed composed of a light zone (LZ) and a dark zone (DZ). The DZ is located closer to the T cell area, where the centroblasts proliferate. In the DZ, B cells undergo somatic hypermutation (SHM) through the upregulation of the expression of activation-induced cytidine deaminase (AID), with the rapidly dividing B cell blasts being characterised as $CXCR4^{Hi}CD83^{low}CD86^{low}$ (96, 97). The LZ is located near the lymph node capsule and in the spleen, closer to the MZ. The LZ can be described as comprising follicular dendritic cells (FDCs), tingible body macrophages (TBM) and T cells. Tfh and FDCs in the LZ play a role in the selection of high affinity B cells (99, 100). Positive selections as high affinity become either plasma cells or memory B cells, whereas those with low affinity are eliminated by apoptosis (101).

In LZ, Tfh and FDC are both needed for the positive selection process. GC-B cells need two an essential step, in order to be positively selected. FDC localised within LZ to form a stromal network, can capture and recognise antigen-bound antibody known as immune complexe (102). FDC then presents immune complexes to the centrocytes; this interaction requires the contact of their expressed CD21 and complement-derived ligands held on FDC (102, 103). Following the initial step, BCR binds Ag via centrocytes, resulting in process and present the captured Ag to Tfh cells, allowing Tfh cells to help and form the GC B cell fate decision. Signals from Tfh cells, via CD40L and IL-21, IL-4 cytokines, provided further survival, differentiation and proliferation signals for GC-B cells (104).

Despite this, immune complexes on FDC have been proposed by some studies, which have noted that they play an important role in the high-affinity selection of B cells and inducing GC affinity maturation (96, 105). In this regard, the present study tests the antibody feedback mechanism by immunising mice with immune complexes of NP-chicken gamma globulin (CGG) and anti-NP IgM. Indeed, the study differentiates between self-generated antibodies and injected antibodies by injection different antibody allotypes. the present study found that all

immune complexes can easily be found in FDC, and low-affinity antibodies has been replaced with self-generated antibodies. The study also found that high-affinity antibodies can replace endogenous antibodies, suggesting that the replacement of antibodies is dependent on their affinity and time. Due to the higher level of competition provided by injected IgM, a higher affinity antibody is produced (105).

As a result of positive selection, plasma cells can secrete high-affinity class-switched antibodies and migrate to the bone marrow. The plasma cells that exit the GCs are high-affinity and can survive in the bone marrow for years if not decades (106). Overall, B-lymphocyte-induced maturation protein 1 (BLIMP-1) is a key factor that drives and associates differentiation into plasma cells and antibody secretion when BCL-6 suppresses BLIMP-1, allowing GC responses to recur until further differentiation of plasma cells occurs (107).

In mice, IgG is sub-divided in IgG1, IgG2a, IgG2b, and IgG3 whereas, C57BL/6 mice are expressing IgG2c but not IgG2a (108, 109). The specificity of Ab isotype is dependent on the process of the germ-line transcription and deletional recombination of the H chain of the Ab resulting in splicing of the VDJ gene segments in the H chain and the Ab undergoes CSR (110). In mice, the class switching of the Ab occurs associated with the response of a specific antigenic stimulus regulated by cytokines such as IL-4, IFN-γ, or TGFβ. IgG1 and IgE produced following the signals provided by IL-4, whereas IFN-γ promotes switching to IgG2a/c and IgG3 and TGFβ regulates IgA and IgG2b production. Generally, IgG1 and IgG3 are following protein responses but IgG2 to polysaccharides (111).

1.3.11. T-Independent (T-I) Antibody Responses

T-I antibody responses are the process that can develop Ab faster than T-D responses as these responses do not require input from Tfh. T-I responses result in the production of low-affinity Ab that can provide rapid protection to the host (112). The B cells involved in this process have been shown to be largely B1 and MZ B cells. MZ B cells are able to differentiate into short-

lived plasma cells and they reside in the spleen at the marginal sinus (113). Nevertheless, MZ B cells show some evidence that they may contribute to the generation of long-lived plasma cells, producing high-affinity antibodies by GC formation through a TD response (114). In mice, B1 cells are located in the peritoneal and pleural cavities and secrete IgM, IgA and IgG3 with no need for external antigens (115-117). These cells can be divided into B1a and B1b; the difference is the expression of CD5 on B1a-cells (118). There are two types of TI antigens, classified as TI-1 and TI-2. An example of the TI-1 antigen is LPS, which can engage both BCR and TLRs in B cells; an example of the TI-2 antigen is bacterial capsular polysaccharides (87). These antigens can directly activate B cells and mediate responses, with no need for the presence of T cells.

1.4. *Salmonella*

Salmonella is a genus of rod-shaped, Gram-negative intracellular facultative organisms that belongs to the *Enterobacteriaceae* family(119) There are two different species of *Salmonella*: *Salmonella bongori* and *Salmonella enterica*. S. *bongori* serovars infect mainly cold-blooded animals, whilst S. *enterica* serovars infect humans, birds and other mammals (119). There are several distinct subspecies for S. *enterica* known as *enterica* (I), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI) and *salamae*. S. *enterica* and these pathogens contains more than 2,500 serovars which can be identified depending on the structures on their surface known as antigens (Ags), flagella (H) and lipopolysaccharide (O). However, most of these serovars belong to the O-antigen groups (A, B, C1, C2, D and E) (119-121). These serovars cause two major groups of diseases known as non-typhoidal and typhoidal *Salmonella*, specifically S. Paratyphi A and S. Typhi, which are restricted to humans (122-124).

1.4.1. *Salmonella* infections in humans

1.4.1.1. Typhoid fever

Typhoid is also termed enteric fever, and it is transmitted by ingesting contaminated water or food. Typhoid is usually associated with poor hygiene and spread via the faecal-oral route by eating or drinking food that is contaminated by an infected person (125). Typhoid fever is widely spread in South and Southeast Asia, resulting in nearly 20 million cases per year and a death rate of 20%, with the majority of affected children under five years old (126-129). Intestinal microfold (M) cells are targeted by S. Typhi and infection may be initially sub-clinical due to a lack of intestinal inflammation (130). In severe cases, symptoms may last for weeks, and the incubation period usually lasts up to two weeks after infection.

People develop symptoms such as persisting fever, cough, abdominal pain, nausea, anorexia, myalgia, constipation or diarrhoea, headache and hepatosplenomegaly (131, 132). S. Typhi can disseminate from the intestine to the liver, spleen and bone marrow, cause death if left untreated

(133). Treatment of enteric fever is usually with antibiotics such as fluoroquinolones, however, antibiotic resistance has led to a serious problem in the forms of delayed treatment and the recovery of individuals with underlying conditions such as anaemia or malaria. In these situations, the bacteria persist for longer which leads to patients taking a protracted period of time to recover after infection (132).

1.4.1.2. Non-typhoidal salmonellosis (NTS)

NTS infections are restricted and affect the gastrointestinal tract without being life-threatening. The incubation period of NTS is as fast as the first six hours after ingesting the pathogen, and the symptoms appear soon after but usually last for less than a week. Typical symptoms of infected individuals are gastroenteritis, abdominal pain, diarrhoea, nausea and fever. NTS infections resolve in a few days, and there is no need to be treated with antibiotics (131-135). However, there is another form of NTS called invasive NTS (iNTS), which is often present in developing countries and infected adults with HIV and children under two years old. iNTS occurs in the absence of major NTS symptoms, such as gastrointestinal symptoms, and it commonly presents as high fever, bacteraemia and hepatosplenomegaly. STm and *Salmonella* Enteritidis (SEn) are the most common bacteria that may cause iNTS, and their transmission could be from human to human rather than from animal to human (134, 136).

1.4.1.3. Pathogenesis of iNTS

Following the ingestion of food or drink contaminated with *Salmonella*, the bacteria survive in the gut and reach the intestinal epithelial barrier to initiate a systemic infection through the M cells of Peyer's patches. Here, the bacteria can invade both the phagocytic and non-phagocytic cells of the host by exploiting a range of virulence factors. Once *Salmonella* reaches the epithelial cells, it induces its uptake through type-3 secretory system 1 (T3SS-1), resulting in the secretion of effector proteins via *Salmonella* pathogenicity island 1 (SPI-1) into the cytosol

(137, 138). As an alternative pathway, *Salmonella* can spread from the gut into the bloodstream by $CD18^+$ phagocytes with no need for help from Peyer's patches (139).

Phagocytic cells, such as dendritic cells, neutrophils or macrophages, uptake the bacteria in the lamina propria. *Salmonella* initially replicates in *Salmonella*-containing vacuoles (SCVs) through SPI-2 encoded T3SS-2, which prevents the formation of the phagolysosome, resulting in the inhibition of oxidative mechanisms and the release of cytokines and chemokines, such as IFNγ, IL-17 and TNF (140, 141). Moreover, recognition of PAMPs or DAMPs by phagocytic cells enhance the innate immune response via the presentation of antigens on their receptors, such as TLRs or NLRs. This leads to the recruitment of innate immune cells, such as neutrophils and macrophages, and the boosting of the inflammatory response (142). Failures to control infection locally by the immune system result in the migration of *Salmonella* to MLNs, spleen and liver via the blood and in the initiation of systemic infection (143).

1.4.2. Disease burden of invasive non-typhoidal salmonellosis

Salmonella infections, especially iNTS, are one of the most fatal causes of infection worldwide; however, the severity of the disease may vary based on bacterial dissemination in the host. In addition, *Salmonella* is one of the most common causes of bacterial gastroenteritis worldwide. The global distribution of iNTS per annum is estimated as 3.4 million cases with the majority of cases in Africa accounting for around 2 million cases (144). iNTS has a death rate of around 20% with approximately 100,000 death/year (123). Estimation of 90% of cases positive of iNTS in adults are associated with HIV infection whereas 20% of children positive with iNTS are diagnosed HIV positive (123, 145, 146).

These data also reveal that the most common pathogens cause bloodstream infections in Africa are NTS alongside with Streptococcus pneumoniae (147). The global distribution has been reported and showed that the incidence rates per year in Sub-Saharan Africa is more than 100 iNTS cases out of 100,000 of population however in South East Asia infections are less than

100 iNTS cases out of 100,000 of population (Figure 1.5 A) (143). Data from Africa showed more than half of the total cases are in Eastern Africa while only 10% occur in Northern Africa (Figure 1.5 B) (148).

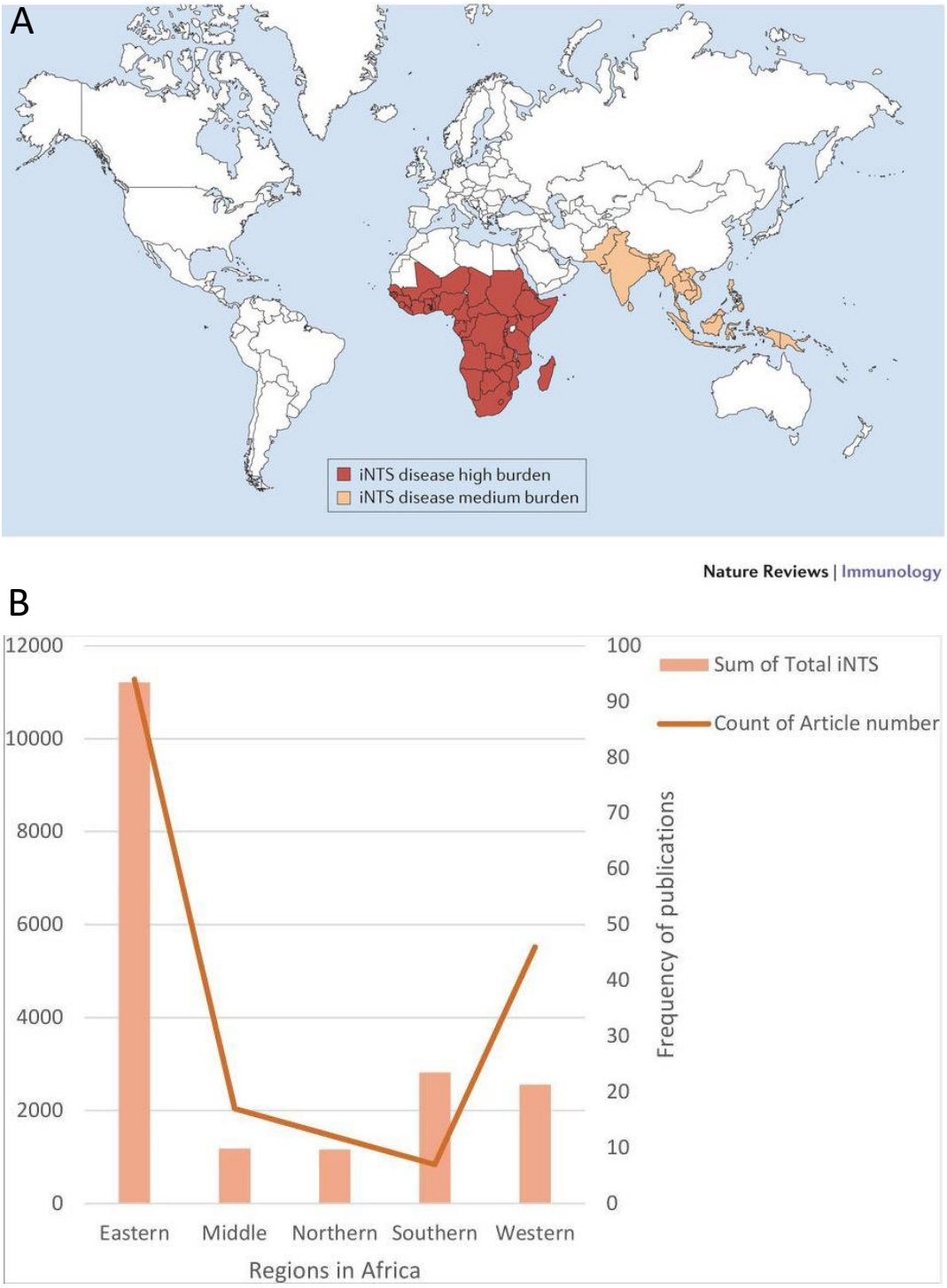

Figure 1.5 The global distribution and frequency of invasive non-typhoidal salmonellosis cases reported in worldwide.

A) this figure reported the geographical majority distribution of iNTS is observed across the world. Adapted from (143). B) The distribution of invasive non-typhoidal salmonellosis cases across different regions in Africa. Adapted from (148).

1.4.3. Structural Features of *Salmonella*

1.4.3.1. Composition of the Cell Wall

The inner and outer membranes form the whole cell wall structure of the *Salmonella*, in which they are separated by a viscous layer called the periplasm (Figure 1.6) (149) . The periplasm contains a high concentration of proteins (including lipoproteins) and peptidoglycan. The inner membrane (IM) or cytoplasmic membrane of *Salmonella* contains a double layer of phospholipids which facilities lipid biosynthesis, ATP production and the transport and secretion of proteins (150). The outer membrane (OM), which forms the surface layer of the bacterium, is composed mainly of lipopolysaccharides (LPS), outer membrane proteins (OMPs), lipoproteins and phospholipids. The OM itself is composed of two layers: the inner leaflet, which contains phospholipids, and the outer leaflet, which contains LPS (151). Lipoproteins are surface exposed of phospholipids into the inner leaflet of OM. In addition, porins are found in the outer membrane and can be distinguished based on their surface-exposed epitopes and monoclonal antibodies and they are known to be important antigenic targets in STm immunity (152-154).

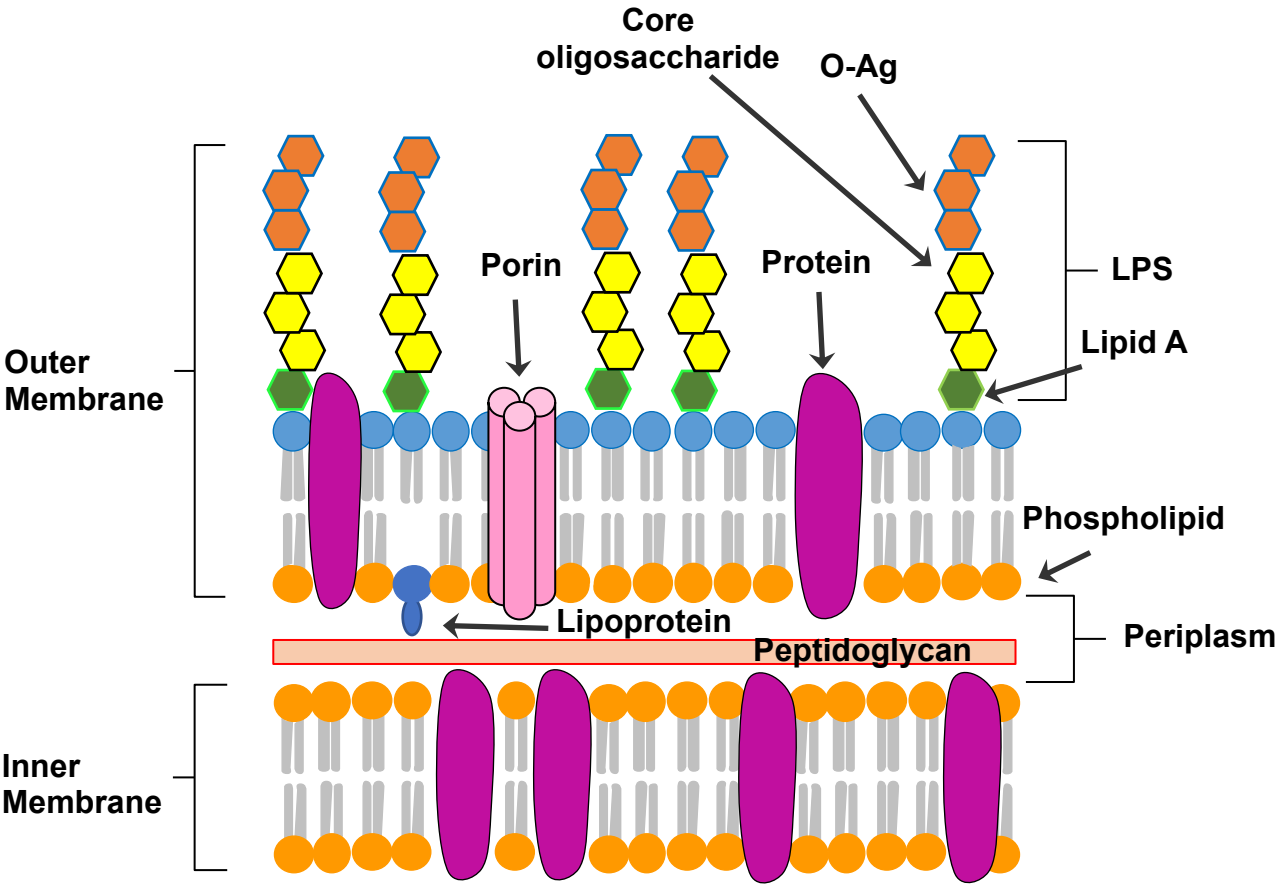

Figure 1.6 Schematic diagram of cell wall structure of Salmonella.
The inner and outer membranes form the whole cell wall structure of the *Salmonella*, in which they are separated by a viscous layer called the periplasm. The inner membrane contains a double layer of phospholipids whereas the outer membrane is composed mainly of LPS, OMPs, porins, lipoproteins and phospholipids. LPS are composed the outer leaflet of the outer membrane and contain three parts: lipid A, core oligosaccharide and O-Ag. Figure modified from (149).

1.4.3.2. Lipopolysaccharides (LPS)

Lipopolysaccharides are composed of lipid A, core oligosaccharide and O-antigen repeats (Figure 1.6) (155, 156). Lipid A consists of a glucosamine disaccharide coated with six hydrophobic long-chained fatty acids in which the lipid A portion is responsible for anchoring LPS molecules into the OM of the cell wall (157, 158). Lipid A is also responsible for the most toxic property of LPS, providing endotoxic activity and inducing innate immunity through TLR4 signalling (157-161). The core is divided into outer and inner cores and provides a surface-exposed, negatively charged oligosaccharide. Lipid A is attached through the inner core, while the outer core connects the O-antigen repeats. These core region and O-antigen repeats are composed of sugars that provide diversity between species and serovars. These diversities and variations of repeating sugar units associated with the O-chain are responsible for the determination of whether LPS can be considered rough or smooth. Therefore, loss or absence of the O-specific region in bacteria results in LPS becoming a rough LPS strain (162).

1.4.3.3. Outer membrane proteins

Porins are proteins found in most OMs of Gram-negative bacteria, and there are approximately 100,000 porin molecules expressed on the cell surface of STm (163). Porins exist as trimeric β-barrels, which form channels to allow the passive diffusion of small molecules inside and outside the cells. Porins allow the transit of nutrients into cells and the excretion of toxic compounds from the bacterium (Figure 1.6) (164, 165). All porins contain several surface epitopes and exist on the outer membrane as either homo- or heterotrimeric forms (164). However, there is evidence that porins have been associated with virulence and antibiotic resistance (163). These porins are extremely permeable and contain 16 β-strands connected by periplasmic and extraplasmic loops (152, 165, 166). This composition of porins can vary with different sizes or numbers in their surface charges and loop topology. Porins that can be found in the OMs of STm are mainly OmpC, OmpF and OmpD. OmpC and OmpF porins which are

expressed on the surface of OM, ST, STm and E. coli. However, OmpD porins are not expressed by ST but they are found on all other serovars of *Salmonella* (167).

1.4.3.4. Flagellin

Flagellin is an antigenic target of the immune system in various hosts, such as vertebrates, plants and invertebrates, and it is situated in the bacterial flagellar filament (168). *Salmonella* expresses flagella, which are important for the acquisition of nutrients and allows the bacterium to swim and is responsible for its chemotactic behaviour (169, 170). There are three distinct parts of each flagella, known as the basal body, the hook and the filament (Figure 1.7) (171).

The basal body is responsible for anchoring flagella molecules into the IM and OM of the cell wall. The basal body consists of a type-3 secretory system (T3SS) needle alongside different rings, such as the P ring, the L ring, the M ring and the S ring. The basal body acts as a rotary motor to turn the flagella either clockwise or counter-clockwise (172, 173). The hook acts as a flexible joint that links the basal structure with the filament. The flagellin monomers are the major component of the flagella filament, where filaments are exported through the T3SS to the surface of the cell wall. The filament is composed of up to 20,000 subunits of flagellar proteins termed flagellin (172). FliC or fljB are the flagellin building-block proteins that are expressed by STm (174).

FliC, which is the fundamental flagellin protein, is a strong immunogen, since this protein has the capability of inducing potent immune responses to itself through its intrinsic adjuvancy and to co-administered Ags through the use of extrinsic adjuvancy. The globular domains known as D0, D1, D2 and D3 are composed of flagellin monomer (20). These domains are recognised by Naip 6 and Naip 5 and D1 domains are recognised by TLR5. Using FliC as an adjuvant is sufficient to drive T and B cell responses, and it leads to microbicidal activities, the production of cytokines and the activation of dendritic cells (168).

Currently, flagellin is being developed as an adjuvant that may aid vaccination due to its adjuvanticity via TLR5 and subsequent induction of a Th2-biased response (175). Flagellin is a molecule that can be engineered in specific ways for vaccine development to retain immunogenicity. For example, flagellin has been designed to display foreign epitopes of specific pathogens such as influenza and Helicobacter pylori (176). Moreover, flagellin fusion proteins have enhanced humoral responses, and they are an attractive alternative for vaccine design due to their greater efficacy and enhanced antigenicity (177).

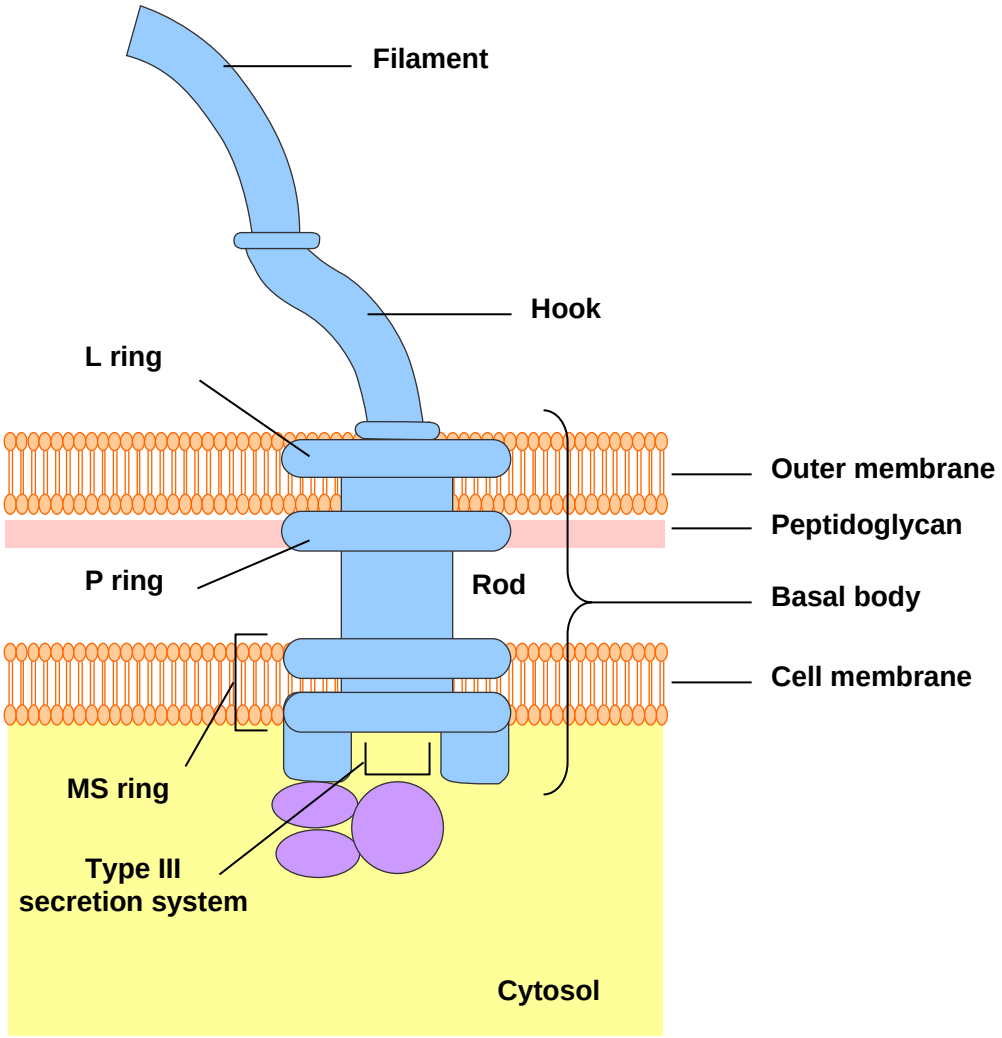

Figure 1.7 Structure of STm flagellin.
Flagella are composed of three distinct parts known as the basal body, the hook and the filament. The basal body is anchoring flagella molecules into the inner and outer membrane of the cell wall. The basal structure consists of T3SS needle alongside different rings, known as the P ring, the L ring, the M ring and the S ring. The hook is known as the flexible joint that links the basal body with the filament. Each filament is composed of up to 20,000 subunits of flagellar proteins termed flagellin. Figure modified from (171).

1.4.4. Immunity to *Salmonella*

1.4.4.1. Innate immune response to STm

Monocytes, DCs, epithelial cells and neutrophils express TLR5, which can recognize flagellin. Recognition results in the dimerization of TLR5 and signalling via MyD88, resulting in the activation of DCs, the upregulation of CD86, MHC II, CD80 and the secretion of various cytokines such as IL-6 and IL-23 (178). In addition, macrophages and neutrophils become involved as they are recruited to the site of inflammation by the local secretion of C5a and IL-8 (179). These cells are effective at controlling bacterial replication in part through the generation of reactive oxygen intermediates (ROS) and reactive nitrogen intermediates (RNI). This is achieved by phagocytosing opsonised bacteria (180, 181). However, failure to control infection induces the upregulation of SPI-2 and the expression of the T3SS-2, which enables *salmonella* to secrete effector molecules into the cytosol, leading to the formation of SCV, which supports bacterial replication and survival (138, 182). This results in bacterial dissemination through macrophages and migratory DCs (183).

If *Salmonella* escapes local immunity in the gut then the bacteria have the capacity to become a systemic infection (183). Salmonella go on to colonise organs, such as the liver, spleen and gallbladder, where the accumulation of DCs, macrophages, neutrophils, monocytes and NK cells takes place (184). DCs and macrophages produce cytokines such as IL-12, IL-15 and IL-18, which then promote the release of IFN-γ from innate cells such as macrophages and NK cells (179, 185). Cytokines, alongside with TLRs activation, lead to the production of ROS and RNI and increases the rate of phagolysosomal fusion, resulting in the killing of intracellular *Salmonella* (58, 186).

These innate immune molecules can facilitate to restrict the bacteria growth during a systemic infection. For example, TLR5 enables flagellin uptake and presentation, which is required to

drive T and B cell responses to flagellin. As a consequence, flagellin stimulates various pathways that are beneficial for vaccine development (187).

1.4.4.2. The role of T cells in immunity to STm

Clearance of a primary infection with STm shows the importance of $CD4^+$ Th1 cells and their production of IFNγ; however, optimal immunity requires both antibody and $CD4^+$ T cells (188). The important role of $CD4^+$ T cells has been demonstrated in mice lack of the IFNγ receptor, TCR β chain and in mice deficient in MHC class II molecules (188). These studies further confirmed that in the absence of $CD4^+$ T cells or IFNγ mice could not clear attenuated *Salmonella* infections from the liver or spleen compared to the control (188). Moreover, T-bet deficient mice that received attenuated *Salmonella* immunisation also displayed increased splenic bacterial burdens and higher death rate (189). These data suggest that Th1 cells and their production of IFN-γ play a crucial role in the clearance of primary STm infection.

Other evidence that suggests the importance of $CD4^+$ T cells to STm clearance was provided by the use of mice lacking OX40 and CD30, where such mice displayed defective activation of $CD4^+$ T cells and resulted in a failure to resolve infection (190, 191). In human studies, it has also been reported that patients with a deficiency in cytokine receptors, such as IFN-γ or IL-12 receptors, are more susceptible to *Salmonella* infections (192, 193).

Taken together observations made in both human and mouse studies support the important role of $CD4^+$ Th1 cells in STm infection. Interestingly, mice deficient in MHC class I molecules, were able to clear a primary infection with STm suggesting that $CD8^+$ T cells were dispensible in this setting (188). In addition, γδ-T cells have also been shown to be not required for the clearance of *Salmonella*, however it cannot be ruled out that either γδ-T cells or $CD8^+$ T cells contribute to immunity while not being required to resolve infection (188).

1.4.4.3. The role of B cells in immunity to STm

It is well known that B cells alongside antibodies can provide protection against secondary *Salmonella* infection. However, B cells do not play a major role in protection during a primary infection, whereas extrafollicular response shows limited protection, fail to reduce the levels of bacteraemia, and do not resolve such an infection (95, 194, 195). However, some studies show that B cells can contribute to clearing T cell responses to *Salmonella* by presenting antigens to Th1 T cells, which results in the secretion of critical cytokines, such as IFNγ (196).

The main role of B cells in *Salmonella* infections is to produce antibodies against significant *Salmonella* antigens such as flagellin and LPS (197). This means antibodies produced from B cells are effective against secondary infection with virulent strains but not against primary infections with attenuated *Salmonella* (195, 197, 198). Both EF and GC help protect against STm in C57BL/6 mice by generating high-affinity, class switched antibody, such as IgG2a and IgG2c, which prevents secondary infection (95).

In summary, B cells and T cells are required for protection against STm infections. Moreover, antibodies contribute to protection against infection by reducing the number of bacteria that might lead to systemic infection (199). This is further corroborated by a study in which Malawian children aged four months to two years were more likely to develop non-typhoidal strains of *Salmonella* when maternal antibodies were lost (200). Therefore, investigating how antibody responses are generated and what antibodies are protective to *Salmonella* infection will provide information that will be key to successful vaccine development.

1.5. Natural killer T (NKT) cells

NKT cells are a subset of innate-like T cells that typically express markers of NK cells and are distinct from conventional αβ T cells despite bearing an αβ TCR. They recognize glycolipid antigenic determinants (not peptides as with conventional T cells) presented by the non-polymorphic MHC I-like molecule CD1d via their TCR (201). They are rapidly activated on recognition of glycolipids releasing cytokines that can activate other immune cells such as DCs. Their peculiar phenotype is highly conserved in both mice and humans (202). iNKT cells are a relatively infrequent immune cells however their frequency has been shown to vary according to anatomical site. For example, NKT cells in the liver form a major population of T cell representing between 20-50% of all lymphocytes.

iNKT cells can be found in many different sites in the body such as liver, gut and other mucosal surfaces as well as in lymphoid tissue such as the spleen, thymus, bone morrow, peripheral blood and peripheral lymph nodes. Importantly, there are two main classes of NKT cells, type I or invariant NKT (iNKT) cells and diverse or type II NKT cells (Table1.2) (203).

1.5.1. Type I or iNKT cells

iNKT cells are the most frequently studied NKT cells as they express a semi-invariant αβ TCR the presence of which can be detected by glycolipid-CD1d tetramers (203). iNKT cells develop from a common lymphocyte progenitor that also gives rise to other T cell populations and like conventional T cells they are selected and develop in the thymus (202). The majority of iNKT express a semi-invariant TCR, composed of a Vα14Jα18 in mice and a Vα24-Jα18 in humans (204-210). Akin to $CD4^+$ T cell effector populations, iNKT cells can be further divided into subsets based on their transcription factor profile and their pattern of cytokine production i.e. iNKT1/ iNKT2/ iNKT17 (211). However, unlike naïve conventional T cells iNKT cell activation results in the rapid release of cytokine without further differentiation or proliferation as iNKT cell subsets are predetermined in the thymus and iNKT cells exist in a semi-activated

state (212). The iNKT TCR recognises certain α- and β-linked glycolipids when presented by CD1d with the glycolipid α-Galactosylceramide (αGalCer, KRN700) the most potent for induction of cytokine release (203).

1.5.2. Type II Natural Killer T cells

Type II NKT cells are also called diverse NKT cells as whilst they are CD1d restricted, unlike iNKT cells, they are polyclonal and express a diverse array of αβ TCRs. Type II NKT cells also express NK surface markers and do not respond to αGalCer (201). Sulfatide has been identified as a potent ligand for type II NKT cells antigen however several other antigens have also been reported as being stimulatory for type II NKT cells including lyso-PC, β-Glucosylceramide (β-GlcCer) (C24:0), lysosulfatide (213).

Table 1.2 iNKT cell subtypes

	CD1d dependent	α-GalCer reactivity	TCR repertoire (TCR α-chain TCR β-chain)
Type 1 NKT (Classical NKT cells)	Yes	Yes	Restricted Vα14-Jα18, Vβ2,7,8.2 (mouse) Vα24-Jα18, Vβ11 (human)
Type 2 NKT (Non-classical NKT cells)	Yes	NO	Diverse
NKT-like (NK1.1+ T cells)	No	NO	Diverse

1.5.3. iNKT Cell Ligands

iNKT cell ligands are characterised based on either the glycerol backbone or the sphingosine chain. The glycolipid structure is composed of an oligosaccharide chain and a linked lipid component. Kawano et al. first discovered the use of α-GalCer as an iNKT cell agonist in 1997 (214). In terms of structure, α-GalCer has fatty acid chains and sphingosine, which are attached to an α-linked carbohydrate moiety (Figure 1.8 A) (215). α-GalCer-CD1d complexes can stimulate iNKT cells, resulting in robust cytokine production, for example IFN-γ and IL-4. α-GalCer and certain analogues have been shown to have a potent effect on tumours, which was recognised in B16 melanoma and EL4 lymphoma models (216). However, α-GalCer has also been reported to evoke other immunomodulatory functions and has been shown to be immunosuppressive in various models of autoimmune disease (217).

A number of α-GalCer analogues, such as OCH and C-glycoside have been synthesised and interestingly such molecules have been shown to induce different functional consequences upon iNKT cell activation. α-GalCer has a sphingosine chain that contains 18 carbons, while OCH only has 9 carbons, and C-glycoside has a carbon instead of an oxygen-based glycosidic linkage (Figure 1.8 A). However, all have a long fatty acid chain, with 26 carbons in α-GalCer and 24 carbons in OCH.

OCH mediated activation of iNKT cells *in vivo* has been reported to elicit a dominant Th2 (IL-4 but little IFNγ) response (218), whereas C-glycoside preferentially induces a Th1 (IFN-γ but low levels of IL-4) response (219-221). Indeed, C-glycoside has also been shown to induce a Th1 response in human studies (222). This is in contrast to the native α-GalCer which elicits both IFNγ and IL-4 secretion by activated iNKT cells (222).

iNKT cells have also been shown to recognise glycolipids during microbial infections (223, 224). There are many microbial ligands, examples of which include Borrelia burgdorferi α-galactosyldiacylglycerols (225), Sphingomonas spp. α-galacturonosylceramides and α-

glucuronosylceramides (226-228), Streptococcus pneumoniae and group B Streptococcus α-glucosyldiacylglycerols (229), mycobacterial phosphatidylinositol mannosides (230), and Helicobacter pylori glycolipids (231). Glycolipids from all these microbials have been shown to stimulate iNKT cells and facilitate bacterial clearance (226).

1.5.4. CD1d and Lipid Presentation

The activation of iNKT cells follows the interaction of the semi-invariant TCR and antigen-loaded CD1d. To better understand iNKT cells, we first need to understand how iNKT cells can specifically recognise self- or exogenous glycolipids present by CD1d. This means it is necessary to understand how CD1d facilitates the binding of self- or exogenous glycolipids, as well as to understand the factors that control the antigenicity of a presented ligand. All these together support a better understanding of iNKT cell function and the types of responses such cells provide either pro-inflammatory or anti-inflammatory responses.

CD1d is encoded by a gene located on chromosome 3, while in humans it is located on chromosome 1 together with CD1a, CD1b, CD1c, and CD1e (211, 232-235). Structurally, CD1d is similar to classical MHC class I, as it is composed of a single chain noncovalently associated with β_2M. There are three extracellular domains, which are termed α1, α2 and α3 and it is the α1 and α2 helices that support glycolipid binding (236). Self- or exogenous glycolipid loading occurs in the CD1d recycle between the endolysosomal compartment and the cell surface (237). This process of endogenous presentation is achieved by lysosomal proteases and the lysosomal trafficking of CD1d molecules (238, 239). Sphingolipid-activating proteins are also well known saposins, are required to bind the α-GalCer to CD1d molecules, resulting in NKT cells activation (240, 241). Since lack of prosaposin has been reported in study that found mice lack of prosaposin showed a serious defect in the presentation process of CD1d, results in lack of NKT cells (240).

The crystal structure of synthetic α-GalCer-CD1d complexes illustrates the way in which the lipid component of glycolipids insert into the CD1d binding groove via pockets formed by α1 and α2 helices (242-244). This means that the 26-carbon acyl chain and an 18-carbon sphingosine chain from α-GalCer are buried within the A' and C' pockets of CD1d (243). The maximum carbon size that fits in pockets A' and C' precisely fits with the number of carbon atoms from α-GalCer (243). Following the binding of α-GalCer and CD1d, three hydrogen bonds are formed after two alkyl chains are positioned over the polar head of the lipid (Figure 1.8 B) (243). The first hydrogen bond is formed between the 2'-OH of the galactose ring with Asp151 and the second, between the 3'-OH on the sphingosine which forms a hydrogen bond with Asp 80. The final hydrogen bond occurs between Thr154 in CD1d with 1'-O from α-GalCer (243). Therefore, the semi-invariant TCR recognises the polar carbohydrate head which the acyl and sphingosine chain serve to anchor the glycolipid in CD1d in the correct orientation for recognition.

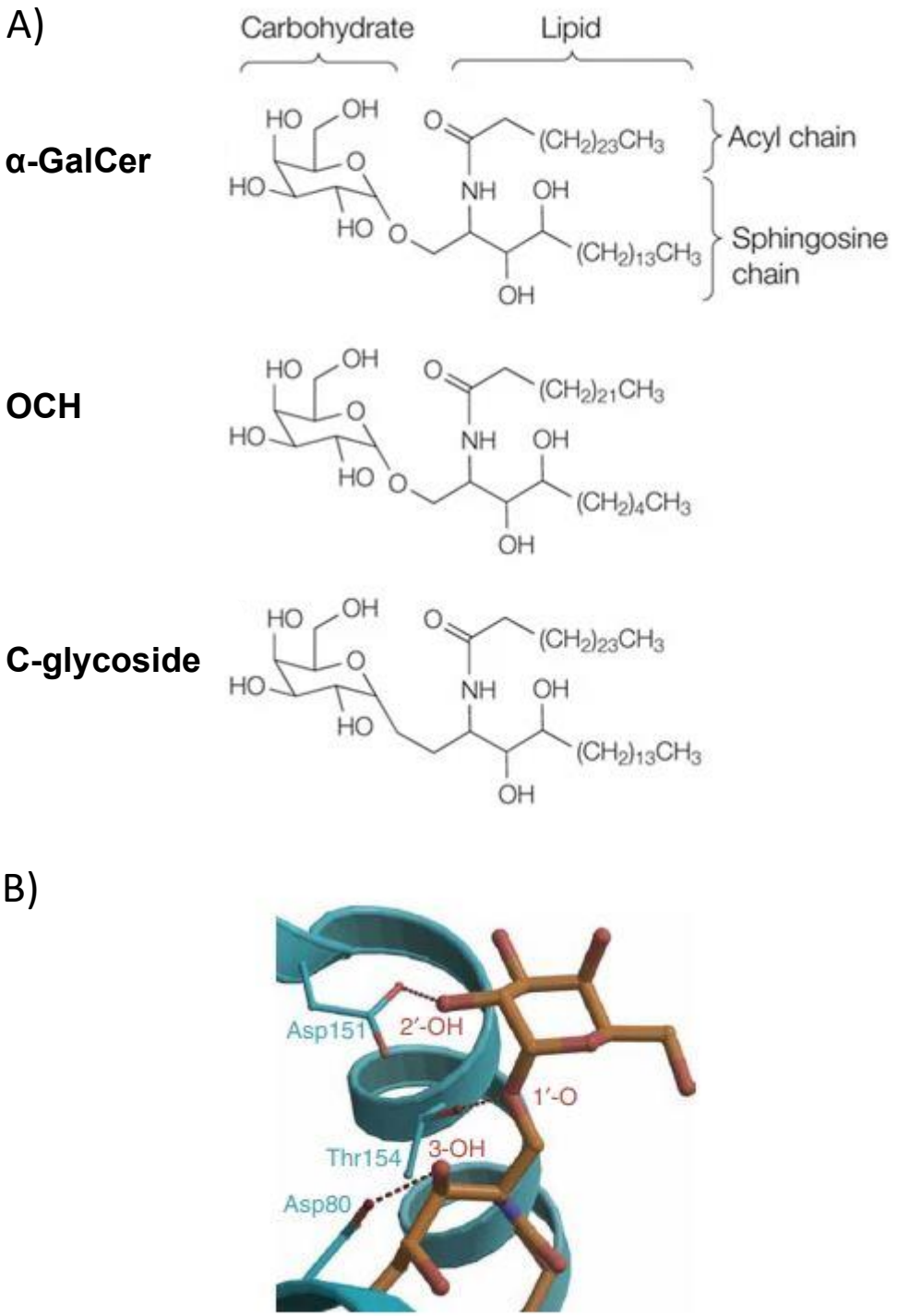

Figure 1.8 Glycolipids structure and α-GalCer interaction with CD1d.
A) Glycolipids have fatty acid chains and sphingosine, which are attached to an α-linked carbohydrate moiety. The compound α-GalCer has a sphingosine chain that contains 18 carbons with 26 carbons fatty acid chain. The compound OCH has a sphingosine chain that contains 9 carbons with 24 carbons fatty acid chain. The compound C-glycoside is similar to α-GalCer however C-glycoside has a carbon instead of an oxygen-based glycosidic linkage. Adapted from (215). B) Three hydrogen bonds are formed between α-GalCer and CD1d. The first hydrogen bond is formed by 2'-OH of the galactose ring with Asp151. Second, the 3'-OH on the sphingosine forms a hydrogen bond with Asp 80. The final hydrogen bond forms between Thr154 in CD1d with 1'-O from α-GalCer. Adapted from (243).

1.5.5. iNKT Cell Development

iNKT cells are like conventional T cells develop in the thymus; however, iNKT cells express a semi-invariant TCR, which is composed of Vα14Jα18 paired with Vβ8.1, Vb8.2, Vβ8.3, Vβ7, and Vβ2 in mice, while in humans, Vα24-Jα18 is paired exclusively with Vβ11 (204, 207-210). During the DP stage and the immature single positive (ISP) stage, α-chain rearrangement takes place, while at the DN3 and DN4 stage, β-chain rearrangement occurs (245, 246). The semi-invariant TCR is similar to other TCR used by conventional T cells in that they are generated by the random recombination of the VDJ region (247).

Due to this, development of iNKT cells requires DP thymocytes for delivering signals for NKT cells generation via expression of their genes RoRγt and Bcl-xL (245, 246, 248). This was revealed in a study where it was found that the deletion of RoRγt and Bcl-xL led to a reduction in the number of iNKT cells whereas over-expression led to increased iNKT cell numbers (246, 249). Moreover, the E protein transcription factor (HEB) that regulates these genes has also been demonstrated to play a significant role in promoting the survival of immature iNKT cells (250).

The positive selection of iNKT cells requires interaction with CD1d (247). Mice lacking CD1d were shown to have a complete absence of iNKT cells (251). This indicates that iNKT cells are selected upon recognition of glycolipid antigens presented by $CD4^{+}CD8^{+}$ thymocytes (249, 252). Glycosphingolipid iGb3 has been proposed as a potential selecting glycolipid that enables the positive selection of iNKT cells however this molecule is not generated in humans raising doubts on the validity of this molecule as the selecting self-antigen (253).

The transcription factor c-Myb has been found to be dispensable for the selection of thymic iNKT cells but its expression has been shown to be important for cell survival and to support iNKT cell proliferation (254, 255). The complete absence of c-Myb in mice resulted in the absence of iNKT cells (255). iNKT cells also require signals provided by lymphocytic

activation molecule (SLAM) receptors (which are expressed at the DP stage), as mice that lack the SLAM-associated proteins (SAP) family failed to generate iNKT cells (256). Taken together, the transcription factor c-Myb is an essential key to promoting the expression of CD1d/TCR and SLAM/SLAM interaction (255, 256).

Following positive selection, iNKT cells undergo maturation from stage 0 to stage 3. Precursor iNKT cells are termed as stage 0 and are first detected as CD1d-glycolipid tetramer positive, $CD44^{lo}$, $CD24^{+}$, $CD69^{+}$, $NK1.1^{lo}$. Then, iNKT cells move through the stages 1 $CD24^{lo}$, $CD44^{lo}$, $NK1.1^{lo}$ to $CD24^{lo}$, $CD44^{+}$, $NK1.1^{lo}$ (stage 2) to $CD24^{lo}$, $CD44^{+}$, $NK1.1^{+}$ (stage 3) (257-259). iNKT cells has been reported in these stages 1,2 and 3 to produce cytokines toward IL-4, IL-10 and IFNγ respectively, additionally most of these iNKT exit thymus before they completing the third stage of their development. More recently, promyelocytic leukaemia zinc finger (PLZF) has been implicated as an important regulator of the development of iNKT cells (260). iNKT1 cells express $PLZF^{lo}$ and produce high levels of IFN-γ with low levels of IL-4; iNKT2 cells express $PLZF^{high}$ and produce high levels of IL-4; and iNKT17 cells express $PLZF^{int}$ and produce IL-17 (261).

It has recently been reported that elevated TCR signals via the calcineurin/NFAT and Ras/MAP kinase pathways lead to enhanced expression of early growth response protein (Egr)1 and (Egr)2, which are important of iNKT cell development (262, 263). These genes are expressed at iNKT thymocytes, as they induce Bcl2 and FasL to promote cell survival as well as the induction of PLZF (262-264). Specifically, Egr2 has been shown to bind to the transcription factor PLZF, leading to it's upregulation of PLZF and the promotion of NKT2 and NKT17 development (264). The absence of PLZF transcription factor results in iNKT cells that produce IL-2 rather than IFN-γ or IL-4 (262-264).

Another transcription factor that is important for the differentiation of iNKT cells is Hobit, which is expressed mainly in iNKT1 cells and promotes the development of NKT1 cells (265).

It has been shown that mice that lack of Hobit have a significantly reduction in the number of iNKT1 cells (265, 266). T Cell Factor 1 (TCF1) and Lymphoid Enhancer Factor 1 (LEF1) are other transcription factors that play a crucial role in iNKT cell development (267, 268). Mice deficient in either TCF1 or LEF1 shows a developmental defect in all NKT cell subsets where LEF1 has been proposed to play a significant role in promoting iNKT cell survival and proliferation (267, 268).

Cytokines are thought to be a master regulator of iNKT effector cell differentiation. This has been seen in iNKT1 cells which required IL-15 during their development whereas TGF- β facilitates differentiation into iNKT17 cells (269, 270). iNKT2 and iNKT17 cells express the IL-25 receptor which is required for the production of IL-17, IL-10, IL-13 and IL-9 after activation of iNKT cells (271). In mice it was observed that more IFN-γ than IL-4 was released via αGalCer activated iNKT cells at stage 3 of thymic maturation (261). iNKT2 cells usually lack NK1.1 but express CD4. This was contrary to the fact that iNKT2 cells in mice release IL-4 exclusively and remain at stages 1 & 2 not moving to stage 3 (261).

At maturation stage 2 iNKT cells can differentiate into either RORγt^{+}CD4^{-} or RORγt^{-}CD4^{+} iNKT cells (202). Stage 2 of thymic maturation is where production of iNKT17 cells also occurs. iNKT17 here arise from iNKT retaining RORγt expression that express CCR6, CD103, CD121 and but lack CD4 and NK1.1 (202). This subpopulation once exported from the thymus show a differential pattern of localisation being predominantly found in skin-draining and other peripheral nodes with a relative minor population being found in the liver and spleen in mice. In humans, iNKT17 cells can be generated from human peripheral blood by using IL-23, TGFβ and IL-1β cytokines and these cells are suggested to be associated with inflammations (202).

1.5.6. iNKT cell activation and effector function

iNKT cells can be directly activated during inflammation or infection. iNKT cells require two distinct signals to be activated: TCR signals and cytokine signals (Figure 1.9). iNKT cells can

be directly activated when APCs provide a dominant TCR signal via the presentation of high affinity glycolipid antigens in the context of CD1d (272). Such CD1d mediated activation leads to the rapid release of specific cytokines, interleukins, interferons, TNF's, CSF, TGF and chemokines such as MIP, RANTES and eotaxin (273). Alternatively, iNKT cells can be activated indirectly upon receipt of dominant cytokine signals such as IL-12, type I interferon and IL-18 along together with weak TCR-CD1d lipid interactions (274).

In some models of infections, endogenous lipid-CD1d complexes alongside PRR-induced cytokines are sufficient to activate iNKT cells. This was seen during *in vivo* STm infection (226, 275). Potent iNKT cells producing IFN-γ can be induced by DC-derived IL-12/IL-18 that have been activated with TLR5/TLR4 ligands like flagellin/LPS, along with weak endogenous lipid-CD1d interaction recognised by iNKT cells (226, 275). Another study by Nagarajan et al., supported the significant role of the distinct cytokines that have been driven by DC IL-18/IL-12 in the activation of iNKT cells producing IFN-γ production and confirmed that such activation could occur in a CD1d-independent manner (276). In addition, a study by Brigl et al. (2011), confirmed that iNKT cells can be activated and produce IFN-γ during microbial infection by innate TLR-driven IL-12/IL-18 and endogenous ligand-derived signals (277).

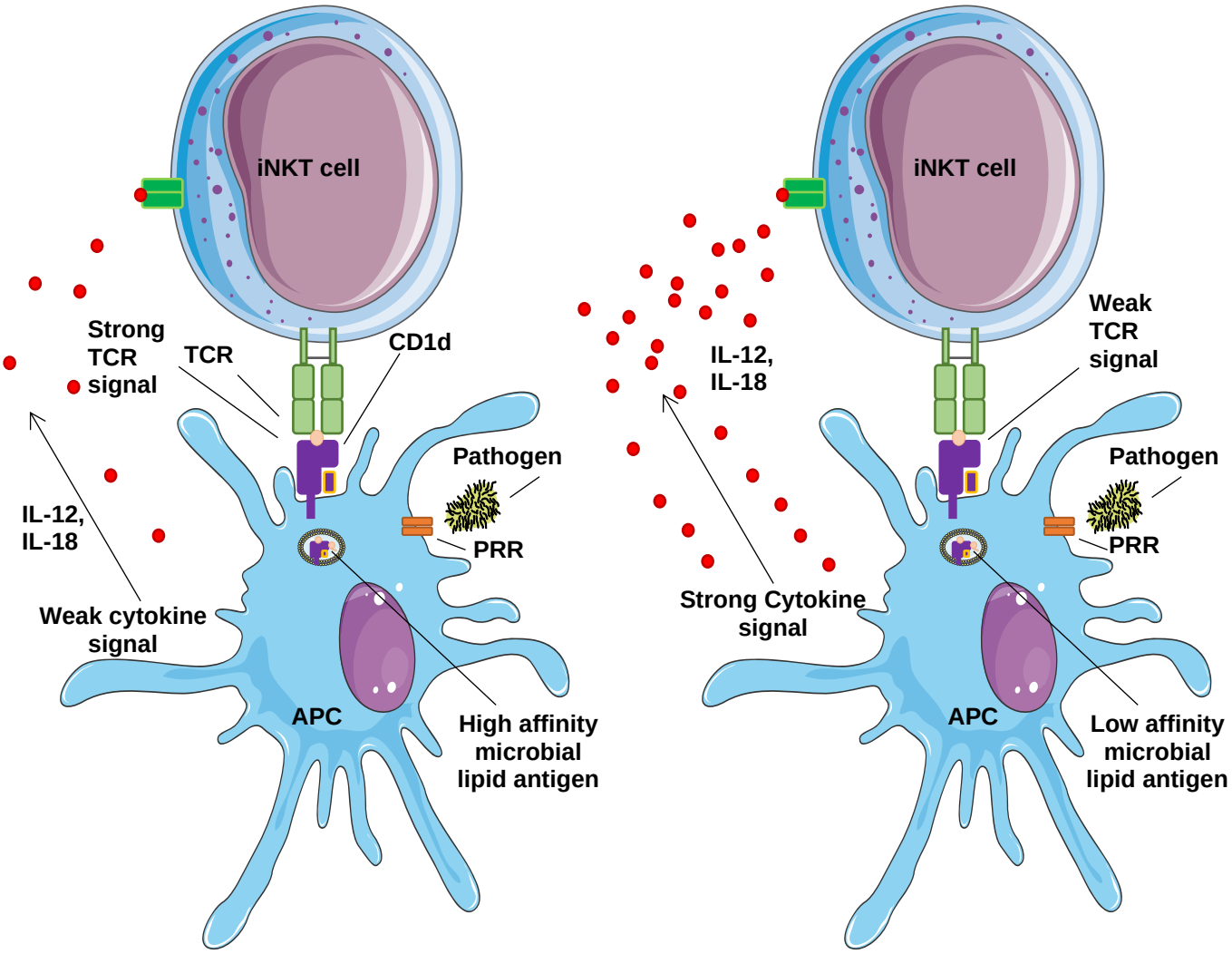

Figure 1.9 Schematic diagram of iNKT cells activation.
Activation of iNKT cells requires two distinct signals; the first signal involves APCs provide a dominant TCR signal that present high affinity glycolipid antigens in the context of CD1d with a weak cytokine signals derived from APCs. The second signal involves a dominant cytokine signals derived from APCs along with a weak TCR-CD1d lipid interaction. Adapted from Brennan et al., 2013, ***Nature Reviews Immunology*** **13, 101-117.**

1.5.7. iNKT cell subtypes

iNKT cells are known to form at least three distinct subsets based on their cytokine production and transcription factor expression (Figure 1.10). These three subsets are iNKT1/iNKT2/iNKT17 (202). In mice it was observed that iNKT1 form the dominant subset in spleen and liver and like Th1 cells, iNKT1 express T-bet and produce the signature cytokine IFN-γ (in addition to a proportion of iNKT1 being able to also secrete IL-4) (261, 271, 278, 279). In absence of T-bet, iNKT2 and iNKT17 cells were found to increase (280). Moreover, iNKT1 cells can be $NK1.1^{+}$, $NK1.1^{-}$, $CD4^{+}$ or $CD4^{-}$ (271).

iNKT2 express high levels of GATA-3 and produce IL-4 in addition to other TH2 associated cytokines such as IL-13, IL-9 and IL-10 (261, 281, 282). The use of GATA-3 deficient mice revealed that iNKT cells are unable to express IL-4 even after α-GalCer stimulation (280). iNKT17 cells are analogous to Th17 cells in that they express the transcription factor RORγT and produce IL-17 when activated (283). Transcription factors Th Poxviruses and zinc finger and Kruppel (Th-POK) are negatively modulated RORγt and mice lacking Th-POK show elevated Th17 associated gene expression like RORγt, CD103, CD121 and IL-17 and increased numbers of iNKT17 cells (202).

Regulatory iNKT cells that produce IL-10 have recently been identified and termed iNKT10 cells. iNKT10 express the E4BP4 transcriptional factor that facilitates IL-10 expression and these cells localise to adipose tissue, spleen and thymus in mice but are present only in very low numbers in human peripheral blood (284). iNKT10 cells express markers such as PD1, CTLA4 and Neuropilin-1 that are associated with Treg cells and produce cytokines as IL-2 and IL-10 (284, 285). Like T follicular helper cells, another subset of iNKT cells has been described as follicular helper NKT (NKTfh) cells and these cells are playing significant role in promoting GCs to produce memory B cells and long-lived plasma cells (286, 287).

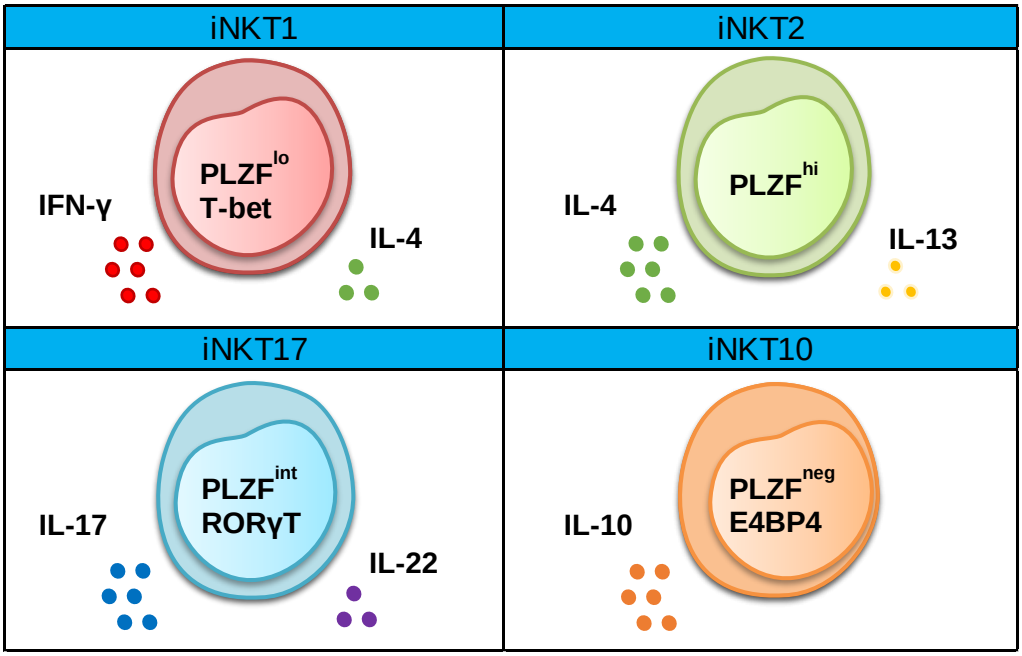

Figure 1.10 iNKT cells subsets.
The subsets of iNKT cells are based on their cytokine production and transcription factor expression. iNKT1 express T-bet as transcriptional factor with low level of PLZF for these cells and dominantly produces IFN-γ with low amount of IL-4. iNKT2 express PLZFhigh as transcriptional factor for these cells and dominantly produces IL-4 with some of other cytokines as IL-13, IL-9 and IL-10. iNKT17 in which they express the transcription factor RORγT with intermediate level of PLZF and preferentially produce IL-17. iNKT10 express E4BP4 as transcriptional factor for these cells and produces IL-10.

1.5.8. iNKT Cell Help for B Cell Antibody Production

The generation of GC, memory B cells, and high-affinity antibodies critically replies on help from primed $CD4^+$ T cells. However, it is now clear that iNKT cells may also participate in this process whereby iNKT cells can also provide help to B cells in certain contexts. For example, Galli et al., were the first to report that iNKT cells could provide help to B cells (288). The study showed that, like conventional $CD4^+$ T cells, human iNKT cells are able to affect the generation of antibodies *in vitro*. In this study, $CD4^+$ iNKT cells were cultured with/without the addition of α-GalCer with autologous purified human B cells for 10 days, results in they found the production of IgM And IgG1 induced with the presence of iNKT cells and absent with the addition of the anti-CD1d neutralizing antibody. The study concluded that the generation of the antibodies was observed with and without the presence of α-GalCer suggesting the involvement of both CD1d-dependent and endogenous ligands (288). iNKT cell help has been described as being either cognate or non-cognate and the outcome of these distinct forms of help has been shown to differentially impact the differentiation of B cells.

Many studies have shown that iNKT cell cognate help requires BCR-mediated uptake of CD1d-restricted antigens and the expression of CD1d on B cells (289, 290) and requires engagement of CD80/86 and CD40 on B cells by CD28 and CD40L expressed by activated iNKT cells. Cytokines released from iNKT cells as IFN-γ and IL-21 also play a role in shaping the B cell response (287, 291). Upon receiving cognate iNKT cell help, high titres of IgM are produced by B cells. This results in a primary response as well as the formation of plasmablasts and short-lived plasma cells (292).

Non-cognate help requires DCs to present glycolipid antigens to iNKT cells in the context of their CD1d (293). This results in DCs' activation and migration to the T-zone of secondary lymphoid organs. The activated DCs are then able to effectively prime conventional T cells to

acquire the capacity to interact with B cells, where they promote the generation of GC, memory B cells, secreting plasma cells, and high affinity antibodies (292).

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1.6. Thesis hypothesis and aims

1.6.1. Hypothesis

We hypothesise that glycolipids that activate iNKT cells have the potential to improve vaccination to FliC.

1.6.2. General Aims

We will aim to: -

I. Determine the phenotype and function of iNKT cell subsets in the small intestine, in terms of their cytokine production and transcription factor expression.

II. Perform phenotypic characterisation of classical DC subsets, in terms of their activation and cell surface markers expression.

III. Determine the impact of an agonist glycolipid (α-GalCer) on DC and iNKT cell subsets in the small intestine.

IV. Co-immunise mice with FliC and the iNKT cell agonist (α-GalCer) and monitor the dynamics of the iNKT cell response, the conventional T cell response and the B cell/antibody response to FliC as compared to responses following FliC immunisation only.

V. Examine the response to FliC in TLR5 deficient mice in the presence or absence of concomitant iNKT cell activation.

VI. Assess whether iNKT cells had any involvement in immune responses following FliC vaccination without exogeneous glycolipid activation as iNKT cells can be activated by proinflammatory cytokines independently of agonist glycolipid.

VII. Co-immunise mice with FliC and α-GalCer or analogues, namely C-glycoside or OCH, and determine the impact on the B cell/antibody response following FliC immunisation.

VIII. Examine the importance of IFN-γ to the enhanced response to FliC seen when concomitantly activating iNKT cells.

CHAPTER 2: MATERIAL AND METHODS

2.1. Mice

Mice were between 7 to 12 weeks of age at the initial of the procedure. All mice were sorted by gender and either sourced from Charles River Laboratories or bred in-house (Appendix I). Also, all mice genetically modified used, were bred in house and they were sourced originally as provided in Table. Mice were kept in SPF conditions at the University of Birmingham's Biomedical Service Unit in the United Kingdom. Animals (Scientific Procedures) Act 1986 and the ethical provisions of the University of Birmingham ethics committee were used for study guidelines.

Table 2.1 Genetically modified mouse strains used and breeds

strain	Origin	Age of experiment
C57BL/6	Bred in house	8-12 weeks of age
C57BL/6	Charles River	8-12 weeks of age
IFN-γ reporter mice (C57BL/6)	Bred in house	8-12 weeks of age
RORγT reporter mice C57BL/6	Bred in house (obtained from David Wither)	8-12 weeks of age
GREATxSMARTxIL -17a reporter mice	Bred in house (obtained from David Wither)	8-12 weeks of age
TLR5-deficient (C57BL/J x 129/Sv background)	Bred in house	8-12 weeks of age
CD1d-deficient C57BL/6	Bred in house	8-12 weeks of age

2.2. Preparation of Antigen and Glycolipids for either Injection or Immunization

2.2.1. Preparation of Glycolipids

Glycolipids prior to injection were sonicated for 5 min and then, heated for 5 min at 40 °C. All glycolipids were either obtained from NIH Tetramer Core Facility as C-glycolipid, OCH or purchased from Abcam as α-GalCer. All glycolipids were diluted in sterile PBS for i.p. injection.

2.2.2. Preparation of Antigen

All immunisations of antigen were applied to mice intraperitoneally and were diluted in sterile PBS as 100µg/ml. Antigen which was used for immunisation was Soluble Recombinant Flagellin (sFliC) in final concentration of 20µg.

2.2.3. Soluble Recombinant Flagellin (sFliC)

The details of the isolation of sFliC from STm were described in Cunningham et al. (2004a); however, we will briefly describe them here. FliC was amplified from *Salmonella* SL3261 and ligated into pET22b+ (Merck Chemicals, Nottingham, UK) to generate pET22b+ FliC Xho1 with a C-terminal His-tag. This is known as a beta-lactamase gene, which provides multi-resistance to ampicillin and over-expresses FliC after the isopropyl β-D-thiogalactopyranoside (IPTG) induction of the Lac operon. Therefore, sFliC was isolated because it is over-expressed in LB.

LB media contained bacteria incubated overnight at 37 °C aeration supplemented with ampicillin. The incubated culture was diluted 10X in fresh LB media and incubated for about another 4 h. Then, IPTG (Promega, Southampton, UK) at a final concentration of 1 mM was added to induce sFliC expression once the mid-log phase was reached (ODλ600 0.6–0.8). The incubated culture was then incubated for a further 2.5 h, and the cell paste was centrifuged at a high speed of 6000 x g for 20 min at 4 °C. Harvested cells were kept at -20 °C until the protein purification step was needed.

For protein purification, the cells were left at room temperature for 20 min after the Bugbuster (Merck Chemicals, Nottingham, UK) was added. Lysis cells were spun at a high speed of 16 000 x g for 30 min at 4 ºC. The supernatant was then incubated with Ni-NTA Sepharose (Qiagen, Hilden, Germany) for 1 h at room temperature. Then, flagellin was twice passed through a disposable Polypropylene Column (Qiagen). After the flagellin was purified using Nickel-affinity chromatography, the column was washed three times with phosphate-buffered saline (PBS). After the addition of 100 mM imidazole (Melford Laboratories) in 5 ml of PBS, the His-tagged protein was eluted. The elution of sFliC was dialysed against PBS in a cold room overnight.

After dialysis, sFliC was filtered via a 0.22 µm filter (Millipore, MA, USA). Then, with the help of Dr Margaret Goodall, sFliC was flowed and purified using an anti-FliC monoclonal antibody via affinity chromatography. Following this step, purified sFliC was dialysed against PBS in a cold room overnight. SDS-PAGE was used to identify the expected molecular weight ~56 kDa of sFliC, and no other protein was detectable after affinity chromatography (Appendix I). A BCA (Thermo Fisher Scientific, MA, USA) assay was used to determine the protein concentration. Sterile protein was aliquoted and kept at -20 ºC.

2.3. Cell isolations

2.3.1. Spleen

Spleens were dissected and put in complete Roswell Park Memorial Institute (cRPMI, Sigma-Aldrich, UK) media containing 1 x penicillin-streptomycin, 1x L-Glutamine and 10% foetal calf serum (FCS; Gibco) (R10). Syringe plunger was then used to mash the dissected spleen before passing it through a 70 µm sterile cell strainer (Thermo Fisher Scientific, UK). Centrifugation of the cells for 5 min at 400 g then followed as red cell lysing buffer (BD Biosciences, Franklin Lakes, USA) was used to lyse the red blood cells. Again, centrifugation was done for 5 min at 400 g. Finally, 10 ml Fluorescence-activated cell sorting (FACS) buffer (1 x PBS, 1 x Azide and 1% FCS) was used as a suspension to suspend the cells.

2.3.2. Mesenteric lymph nodes

Mesenteric lymph nodes (MLNs) were dissected, put in R10 after removal of fat. Digestion of the MLNs in 3 mL R10 with 7.5 µl Collagenase/Dispase (100 mg/ml; Roche, Germany) and 7.5ul DNase 1 (100 mg/ml; Roche, Germany) for 20 min followed at 37° C. Enzymtic reactions were eliminated by the addition of 60 uL EDTA. The mashing of the digested tissue is followed by filtration through a 70 µm sterile cell strainer. A 2.5 ml FACS buffer was then used to suspend the cells.

2.3.3. Small intestine

The small intestine was dissected and fat, and Peyer's patches were removed. The intestine was sliced into approximately 5 mm- sized pieces. 20 ml Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich, UK) with 2% FCS was used to clean the cut pieces. Twenty minutes incubation at 37° C then followed. During incubation, the intestines pieces were put in 20 ml HBSS with 2 mm EDTA and shaken at a speed of 220 rpm. Washing of the tissue with 20 ml HBSS only, no EDTA, followed. Again, the tissue was placed in 20 ml HBSS with 2 mm EDTA, incubated for 20 minutes at a temperature of 37° C and shaken at a speed of 220 rpm. Digestion of the

tissue in 15 ml R10 with 0.015g Collagenase P (Sigma-Aldrich, UK) was allowed for 15 minutes at 37°C after which, another 220-rpm shaking was done. The mixture was subjected to a two-stage filtration; first through a 100 µm then 70 µm sterile cell strainer, while being washed with 5mL FACS buffer. Finally, the cells were suspended in 5 mL FACS buffer.

2.3.4. Bone marrow (BM)

Bones were dissected from the femora and tibiae of mice and put in R10. A 25-syringe gauge needle (Terumo) was then used to flush the BM from the bone cavities of the femora and tibiae using R10. Centrifugation of the cells for 5 min at 400 g then followed as red cell lysing buffer was used to lyse the red blood cells. Again, centrifugation was done for 5 min at 400 g. Finally, 10 ml FACS buffer was used as a suspension to suspend the cells.

2.4. Flow Cytometry

2.4.1. Controls

Intracellular staining was done using isotype and Fluorescence minus one (FMO) controls. The isotype controls and FMOs enabled the application of gates and quadrants such that the percent positive cells were less than 1%. Unloaded tetramer (NIH Tetramer Core Facility) was used to apply the correct gate for iNKT cells.

2.4.2. Surface Staining

Zombie Aqua (ZA) (Life Technologies, USA) was used to stain cells to discriminate between live/dead cells for 10 minutes in the dark at room temperature (RT). Anti-Mouse CD16/32 was used to block Fc receptors and was incubated with cells for 10 minutes in the dark at RT. FACS buffer was used to dilute antibodies, after which 50 µl was added to each sample. Resultant samples underwent 30 min incubation at 4°C. After incubation, 1ml FACS buffer of was used to wash the samples twice.

2.4.3. Intracellular Cytokine Staining

250 µl of intracellular (IC) fixation buffer was used to fix samples followed by incubation of cells at 40C for 30 mins. After incubation, cells were washed with 1 ml of permeabilization buffer (PB). Intracellular antibodies were diluted in PB and added to each sample which were left for 45 mins at 4 ºC. Cells were washed twice in PB and once in FACS buffer before being acquired on a BD Fortessa flow cytometer (Becton Dickinson). BD FACSDiva software version 6.0 was used for acquisition and FlowJo, LLC V10.0.7 software (Tree Star, Inc.) was used to analyse the obtained data.

2.4.4. Ex-vivo Re-stimulation of iNKT Cells

Para-Methoxyamphetamine (PMA, Sigma) (500 ng/ml) and ionomycin (Sigma) (2500 ng/ml) were used to stimulate cells. Stimulation was followed by incubation at 37°C. The stimulated cells were incubated for 1h, after which 1 µl of Brefeldin A (BFA) (3.0 µg/ml) (e biosciences), and 1 µl of monensin (ebioscience) (2 µM), were added and the cells underwent a further 3h incubation. After that, cells were put in FACS tubes. Transferred cells were washed for 5 minutes with 1 mL of FACS buffer at 400 g. Finally, the cells were suspended in 1mL FACS buffer.

2.5. Cell Count

Beads (Spherotech inc.) were used in order to determine the number of cells which is been used ($1x10^4$ particles/10 µl). However, for all of our cell counts, we were used the equation below to determine them.

$$\frac{\text{Leukocytes number}}{\text{Bead number}} \times 10^4$$

Table 2.2 Antibodies were used to stain surface marker for flow cytometry analysis.

Marker	Fluorochrome	Clone	Company	Stock Conc.	Dilution
B220	FITC	RA3-6B2	Invitrogen	0.2 mg/ml	(1/200)
CD4	BV-711	GK1.5	Biolegend	0.2 mg/ml	(1/300)
CD8	BV-786	53-6.7	BD Pharmingen	0.2 mg/ml	(1/400)
CD11C	FITC	HL3	BD Pharmingen	0.2 mg/ml	(1/200)
CD11b	BV-421	M1/70	BD Pharmingen	0.2 mg/ml	(1/100)
CD1d tetramer (PBS57)	APC		NIH Tetramer core facility	0.2 mg/ml	(1/200)
CD19	BV-786	6D5	Biolegend	0.2 mg/ml	(1/800)
CD25	BV-605	PC61	Biolegend	0.2 mg/ml	(1/100)
CD38	BV-421	90	Invitrogen	0.2 mg/ml	(1/100)
CD40	PerCP	(3/23)	eBioscience	0.2 mg/ml	(1/200)
CD44	Alexa700	IM7	Invitrogen	0.2 mg/ml	(1/200)
CD62L	PerCP	MEL-14	Biolegend	0.2 mg/ml	(1/200)
CD86	PECy7	GL1	eBioscience	0.2 mg/ml	(1/200)
CD95	PECy7	J02	BD Pharmingen	0.2 mg/ml	(1/200)
CD103	PE	M209	eBioscience	0.2 mg/ml	(1/100)
CD138	BV-711	281-2	Biolegend	0.2 mg/ml	(1/200)
CXCR5	APC	Purified	BD Pharmingen	0.5 mg/ml	(1/150)
F4/80	FITC	BM8	eBioscience	0.2 mg/ml	(1/200)
Gr1	FITC	RB6-8C5	eBioscience	0.2 mg/ml	(1/200)
hCD271	PECy7	ME20.4	Biolegend	0.2 mg/ml	(1/200)
MHCII	Alexa700	M5/114.15.2	Biolegend	0.2 mg/ml	(1/200)
TCRb	APCCy7	H57-597	Biolegend	0.2 mg/ml	(1/200)
CD1d tetramer (PBS57)	PE		NIH Tetramer core facility	0.2 mg/ml	(1/200)
PD1	BV-421	29F.1A12	Biolgend	0.2 mg/ml	(1/200)
ZA	BV-510		Biolegend	0.2 mg/ml	(1/1000)

Table 2.3 Intracellular antibodies and their isotypes were used to stain for flow cytometry analysis.

Marker	Fluorochrome	Clone	Company	Stock Conc.	Dilution
RORγt	PE	B2D	eBioscience	0.2 mg/mL	(1/50)
GATA-3	APC	TWAJ	eBioscience	0.125 μg/mL	(1/50)
T-bet	PerCP Cy - 5.5	4B10	Biolegend	0.2 mg/mL	(1/50)
PLZF	PE Cy-7	9EE12	Biolegend	0.2 mg/mL	(1/50)
E4BP4	PE	S2M-E19	eBioscience	0.2 mg/mL	(1/50)
Foxp3	PE RED	FJK-16s	Invitrogen	0.2 mg/mL	(1/50)
IFN-γ	PE	XMG1.2	eBioscience	0.5 mg/mL	(1/50)
IL-17A	FITC	TC11-18H10.1	Biolegend	0.5 mg/mL	(1/50)
IL-4	FITC	11B11	eBioscience	0.2 mg/mL	(1/50)
IL-10	PE Cy-7	JES5-16E3	eBioscience	0.2 mg/mL	(1/50)
IL-13	PE	EBIO13A	eBioscience	0.2 mg/mL	(1/50)
Rat IgG1,k	PE	RTK2071	Biolegend	0.2 mg/mL	(1/50)
Rat IgG2b	APC	EB149/10H5	eBioscience	0.2 mg/mL	(1/50)
Mouse IgG1,k	PerCP Cy - 5.5	MOPC-21	Biolegend	200 μg/mL	(1/50)

2.6. Enzyme-Linked ImmunoSpot (ELISPOT)

Plates (MultiScreen; Millipore) were coated with FliC at 5 μg/ml in 1X PBS overnight at 4°C. The plates were then washed with 1X PBS and blocked with R10 media for 1 h at 37°C. Following incubation, cells were added in triplicate at $5x10^5$ or $3x10^5$ cells per well and cultured at 37°C for 6 h. The cells were lysed, washed three times before addition of either an AP-conjugated goat anti-mouse IgG or IgA (Southern Biotech) and the plates left overnight at 4°C. Sigma FAST BCIP/NBT (Sigma Aldrich) was added to develop the reaction after washing the plate three times. AID ELISPOT Reader System and AID software version 3.5 (Autoimmune Diagnostika, Germany) were used to count spots and counts were expressed as SFUs/5×10^5 cells.

List of Buffers used for ELISPOT assay, supplied from (Sigma-Aldrich, UK).

Coating Buffer	1X PBS dissolved in sterile distilled H2O to make up the Final volume to 500ml.
Blocking Buffer	sterile R10 medium contains RPMI with 10% FCS, 0.01 μm 2-ME, 1% penicillin, 1% L-Glutamine and streptomycin
Wash Buffer	make up 1X PBS, one tablet of PBS dissolved in 500ml of sterile distilled H2O, then add 0.01% Tween-20 in 1X PBS.
Dilution Buffer	0.05% Twwen-20 dissolved in 1X PBS make up final volume of 500ml.
ELISPOT Substrate	use of 10ml distilled H2O to dissolve 1 tablet Sigma FAST BCIP/NBT tablets.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

At time of sacrifice, cardiac puncture was performed, and blood withdrawn. Serum was separated from blood by centrifugation at 400g for 15 minutes. Serum was frozen at -80˚C until further required. ELISA plates (flat-bottomed, Thermo Fisher Scientific) were coated with FliC at 5 μg/mL in carbonate coating buffer (Na2HCO3/NaH2CO3/0.1% NaN3/dH2O) overnight at 4°C. The plates were then blocked (1% BSA/PBS) for 1 h at 37 ºC and washed (PBS/0.05% Tween 20) and serial dilutions of sera added. The plates were incubated for 1 h at 37 ºC, washed and Alkaline Phosphatase (AP) conjugated antibodies added (either AP-conjugated goat anti-mouse IgG (1:1000), IgG1 (1:1000), IgG2c (1:1000), or IgA (1:800); Southern Biotech) were added. The plates were washed and p-Nitrophenylphosphate (pNPP) added (Sigma) for 15 mins at 37 ºC to develop. ODs were read using the softmax pro programme at405 nm on SpectraMax ABS/ABS Plus Microplate Readers. Relative antibody titres were determined by plotting the dilution against the OD values. The dilution of each sample was taken at a constant OD value, set in the mid-exponential phase of the curve.

List of Buffers used for ELISA assay, supplied from (Sigma-Aldrich, UK).

Coating Buffer	1.42 g NaH2CO3, 0.83 g Na2HCO3, 0.1% NaN3, all chemicals were weighed out and dissolved in 450ml of distilled H2O to make up the Final volume to 500ml.
Wash Buffer	make up 1X PBS, one tablet of PBS dissolved in 500ml of distilled H2O, then add 0.05% Tween-20 in 1X PBS.
Blocking Buffer	%1 Bovine Serum Albumin (BSA) dissolved in 1X PBS, make up final volume of 500ml.
Dilution Buffer	%1 BSA, 0.05% Twwen-20 dissolved in 1X PBS make up final volume of 500ml.

ELISA Substrate use 20 ml of distilled H2O to dissolve 1 tablet SIGMAFAST p-Nitrophenyl phosphate plus 1 tablet Tris buffer.

Table 2.4 Antibodies were used for ELISPOT and ELISA analysis and their sources

Specificity	Conjugate	Company	volume	Dilution
Goat Anti-Mouse IgM	Alkaline Phosphatase	Southern Biotech	1.0 ml	(1/5000)
Goat Anti-Mouse IgG	Alkaline Phosphatase	Southern Biotech	1.0 ml	(1/1000)
Goat Anti-Mouse IgG1	Alkaline Phosphatase	Southern Biotech	1.0 ml	(1/1000)
Goat Anti-Mouse IgG2C	Alkaline Phosphatase	Southern Biotech	1.0 ml	(1/1000)
Goat Anti-Mouse IgA	Alkaline Phosphatase	Southern Biotech	1.0 ml	(1/1000)

2.8. Statistical Analysis

GraphPad Prism 8 (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA) was used to analyse data. The significance of the difference between data sets was determined by student t-test (unpaired; two-tailed). The data were interpreted as being statistically significant when the p-value was <0.05. The mean ± standard error of the mean (SEM) was used for data presentation.

CHAPTER 3: CHARACTERISATION OF INTESTINAL INKT CELLS AND CLASSICAL DC SUBSETS

3.1 Introduction

NKT cells are a subset of innate-like T cells that typically express markers of NK cells and are distinct from conventional αβ T cells despite bearing an αβ TCR. They recognize glycolipid antigenic determinants (not peptides as with conventional T cells) presented by the non-polymorphic MHC I-like molecule CD1d via their TCR (201). They are rapidly activated on recognition of glycolipids releasing cytokines that can activate other immune cells such as DCs. Such CD1d mediated activation leads to the rapid release of specific cytokines such as IFN-γ. iNKT cells can be further divided into subsets based on their transcription factor profile and their pattern of cytokine production i.e. iNKT1/ iNKT2/ iNKT17 (211). However, unlike naïve conventional T cells iNKT cell activation results in the rapid release of cytokine without further differentiation or proliferation. Moreover, there are other subsets that have been identified in periphery known as NKT10 and NKTfh cells. These cells are induced in tissue following antigenic stimulation such as αGalCer (285, 294). The iNKT TCR recognise certain α- and β-linked glycolipids when presented by CD1d with the glycolipid αGalCer the most potent for induction of cytokine release (203).

It has been found that all intestinal DCs express CD1d but it is higher in cDC2 ($CD103^+$ $CD11b^+$) (295). These cells are described as the main cells that migrate to the MLN from the lamina propria of small intestine (296). Therefore, cDC2 in MLN and small intestine may present glycolipids to iNKT cells and influence immunity. This was confirmed by a study that found glycolipid presentation by intestinal DCs can activate and shape local iNKT cells in intestinal lamina propria (295, 297, 298). The presence of intestinal iNKT cell populations are provided a significant role of regulation the mucosal immunity as well as the development of intestinal inflammation (299).

In mice, NKT cells are found in the small intestine where they increase in frequency over the first month of age (297, 300). There has been evidence that the number of intestinal iNKT is

higher in the lamina propria of small intestine than in the epithelial layer as the number of iNKT cells were found to inversely correlate with bacterial proximity and bacterial density (295, 301).

The make up of iNKT cell subsets in the intestinal tract has been reported to be dominated by NKT1 cells which have been reported to constitute up to 85% of iNKT cells in lamina propria, Peyer's patches and colon of naïve B6 mice. In addition, NKT17 constitute up to 10% and NKT2 around 5% of the iNKT population residing in gut (295, 301). Moreover, NKT1 cells are also found in gut-draining lymph node but with more of a mixture of NKT2 and NKT17. Therefore, although NKT1 are dominant in the small intestine at steady state, there is a possibility that other subsets of iNKT cells may also confer different functional qualities to subsequent immune responses under certain inflammatory conditions. Therefore, combinations of glycolipids that activate iNKT cells and the agonists that induce immune cells via TLRs have a high chance of providing efficient adjuvant vaccines.

FliC which is the fundamental flagellin protein is a strong immunogen since this protein has the capability of inducing potent immune responses to itself through its intrinsic adjuvancy and to co-administered Ags through the use of extrinsic adjuvancy. Using FliC as an adjuvant is sufficient to drive T and B cell responses that lead to microbicidal activities, the production of cytokine, and the activation of the dendritic cells (168). Currently, flagellin is being developed as an adjuvant that may aid vaccination due to its adjuvanticity via TLR5 and subsequent induction of a Th2-biased response (175).

Flagellin is molecule that can be engineered in specific ways for vaccine development to retain immunogenicity. For example, Flagellin has been designed to display foreign epitopes of specific pathogens such as influenza and Helicobacter pylori (176). Moreover, flagellin fusion proteins has been enhanced the humoral responses and it is been attractive alternative for vaccine design due to its greater efficacy of enhanced antigenicity (177).

It is been shown that DCs play an important role in induction of specific immune responses and the maintenance of self-tolerance (302). Targeting such DCs are key initiators and modulators of systemic immune system and enhancing the vaccine responses (303, 304). Moreover, there are several DC subsets in lymphoid and non-lymphoid tissues which suggests that DC function may be influenced by their specific location. These subsets are classified as cDC1 or cDC2. cDC1 require transcription factors such as IRF8, BATF3, and ID2 for their development. In contrast, cDC2 develop independently of these transcription factors but rather require the transcription factor IRF4 for their function and survival.

These subsets of DCs are significant as they clearly identified the DCs based on their anatomical location either in lymphoid or non-lymphoid tissues (Table 1.1) (305, 306). For instance, the intestinal mucosal of cDCs are sub populations as cDC1 ($CD11c^+MHCII^+CD103^+CD11b^-$), cDC2 ($CD11c^+MHCII^+CD103^+CD11b^+$) and $CD103^-$DC ($CD11c^+MHCII^+CD103^-CD11b^+$). Each of these subsets can provide a significant role by supporting the control of immune homeostasis of the intestinal mucosa (307-309).

Among systematic lymphoid tissues like the spleen, DCs presenting foreign antigen usually stimulate the development of effector T cells (310). However, in other anatomical locations like the gut, it has been found that $CD103^+$ DCs in the small intestine and mesenteric lymph nodes show regulatory functions (311). Upon activation, the $CD103^+$ lamina propria DC (LPDC) are known to move from the small intestinal lamina propria to the mesenteric lymph nodes in a CCR7-dependent manner (312). This is to ensure that there is the promotion of the generation of $Foxp3^+$regulatory T cells via retinoic acid (312). However, other studies have reported that $CD11b^+F4/80^+CD11c^-$ macrophages in the small intestinal lamina propria are more effective stimulator of regulatory T cells than the LPDCs (311). Furthermore, the $CD11b^+$ LPDCs are responsible for generating T cells which produce IL-17. All these studies together suggest that the LPDCs stimulate Treg development as well as Th17 cells (311). Despite these studies, it is

still indecipherable which form of stimulation enables LPDC to facilitate the generation of Th17 cells.

TLR5 is expressed by specific DC populations in particular, the $CD11c^{+}$ lamina propria DCs situated in the small intestine (313). Therefore, in small intestine, TLR5 signalling may play a dominant role in the recognition of certain bacterial species, particularly gram negative, flagellated bacteria. This is significant as TLR5 triggering results in the induction of CD103 and IL-23 expression which leads to resistance to bacterial colonization (314). In the absence of exogenous adjuvant, flagellin proteins like FliC can have immunomodulatory roles and can lead to an immune response against themselves (312). Also, $TLR5^{-/-}$ mice display resistance to oral Salmonella typhimurium infection since these bacteria use TLR5 expressed by $CD11c^{+}$ LPDCs to mediate systemic infection (313).

3.2 Aims and Hypothesis

We hypothesise that glycolipid administration will activate iNKT cells in the small intestine and will act as an adjuvant to improve FliC vaccination.

3.2.1 Aims

Overall aim

- To use the iNKT cell activator (α-GalCer) together with soluble flagellin protein (FliC) in order to induce long-lived, systemic and mucosal humoral immunity.

Aims of this study

- Determine the phenotype and function of iNKT cell subsets in the small intestine, in terms of their cytokine production and transcription factor expression.
- Perform phenotypic characterisation of classical DC subsets, in terms of their activation and cell surface markers expression.
- Determine the impact of an agonist glycolipid (α-GalCer) on DC and iNKT cell subsets in the small intestine.

3.3 Results

3.3.1 iNKT cells are present in the small intestine and are predominately NKT1

The project proposes to use the activation of iNKT cells to enhance vaccination responses to flagellin (FliC) which is known to require the activation of cDC2 in the small intestine (315). We first needed to confirm the presence and the dominant subset of iNKT cells in the small intestine.

To this end, we analysed the small intestine, spleen and MLN of naïve B6 mice and assessed the proportion and number of iNKT cells in each tissue (Figure 3.1 A and B). iNKT cells were first gated as singlet live lymphocytes and then identified by staining with a PBS57-Tetramer as being tetramer^{+} TCRβ^{+}. iNKT cells represented around 0.2% of all small intestine lymphocytes and around 0.55% in spleen (Figure 3.1 A and B). Whilst in MLN, iNKT cells represented around 0.05% of all MLN lymphocytes (Figure 3.1 A and B). Analysis of iNKT cell confirm the presence of iNKT cells in the small intestine (Figure 3.1 A and B).

To determine the phenotypes of iNKT cells, the expression levels of lineage-defining transcription factors was assessed. As has been previously reported, the majority of iNKT cells in the spleen were T-bet^{+} with only a small population expressing RORγt (271, 278, 295). In small intestine, 65-75% of iNKT cells expressed T-bet with 7-10% expressing RORγt (Figure 3.2 A and B). The majority of iNKT cells in spleen expressed T-bet (85-90%) with 2-3% expressing RORγt. In contrast, 35-40% of iNKT cells in the MLN expressed T-bet with 10-15% expressing RORγt (Figure 3.2 A and B).

In addition to defining the subset composition in tissue by transcription factor expression the subset distribution was also defined using differential cytokine production. iNKT cells obtained from small intestine, spleen and MLN were stimulated with PMA and ionomycin, followed by BFA and monensin, and analysed for the production of IFN-γ, IL-4, IL-10 and IL-17A (Figure

3.2 C and D). Upon activation, the majority of iNKT cells in the small intestine (NKT1) produced IFN-γ (~65-70%) with small populations of 10–15% producing either IL-17A (NKT17) or IL-4 (NKT2). In spleen, the majority of iNKT cells produced IFN-γ (NKT1) as well with around 5% producing IL-4 only (NKT17) (Figure 3.2 C and D). However, MLN iNKT cells produced a mixture of cytokines. 35-40% of MLN iNKT cells produced IFN-γ (NKT1) and around ~20% produced IL-4 only (NKT2). Also, 15-25% of MLN iNKT cells produced IL-17A (NKT17) and very few producing IL-10 (NKT10) (Figure 3.2 C and D).

3.3.2 Reporter mice confirm the dominance of the NKT1subset in the small intestine

Due to our observation that IFN-γ constituted the dominant population in the small intestine (Figure 3.2 C and D), we decided to use IFN-γ reporter mice which label IFN-γ producing cells with yellow fluorescent protein (YFP) to measure the steady state IFN-γ transcription in vivo. Furthermore, although NKT17 cells were found to be a minor population in small intestine and spleen we also investigated NKT17 cells using a RORγt reporter mouse. In addition, IL-17A was further investigated using GREATxSMARTxIL-17a reporter mice (IL-17A reporter). This mouse fate maps cells that have expressed IL-17A in red (RFP^+) as well as identifying cells actively expressing IL-17A (via the expression of human CD271; Figure 3.3 A and B).

As expected most NKT cells in the small intestine and spleen expressed IFN-γ as judged by reporter expression even under homeostatic conditions (Figure 3.3 A and B) whereas approximately 55% of iNKT cells in the MLN were YFP^+ (Figure 3.3 A and B). In contrast, the RORγt reporter mice showed a minor 5-10% population of RORγt expressing iNKT cells in all tissues. Consistent with this observation, IL-17A fate mapped iNKT cells were also a minor population and less than 4% of iNKT cells were found to actively be expressing IL-17A confirming the paucity of NKT17 cells in these tissues (Figure 3.3 A and B).

3.3.3 α-GalCer iNKT cell activation results in the modulation of cell surface proteins within 48 hr.

Next, we sought to determine whether iNKT cells could be activated in the small intestine in addition to spleen and MLN following injection with α-GalCer. To this end, B6 mice received 2µg α-GalCer i.p. and iNKT cells derived from the small intestine, spleen and MLN of were analysed 48 hours later (Figure 3.4). Initially we determined the proportion and absolute number of iNKT cells in each tissue. This revealed that the number and percentage of small intestine and spleen iNKT cells in α-GalCer mice were dramatically decreased compare to control mice (Figure 3.4 A and B). However, the number and percentage of MLN iNKT cells in control mice were lower than treated mice (Figure 3.4 A and B).

In pilot studies we found that the most robust correlate of activation of iNKT cells was the upregulation of PD-1 and CD25. Therefore, we also assessed the expression of PD-1 and CD25 on iNKT cells in the small intestine following α-GalCer injection as markers of activation (Figure 4.3 C and D). In the small intestine, iNKT cells were found to have upregulated both PD-1 and CD25 in activated mice compared to control mice (Figure 3.4 C and D) to a similar degree to iNKT cells found in the spleen following α-GalCer injection (Figure 3.4 C and D). Interestingly, despite α-GalCer administration, MLN iNKT cells were found to have similar proportions of PD-1^+ and $CD25^+$ cells as in unactivated controls (Figure 3.4 C and D).

3.3.4 cDC2 found in the small intestine express CD1d.

iNKT cells will only respond to glycolipid if it is presented by CD1d therefore the CD1d expression of different cDC subsets found in the small intestine was investigated in naïve B6 mice (Figure 3.5). cDCs subsets were gated as singlet live leukocytes and were identified as Lin^- (CD3, B220, F4/80, Gr1) $CD11c^{hi}MHCII^+$. cDC subsets were further defined as mucosal

cDC2 ($CD103^+$ $CD11b^+$), mucosal cDC1 ($CD103^+$ $CD11b^-$) and mucosal $CD103^-$ cDC ($CD103^-$ $CD11b^+$), and splenic cDC1 were gated as $CD8\alpha^+$ cells and cDC2 as $CD4^+$ cells (Figure 3.5 A).

In small intestine, the CD1d expression was ~ 95% in cDC2 cells and the level of CD1d expression showed a significant increase in cDC2 in comparison to other cDC subsets. In contrast, the number of cells that expressed CD1d in different DC subsets were similar (Figure 3.5 B and C). In MLN, 90% in cDC2 cells were found to express CD1d expression and the level of CD1d expression was higher than other cDC subsets. However, the number of cells that expressed CD1d in cDC2 and cDC1 were similar but significantly lower than of $CD103^-$DC (Figure 3.5 B and C). In the spleen, CD8 and CD4 DC expressed at around ~90% of CD1d. The number and proportion of cells that expressed CD1d in spleen were similar. However, the level of CD1d expression showed a significant loss of CD4 in comparison to CD8 (Figure 3.5 B and C). Despite the selective increase in the cDC2 frequency in small intestine and MLN, cDC2 reflected the upregulation of MFI-CD1d in small intestine and MLN (Figure 3.5 B and C).

3.3.5 α-GalCer induces the accumulation of DCs in the MLN after 24hr

Given that most cDC subsets express CD1d we next sought to determine the impact of iNKT cell activation on cDC subsets. cDC subsets were assessed in small intestine, MLN and spleen 24 hours post α-GalCer i.p. injection (Figure 3.6). A significant increase in cDC2 frequency and number was observed in MLN of mice that received α-GalCer compared to control mice (Figure 3.6 A and C). In contrast, the cDC2 frequency and number in the small intestine after the α-GalCer injection were not altered (Figure 3.6 A and B). However, cDC1 and $CD103^-$ DC showed a significant decrease in the frequency of MLN following iNKT cell activation, while the MLN numbers and the small intestine frequency and number were comparable to those of the control mice (Figure 3.6 A, B and C).

In mLN, the CD40 expression was significantly decreased in the α-GalCer injected group, while the expression of both CD86 and CD1d were comparable to those of control DC. The MFI in CD40, CD86 and CD1d of small intestine was comparable to control (Figure 3.6 B and C). In the spleen, CD4 in DCs was loss following α-GalCer administration, showing that the frequency and numbers did decrease in the α-GalCer injected group in comparison to the control mice group (Figure 3.6 D). Importantly, α-GalCer was able to induce the MFI in CD1d, CD86 and CD40 in spleen (Figure 3.6 D). This increase of the level of expression of CD1d, CD86 and CD40 showed that the DCs were activated following α-GalCer injection (Figure 3.6). Taken together these data suggest that the administration of α-GalCer induces an increase in frequency and numbers of cDC2 in MLN and induce the CD1d, CD86 and CD40 level of expression in spleen (Figure 3.6).

3.3.6 α-GalCer induces the accumulation of cDCs in the small intestine after 48hr

We found that cDC2 were activated and increased in the frequency and number in MLN 24 hr after mice were injected with α-GalCer. These finding suggest that cDC2 might migrate from the small intestine lamina propria and accumulate in MLN. We now examined whether such DCs returned to the small intestine or migrated elsewhere. To this end, cDC subsets were assessed in small intestine, MLN and spleen 48 hours after α-GalCer injection (Figure 3.7). We found that the frequency of cDC2 was significantly increased in the small intestine of injected mice compared to control mice (Figure 3.7 A, and B). In contrast, the cDC2 frequency and numbers following an α-GalCer injection were comparable to those of the control mice in MLN and spleen. However, α-GalCer caused a decrease cDC1 in MLN and spleen at this time (Figure 3.7 C and D). This activation of DCs showed that there was still a response to α-GalCer after 48 hr. Taken together these data suggest that the injection of α-GalCer induces increased numbers of cDC2 in the mLN after 24 hours and in the small intestine after 48 hours.

3.4 Discussion

Flagellin can drive immune responses in gut post systemic immunization. Mucosal $CD103^+$ DCs have been to be present in MLN and small intestine following systemic FliC immunization (295, 315, 316). Targeting these cells with a glycolipid as α-GalCer would be an effective way to drive immune responses in mucosal tissue (295, 315, 316) and improve the efficacy of vaccines. The overall aim of this project is to use the iNKT cell activator (α-GalCer) together with FliC in order to induce long-lived, systemic and mucosal humoral immunity (315, 316).

Given that we propose that iNKT cells in mucosal tissues might aid mucosal antibody responses upon vaccination we first sought to define the functional characteristics of iNKT cells in the small intestine. It is known that iNKT cells can rapidly secrete a massive amount of cytokines once they are activated (271). Therefore, the frequencies, number and phenotype of intestinal iNKT cells was investigated and characterized.

iNKT cells have previously been reported in mucosal tissues and they account for up to 5% of $TCR\beta^+$ T cells in the whole gut of mice (295, 301). Our data showed that in lamina propria of the small intestine, iNKT cells make up approximately 1% of $TCR\beta^+$ T cells, with varying frequencies across mice (Figure 3.1 A and B). It has been previously demonstrated that the dominant subset of iNKT cells in the small intestine and spleen are NKT1 cells that express T-bet and produce IFN-γ upon activation (271, 278, 295). In the studies presented here we confirmed that the majority of the intestinal iNKT cells secreted IFN-γ when activated in B6 mice (Figure 3.2 C and D). Moreover, analysis of lineage defining transcription factors also revealed that T-bet expressing cells were the most frequent iNKT cell population in the small intestine (Figure 3.2 A and B). IFN-γ reporter mice were also used to confirm that NKT1 cell dominate the pool of intestinal NKT cells (Figure 3.3 A and B).

Another location important for the generation of immune responses to FliC is the gut-draining MLN. Here, iNKT cell subsets showed more of a mixture picture with a sizeable population of

NKT1 but with a significant population of NKT2 cells (Figure 3.2 and 3.3) but again few NKT17 cells (Figure 3.2 and 3.3). Overall, these data demonstrate that the phenotype and function of the majority of intestinal iNKT cells in steady state are Th1, we therefore postulated that activating gut iNKT cells may enhance both clearance of STm infection and temper the Th2 response generated by FliC alone (317, 318) whilst promoting Th1 responses that have been shown to provide long term protection against STm (318).

Next, we sought to determine whether the i.p. administration of α-GalCer would activate iNKT cells in the small intestine as well as in the spleen. It has been shown that cDC2 are capable of processing and presenting α-GalCer (274, 295, 319), subsequent to which α-GalCer can activate iNKT cells following administration. α-GalCer was administered in vivo for 48 hr. The number and percentage of intestinal iNKT cells in α-GalCer mice were dramatically decreased compare to control mice which was likely due to TCR downregulation following activation. In order to visualise iNKT cell activation we used surrogate markers of activation, the modulation of CD25 and PD-1, which have been described as being upregulated following iNKT cell activation (274, 319, 320). We found that the majority of iNKT cells in the small intestine had upregulated PD-1 and to a lesser extent CD25 by 48 hours after administration of α-GalCer. These data suggest that indeed iNKT cells were activated by α-GalCer in the small intestine by 48 hr after α-GalCer administration (Figure 3.4). Alternatively, it is possible that PD-1 activated cells might have homed to the small intestine following activation in SLOs. These cells showed a small portion of negative expression in small intestine among high expression of PD-1 in small intestine while spleen revealed indeed high levels of PD-1 expression (Figure 3.4 D).

Thus far we had shown that iNKT cells in the small intestine are predominately NKT1 cells and that they can be activated following i.p. administration of α-GalCer (295, 321). Next it was important to determine the CD1d expression on cDC subsets in the small intestine and MLN and assess whether the activation of iNKT cells had any impact on the relative frequency and

number of these cDC subsets. These analyses showed that cDCs in small intestine, MLN and spleen all express CD1d (Figure 3.5). Furthermore, cDC2 in all tissues showed a higher level of CD1d expression in comparison with cDC1 and $CD103^-$ DC (Figure 3.5) (295). This was also suggested in a previous study that showed that all intestinal DCs express CD1d but it is higher in cDC2 ($CD103^+$ $CD11b^+$) (295). These findings support our hypothesis that exogenous glycolipid could be presented to gut iNKT cells by cDC2 which would be likely necessary in order to boost responses to FliC where cDC2 are absolutely essential (295, 321). Experiments have bene conducted to try to detect the presence of α-GalCer/CD1d on CDC2 *ex vivo* however no complexes where detected. This may be due to the poor efficacy of the anti-α-GalCer/CD1d used therefore in future experiments cDC2 could be isolated from small intestine and cultured with α-GalCer in vitro. The ability of such cells to activate iNKT cells in vitro could then be assessed.

The response of cDCs in small intestine, MLN and spleen was assessed 24 and 48 hrs after α-GalCer administration. cDC2 in MLN were recruited in response to α-GalCer 24 hr after administration, suggesting that cDC2 migrated from SI to MLN (Figure 3.6). Therefore, in this time period, the administration of α-GalCer was shown to induce migration of cDC2 from the SI to MLN even in the absence of TLR5 triggering. Such migrated cDC2 were also found to have upregulated CD1d and CD86 by 24 hr post injection in the MLN consistent with activation (295, 315, 321). Taken all together suggest that whether 48hr post injection mice with α-GalCer would activate DCs in small intestine. DCs responses in small intestine, MLN and spleen were analysed 48 hr after injecting mice with α-GalCer administration. Consequently, we found that cDC2 increased in the small intestine of injected mice in comparison to control mice which correlated with a normalising of numbers in the MLN (Figure 3.7). These finding support that 48 hr subsequent to α-GalCer administration activation of cDC2 can be induced in the small

intestine (Figure 3.7). However, the accumulation of cDC2 in the MLN cells arise as a result of migration from other sites in addition to the small intestine (295, 321).

In conclusion, the dominant population of iNKT cells in the small intestine are NKT1 cells which can be activated following the administration of α-GalCer i.p (295, 301, 320, 321). The activation of small intestine iNKT cells is likely to occur in situ as cDC (in particular cDC2 that are also responsible for the presentation of flagellin) isolated from the small intestine express high levels of CD1d (295, 315). The activation of iNKT cells was found to affect cDC populations in both small intestine and MLN however whether this is a consequence of iNKT cell activation in the small intestine remains to be determined. Taken together the data suggest that iNKT cells activated in the small intestine are likely to influence adaptive mucosal immune responses to flagellin and therefore glycolipids that activate iNKT cells may be a useful adjunct strategy to boost immune responses after vaccination (295, 315, 321).

3.5 Figures

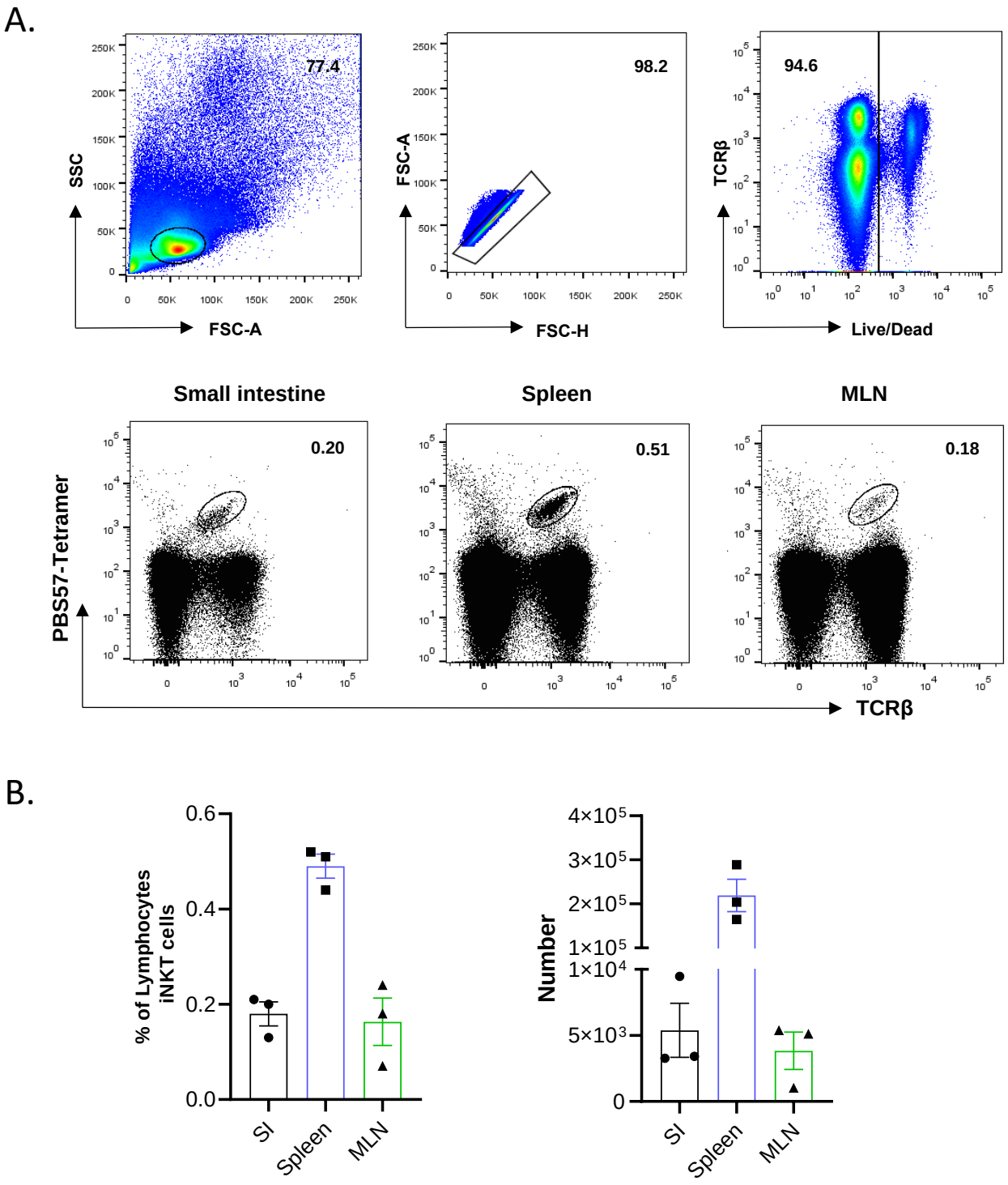


Figure 3.1 iNKT cell can be identified on small intestine.
A) iNKT cells were selected as singlets live cells then all tissues were identified as PBS57-Tetramer^{+} TCRβ^{+}. B) Percentage and number of iNKTs in lymphocytes of different tissues. Data are of 3 mice.

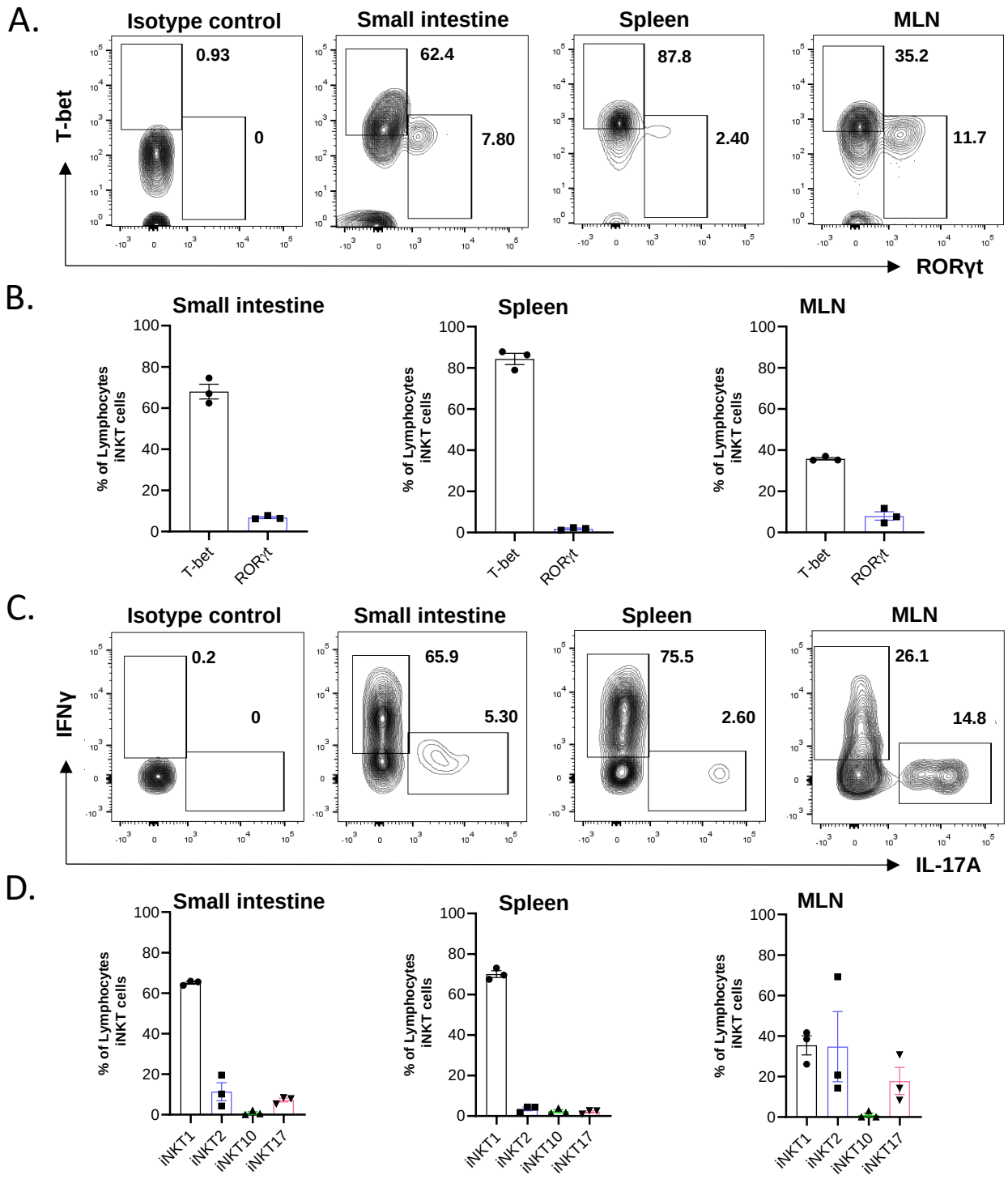


Figure 3.2 iNKT cell subsets can be distinguished based on their cytokines and transcription factor expression.

Freshly isolated of small intestine, splenocytes and MLN were stimulated with PMA and ionomycin then culture in 37°C for 1hr and following with BFA and monensin then incubated for 3 hr. Then cells were analysed by flow cytometry after fix them with IC fixation buffer A) iNKT cells were identified as PBS57-Tetramer^{+} TCRβ^{+}. B) Percentage and number of iNKTs in lymphocytes of different tissues. C) The expression levels of transcription factors iNKT cells in small intestine, splenocytes and MLN. D) Intracellular expression of iNKT1, iNKT2, iNKT10 and iNKT17, which were representative of IFN-γ, IL-4, IL-10 and IL-17A respectively, were assessed in spleen, small intestine and MLN. Data are of 3 mice.

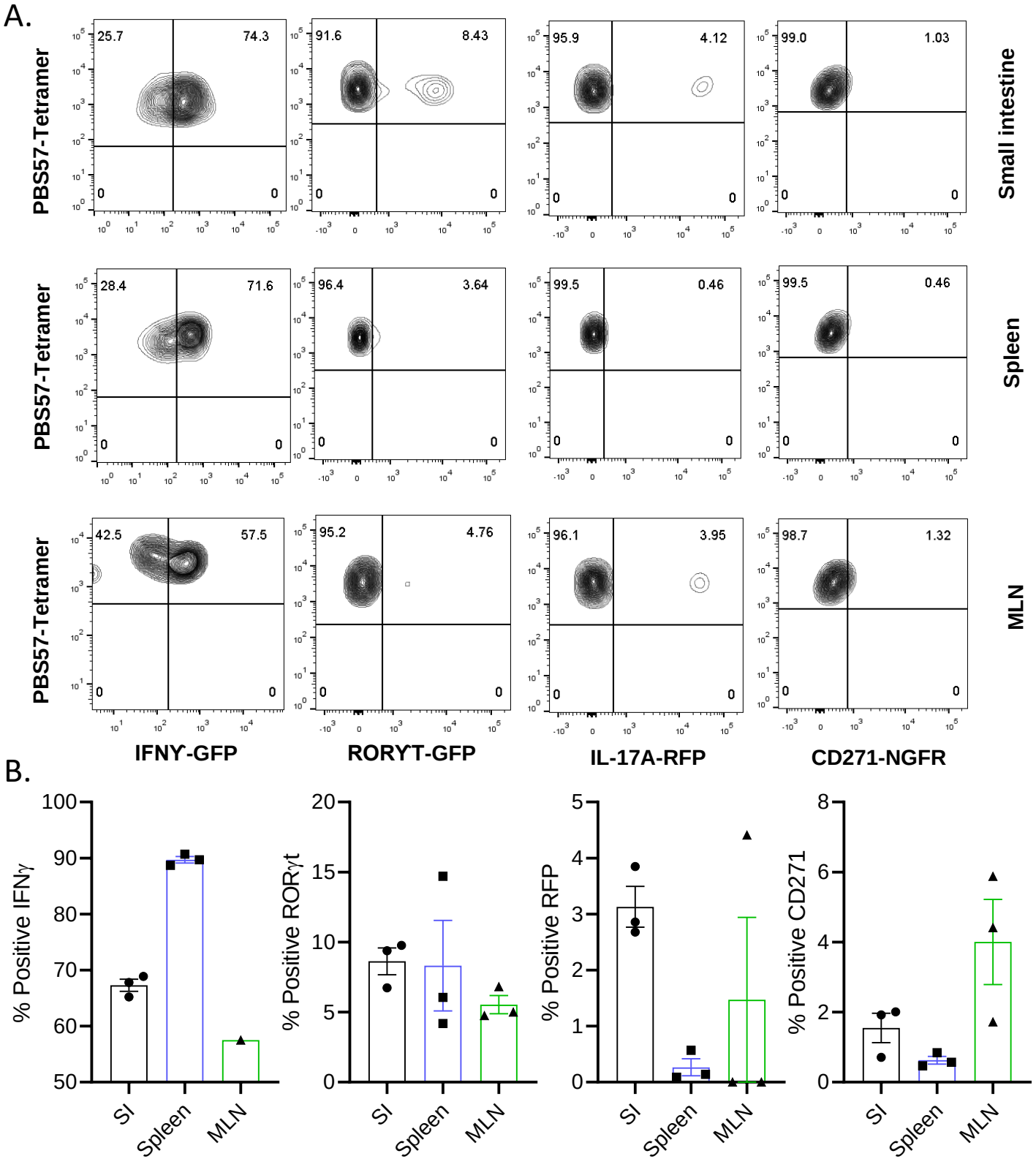


Figure 3.3 In vivo, iNKT cells are predominantly NKT1 with NKT17 cells being a minor population. small intestine, splenocytes and MLN were isolated from mice reporter (IFN-γ reporter, RORγt reporter and IL-17A reporter) then cells were analysed by flow cytometry for endogenous expression of IFN-γ, RORγt and IL-17A production. A) Representative dot plots of IFN-γ, RORγt and IL-17A fate mapped and CD271 (NGFR) were shown in small intestine, spleen and mLN. B) Percentage of positive IFN-γ, RORγt and IL-17A fate mapped and CD271 (NGFR) were shown. Data are of 3 mice.

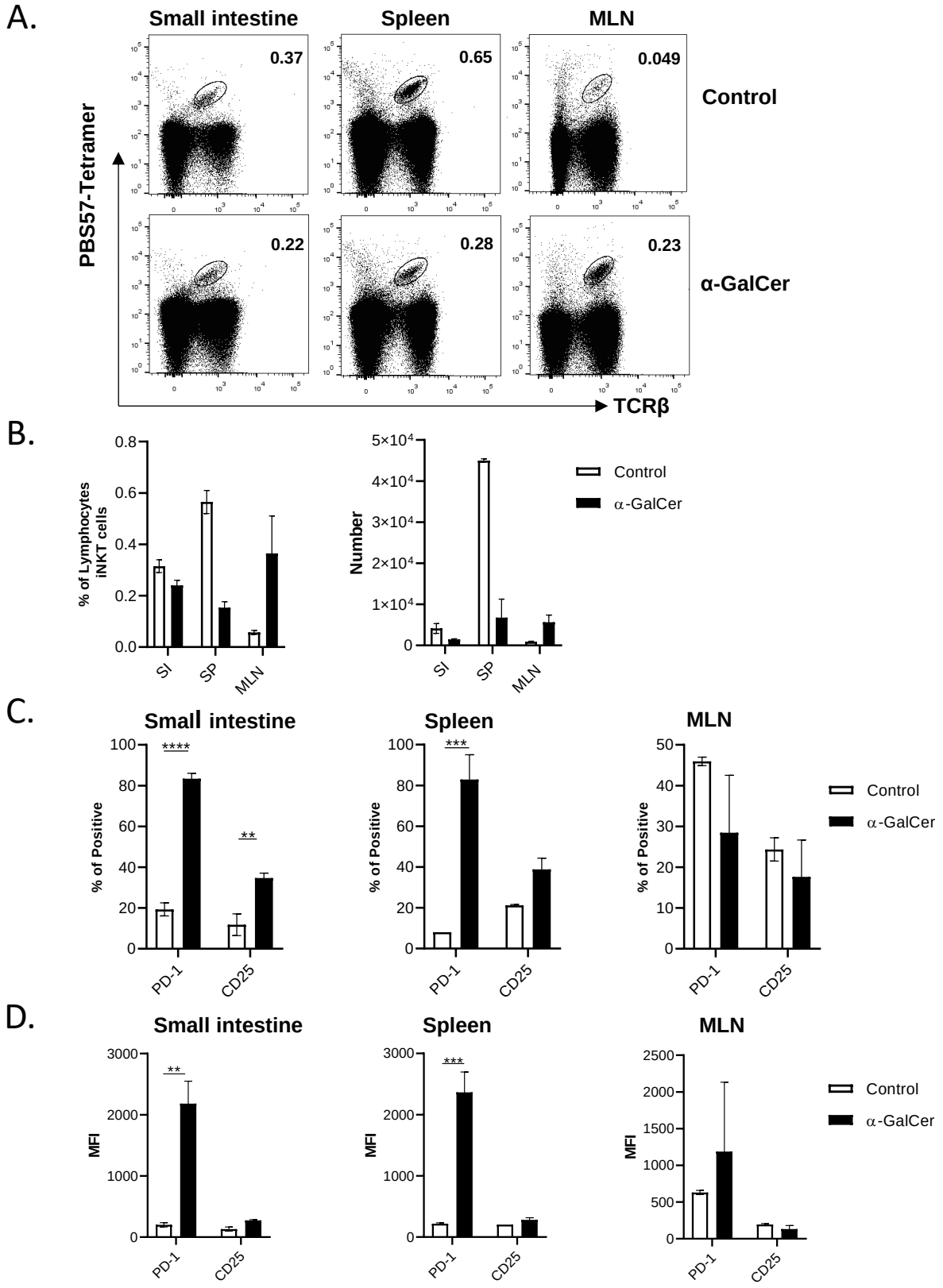


Figure 3.4 iNKT cells in the small intestine upregulate CD25 and PD-1 48hr of α-GalCer injection.

A) INKTs were identified as PBS57-Tetramer+ TCRβ+. B) Percentage and number of iNKTs in lymphocytes of different tissues. C) Percentage of PD-1 and CD25 in iNKT cell subsets in small intestine, spleen and MLN. D) Bar-charts of MFI of PD-1 and CD25 expression in small intestine, spleen and MLN. Data are representative of mice (n=3), expressed as mean ± SEM (error bars; unpaired t-test, two-tailed test). **P < 0.01, ***P < 0.001, ****P < 0.0001.

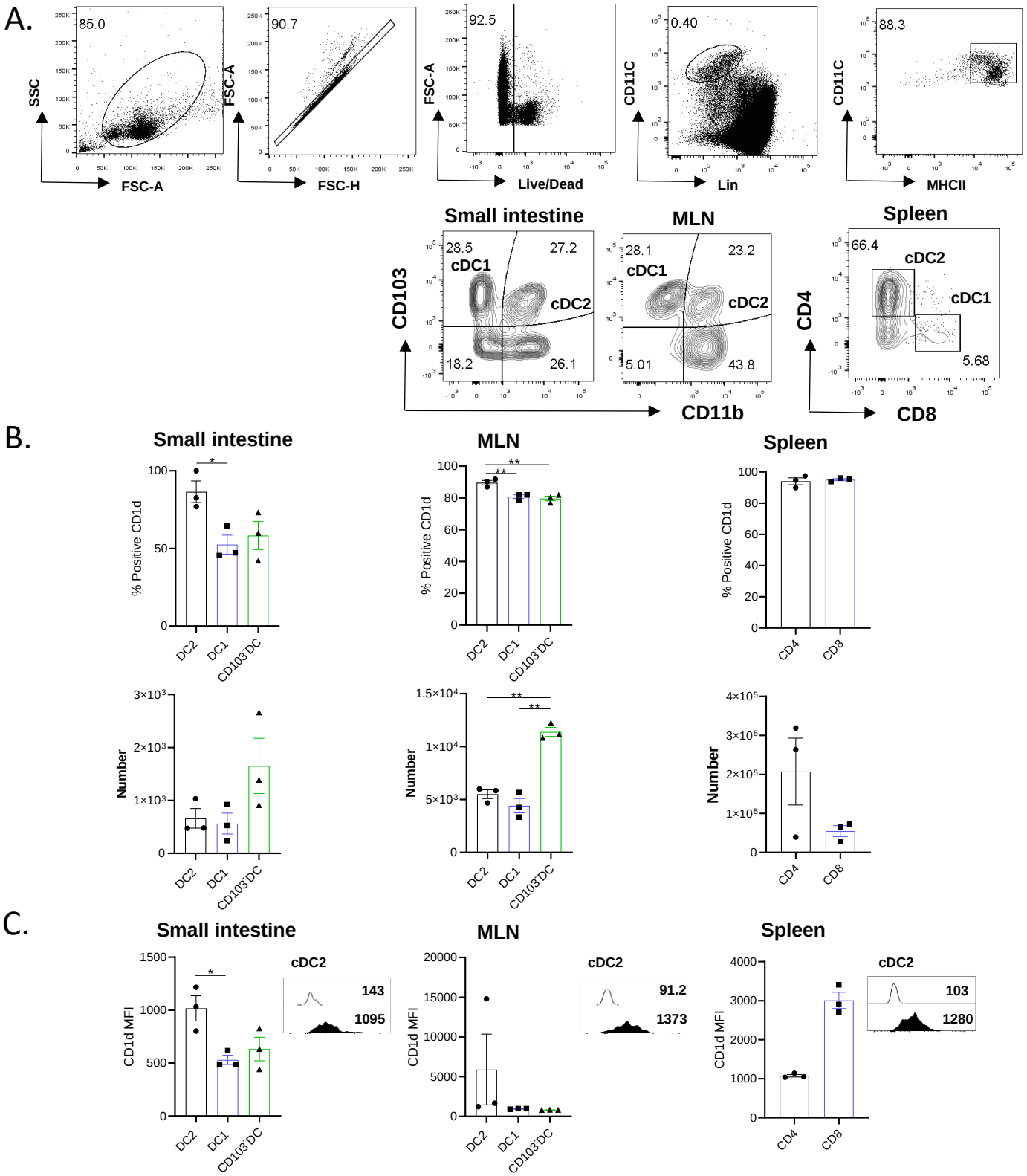


Figure 3.5 cDC2 expresses increased levels of CD1d expression in comparison with other cDC subsets
cDCs were selected as singlets live cells, cDC were identified in all tissues as Lin$^-$ (CD3, B220, F4/80, Gr1) CD11c^{high}. cDC were selected as CD11c^{high} MHCII$^+$ then cDC subsets were shown as mucosal DC2 (CD103$^+$CD11b$^+$), mucosal DC1 (CD103$^+$CD11b$^-$), mucosal CD103$^-$(CD103$^-$CD11b$^+$), splenic DC1 were shown as CD8α$^+$ and DC2 as CD4$^+$cells. A) Representative plots with percentages for DC2, DC1 and CD103$^-$ DC subsets were shown. B) Percentage and number of positive CD1d expression on DC1, DC2 and CD103$^-$ DC subsets. C) Bar-charts and representative histograms of MFI of CD1d expression on DC1, DC2 and CD103$^-$ DC subsets. Data are of 3 mice, expressed as mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P < 0.01.

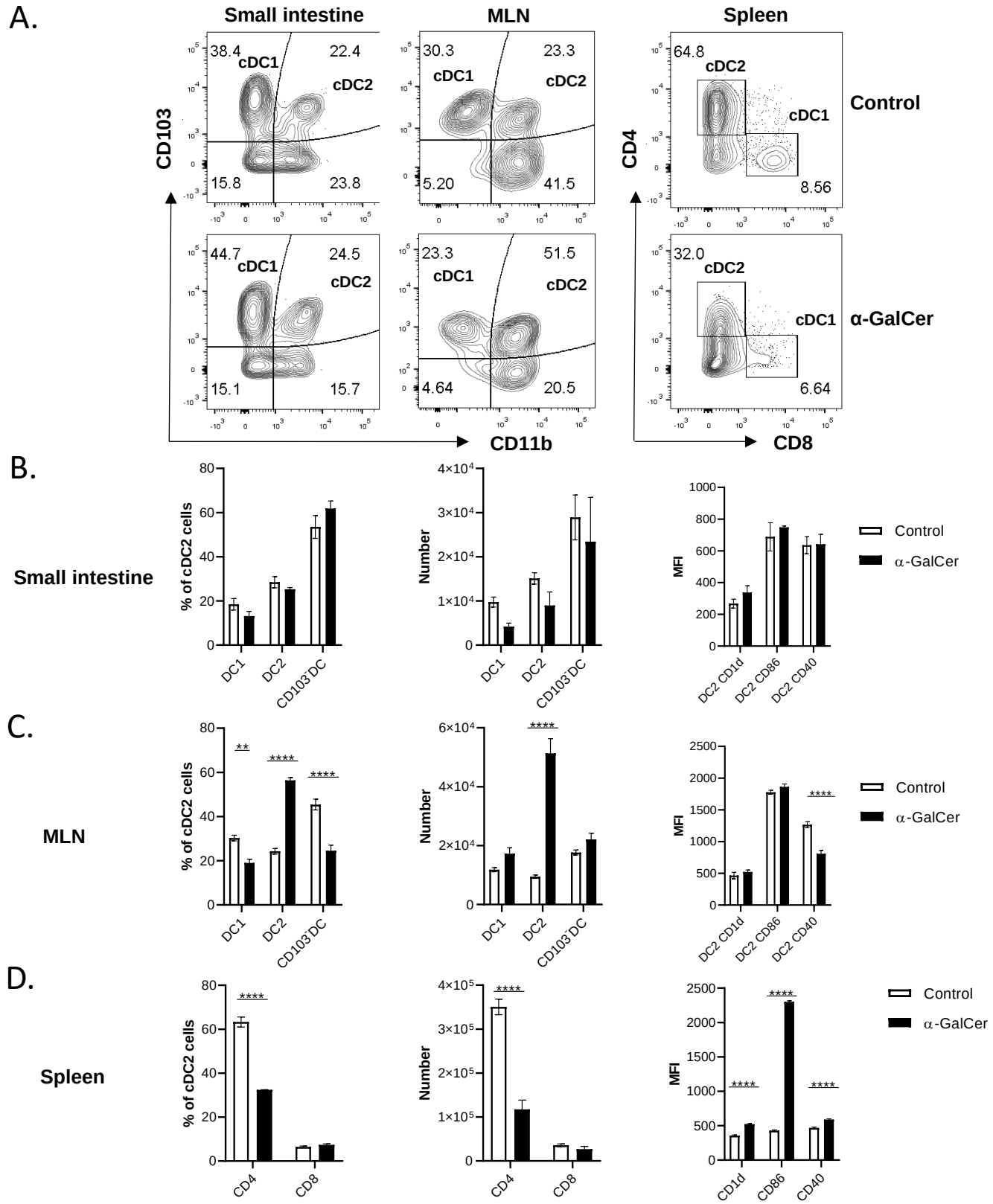


Figure 3.6 Ip injection with α-GalCer after 24h induces the accumulation of DC subsets in mLN. A) Representative plots with percentages for DC2, DC1 and $CD103^-$ DC subsets were shown. B) Percentage and number of DC1, DC2 and $CD103^-$ DC subsets and MFI of CD1d, CD86 and CD40 expression on DC2 in small intestine C) In MLN. D) Percentage and number of splenic $CD8^+$ and $CD4^+$ and MFI of CD1d, CD86 and CD40 expression on cDC2. Data are representative of mice (n=3 per group). Mean ± SEM (error bars; unpaired t-test, two-tailed test), **P < 0.01, ***P < 0.001, ****P < 0.0001, versus control.

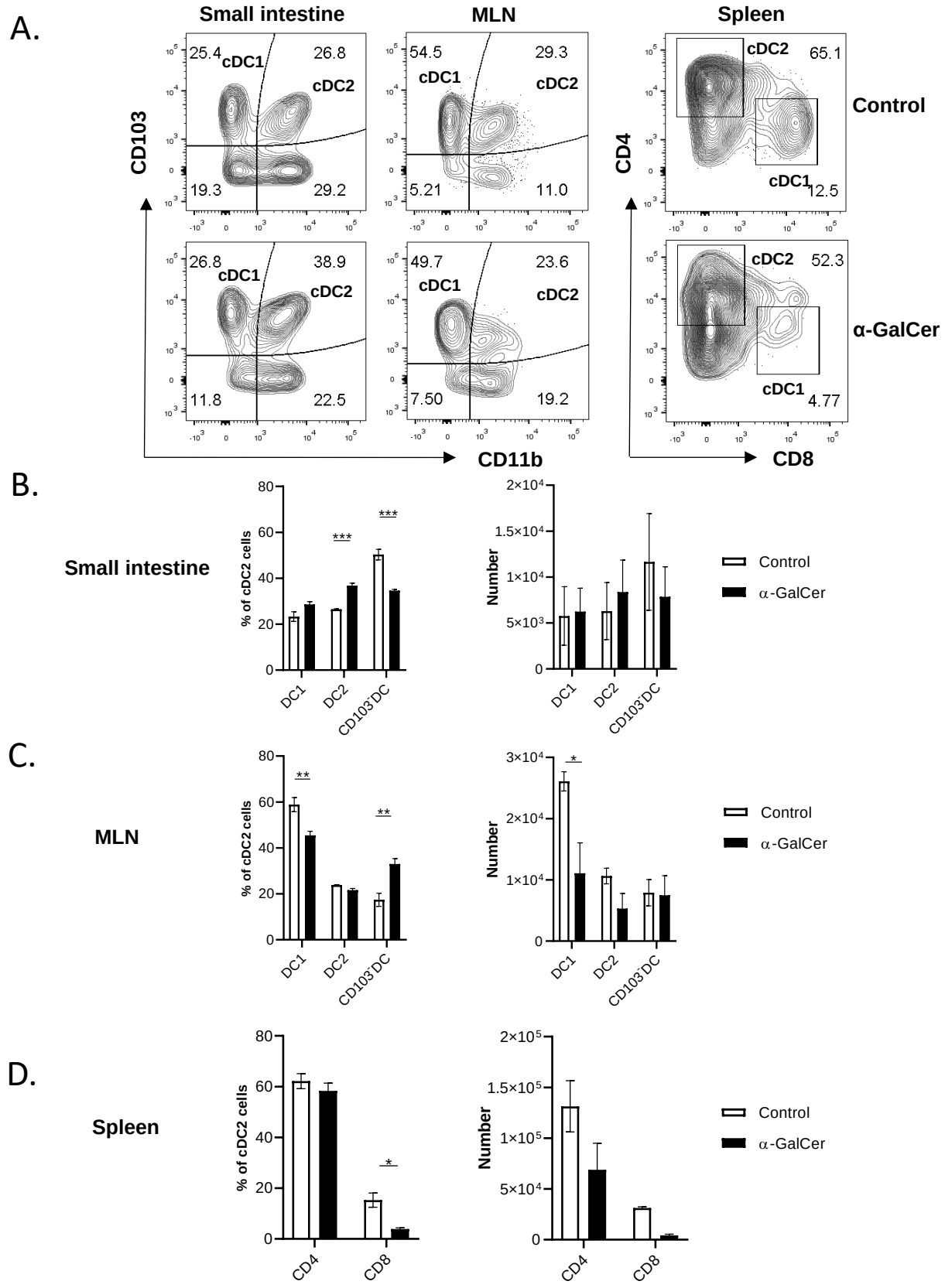


Figure 3.7 Ip injection with α-GalCer induces the accumulation of DCs in the small intestine after 48hr.

A) Representative plots with percentages for cDC2, cDC1 and CD103- DC subsets were shown. B) Percentage and number of DC1, DC2 and CD103- DC subsets in small intestine. C) MLN. D) spleen. Data are representative of mice (n=3 per group). Mean ± SEM (error bars; unpaired t-test, two-tailed test), *P < 0.05, **P < 0.01, ***P < 0.001, versus control.

CHAPTER 4: INKT CELL ACTIVATION COINCIDENT WITH FLIC VACCINATION BOOSTS IMMUNITY FOLLOWING FLIC VACCINATION

4.1 Introduction

The flagellin molecule, FliC, is the fundamental building block of the flagella of STm, which has potential to contribute to vaccine formulations due to it acting as its own adjuvant following recognition by TLR5 with negligible safety concerns. Binding of FliC to TLR5 leads to dimerization and signalling via MyD88/IRAK1-4/TRAF6/IKK-NF-κB pathway, resulting in the activation of DCs and the subsequent upregulation of MHC II and costimulatory molecules such as CD80/86 and the secretion of various chemokines and cytokines like IL-6, IL-12 and IL-23 (168, 322). Furthermore, TLR5 enables the preferential sequestration of FliC by TLR5 expressing cells (such as intestinal DC2) which initiates productive T cell help for B cell responses to flagellin (315, 316, 323).

Indeed, STm via a type III secretion system able to deliver flagellin into the host cell, resulting in recognition of the intracellular flagellin by NAIP5 and NAIP6. This binding of flagellin to NAIP5/NAIP6 results in dimerization and signalling via the formation of NLRC4 inflammasome leads to activation of caspase-1 resulting in either cell death by pyroptosis or maturation and secretion of IL-1β and IL-18 respectively (324-328).

Flagellin administration has been shown to be sufficient to generate both T and B cell responses to itself and leads to a degree of protection from subsequent STm infection in mouse models (178, 315, 316, 323). The potent effect to flagellin is not only refer to its ability to provide immune response through TLR5 and NLRs, but it also provides proteinaceous nature that can stimulate specific CD4 T cell responses leading to class-switched, high affinity antibodies (329, 330). It has to be mentioned that this featured of proteinaceous nature is restricted to flagellin where it is not seen in other bacteria-derived PAMPs such as peptidoglycans and LPS (329, 330). These endocytosis activity by flagellin leads to process and present peptide on MHC class II resulting in optimal presentation of flagellin to CD4 T cells (331). Surprisingly, the optimal presentation of flagellin is not required MyD88 signalling but DCs indeed depend on sky

activity for delivering optimal presentation to $CD4^+$ *in vivo and in vitro* (332). However, such a dependant on Syk-mediated signalling pathway for optimal presentation by DCs remains to be further investigated.

Many studies have shown that iNKT cells enhance the production of antibodies (288, 333-337). This was supported by a study done on human peripheral blood ex-vivo (288). It was shown that after stimulating iNKT cells with α-GalCer, iNKT cells enhanced Ab production through autologous B cells. This study used human B cells co-cultured with iNKT cells, resulting in enhance production of IgM and IgG1 without the presence of T cells. The present study concluded that IgM/IgG1 production from B cells was CD1d-NKT cell dependent. In a model of experimental asthma it was shown that IgE antibody production was reduced in the presence of CD1d-blocking antibodies and deficient in $J\alpha18^{-/-}$ mice (338). Importantly, these findings provide clear evidence that iNKT cells are implicated in antibody-mediated protection against pathogens (286, 339). Antigens derived from some pathogens, such as *Streptococcus pneumoniae*, are CD1 restricted and have been reported to produce antibodies, suggesting that the production of antibodies is CD1d dependent (340).

The ability of α-GalCer to improve the efficacy of vaccines against certain microbial pathogens has been investigated widely. The addition of α-GalCer to the malaria vaccine resulted in increased secretion of IFN-γ by iNKT cells and enhanced protective anti-malarial immunity that had been previously demonstrated to be dependent on IFN-γ (341, 342). The critical role of the CD8 T cells offered at liver stages of the infection explains the need for IFN-γ (342). α-GalCer has also been used as part of an HIV-1 DNA vaccine, where α-GalCer enhanced the immunogenicity of the vaccine, resulting in boosted CD8/CD4 T cell responses alongside humoral immunity (343). In cancer vaccines, mice immunized by coadministration of α-GalCer with a viral vector containing the OVA antigen demonstrated that α-GalCer enhanced the

immune response to the OVA antigen by improving both priming and boosting of CD8 T cells (344).

There are many studies focusing on iNKT cells and the use of α-GalCer as adjuvant to activate iNKT cells in some mouse models. In influenza infection, the combination of the antigen PR8 HA with the α-GalCer administration increases the level of IgG Abs, as well as the levels of both cytokines IL-4/ IFN-γ and Ag-specific CTLs (345). This study found that the addition of α-GalCer induces a high degree of protection against the influenza A virus (345). Another study confirmed that co-administration of α-GalCer with the vaccine provides a strong influenza Abs response (346, 347). Another example of the use of NKT cells in vaccines is found in the study that injected α-GalCer during murine cytomegalovirus infection; the study's findings demonstrate an increase in the cell frequency of $CD8^+$ supporting the critical effect of the NKT cells provided for memory responses (348).

In the previous chapter we found that the dominant population of iNKT cells in the SI are NKT1 cells that secrete IFNγ upon stimulation and provided evidence to suggest that such cells were activated following the administration of α-GalCer i.p. The activation of iNKT cells was found to affect cDC populations in both SI and MLN however whether this is a consequence of iNKT cell activation in the SI remains to be determined. Nevertheless, taken together the data suggest that iNKT cells are activated in the SI following α-GalCer administration and as such are ideally placed to influence an adaptive mucosal immune response to FliC. Therefore, glycolipids that activate iNKT cells may be a useful adjunct strategy to boost immune responses after vaccination.

4.2 Aims and Hypothesis

We hypothesise that glycolipids that activate iNKT cells have the potential to improve vaccination to FliC. We therefore aimed to co-immunise mice with FliC and the iNKT cell agonist (α-GalCer) and monitor the dynamics of the iNKT cell response, the conventional T cell response and the B cell/antibody response to FliC as compared to responses following FliC immunisation only.

4.3 Results

4.1.1 INKT cell activation coincident with FliC vaccination boosts immunity following FliC vaccination.

The Ab response to FliC is T-dependent, and it has been reported that i.p. immunisation of FliC can drive long term mucosal adaptive responses (315, 316). Furthermore, immunization with FliC has been shown to drive a dominant IgG1 isotype Ab response which is associated with Th2-like responses (349, 350). Therefore, we sought to examine whether iNKT cell activation can influence the generation of high-affinity, class switched antibody to FliC detectable in the serum of immunised mice (figure 4.1). To this end, mice were immunised with FliC with and without α-GalCer administration (or with α-GalCer alone or PBS). 14 days post immunisation mice received a further dose of FliC and serum and tissues were harvested 4 days later (i.e. day 18).

Assessment of the anti-sFliC IgG response revealed mice co-immunized with α-GalCer and sFliC (α-Gal/FliC) demonstrated a significant increase in IgG titre in serum compare to FliC alone (figure 4.1 A). This was also apparent on examining IgG subclasses with anti-sFliC IgG1 Ab (figure 4.1 B) and anti-sFliC IgG2C Ab (figure 4.1 C) titres increased in mice co-immunized with α-Gal/FliC compared to mice immunized with FliC alone. However, FliC-specific IgA titres were similar in both groups at this time (figure 4.1 D). These data suggest that a single dose of α-GalCer improves the antibody response to FliC following FliC immunisation.

To assess whether iNKT cell activation influenced the generation of Ab-secreting cells the number of IgG and IgA FliC-specific Ab-secreting cells generated following FliC administration with and without the activation of iNKT cells was assessed in the spleen, MLN and SI (figure 4.2). Mice were primed for 14 days with and without iNKT cell activation and a day 14 boost with FliC as previously mentioned.

The relative frequency of FliC-specific IgG and IgA Ab-secreting cells was significantly increased upon FliC immunisation with or without iNKT cell activation in the spleen, MLN and SI (figure 4.2 B-C). However, in the spleen, the frequency of FliC-specific IgG secreting cells was significantly increased in mice that had received both α-Gal/FliC compared to mice that had received FliC alone (figure 4.2 A-B).

4.1.2 The co-incident activation of iNKT cells results in increased germinal centre B cells following vaccination.

FliC vaccination has been shown to induce the robust generation of sFliC-specific germinal centre B cells in spleen with a limited extrafollicular plasma cell response and Tfh cells (349, 351). Therefore, we sought to examine whether concomitant iNKT cell activation affected the generation of GC B cells, plasma cells and Tfh cells following FliC administration (figure 4.3-5).

Analysis of GC B cells in the spleen 18 days post-FliC immunisation showed that the frequency and number of GC B cells was significantly increased compared to controls (figure 4.3 A). Co-administration of α-Gal/FliC was found to induce a significant increase in the frequency of GC B cells in the spleen compared to sFliC only although absolute numbers were similar (figure 4.3 A). Assessment of plasma cells in spleen 18 days post-FliC immunisation demonstrated that the frequency and number of plasma cells was significantly increased in co-immunized mice of α-Gal/FliC compared to controls (figure 4.3 B). However, co-administration of α-Gal/FliC showed similar response in plasma cells compare to FliC alone (figure 4.3 B).

Tfh cells were identified as being pre-gated on memory T-cells ($CD4^+CD44^+$ $CD62L^-$ cells). Therefore, memory T-cells are being investigated in lymphoid tissues 18 days post-FliC immunisation. Analysis of memory T-cells in spleen showed that immunisation with FliC increased the proportion and number of $CD44^+$ $CD62L^-$ compare to the controls (figure 4.3 C).

Co-immunized α-Gal/FliC demonstrated significant decrease in frequency compare to FliC (figure 4.3 C) although absolute numbers were significantly higher than the controls. Analysis of Tfh in spleen showed that co-administration of α-Gal/FliC increased the proportion of Tfh amongst controls (figure 4.3 D). In contrast, the frequency of Tfh cells did not alter with or without the activation of iNKT cells. The number of Tfh cells remains the same between all groups (figure 4.3 D).

Next, FliC administration has also been shown to increase Treg 5 days post-immunization in MLN (316) and the responses to FliC directs polarisation of B cells to IgG1, reflects the Th2 activity (349). This marks the kinetics response to $CD4^+$ T cells are important to protect against STm. Given that Treg can modulate Tfh and therefore subsequent antibody responses we sought to both confirm previous observations and extend them to assess the impact of iNKT cells on the process (figure 4.3-5).

18 days post FliC immunisation in spleen we found that the proportion and number of $CD4^+$ T cells in α-Gal/FliC immunised mice showed significantly increase compared to FliC, α-GalCer or control animals (figure 4.3 E). Analysis of Treg found that indeed the percentage and number of Treg was increased in multiple tissue sites compared to PBS control animals (figure 4.3-5). Interestingly, the increase in the proportion of Treg in the spleen following FliC injection was attenuated by co-administration of α-GalCer whereas the number of Treg was similar after α-Gal/FliC injection in the spleen (figure 4.3 F).

In MLN, analysis of GC B cells showed that the frequency and number of GC B cells was significantly increased compared to α-GalCer/control animals (figure 4.4 A). However, co-administration of α-Gal/FliC did not impact GC B cells generation at this time (figure 4.4 A). The frequency and number of plasma cells was significantly increased in α-Gal/FliC compared to controls (figure 4.4 B). However α-Gal/FliC showed similar response in plasma cells compare to FliC alone (figure 4.4 B).

Analysis of the frequency and number of $CD44^+$ $CD62L^-$ cells in MLN were significantly increased in FliC or α-Gal/FliC compared to control animals (figure 4.4 C). However, co-administration of α-GalCer did not alter memory T cells in compare to FliC administration (figure 4.4 C). Assessment of Tfh in MLN showed that co-administration of α-Gal/FliC increased the proportion of Tfh amongst controls. In contrast, the frequency of Tfh cells did not alter with or without the activation of iNKT cells. The number of Tfh cells remains the same between all groups (figure 4.4 D).

Interestingly in MLN, assessment of the percentage of $CD4^+$ T cells of either FliC only or α-Gal/FliC was significantly lower than the PBS/α-GalCer only animals. However the number of $CD4^+$ T cells remain increased after α-Gal/FliC or FliC alone (figure 4.4 E). Analysis of Treg found that indeed the increase in Treg in the mLN remained unaltered by iNKT cell activation at this time (figure 4.4 F).

Analysis of the small intestine revealed no increase in the proportion or number of GC B cells upon FliC immunisation indicating that germinal centre formation following FliC immunisation is restricted to the lymphoid tissue (figure 4.5 A). Furthermore the frequency of plasma cells was significantly increased in α-Gal/FliC compared to controls (figure 4.5 B) but the number were the same between all groups. Assessment of $CD4^+$ T cells in small intestine display that co-administration of α-GalCer and sFliC revealed significant increase in the frequency and number compare to other groups (figure 4.5 C). Analysis of the small intestine revealed no increase in the proportion Treg cells upon FliC immunisation however the number of Treg was increased after α-Gal/FliC injection in the small intestine (figure 4.5 D).

Collectively, these data demonstrated that activation of iNKT cells do induce germinal centre formation at the time of FliC immunisation in spleen. However, activation of iNKT cells did not impact extrafollicular plasma cell response following FliC administration. Similarly, Tfh cells responses following FliC administration did not alter after the addition of iNKT cells at

this time. In addition, $CD4^+$ T cells in spleen or small intestine at this time plays a crucial role of modulating the effect of FliC after activation iNKT cells following FliC administration and iNKT cells influence the induction/expansion of $CD4^+$ T cells and Treg seen following FliC immunisation but that the modulation may be different in different tissues.

4.1.3 Increased immunity to FliC by the co-administration of α-GalCer at the point of FliC vaccination persists for over a month.

To test, whether the impact of α-GalCer on FliC responses is maintained in the longer-term, we examined the serum of immunised mice for anti-FliC antibody (figure 4.6). To this end, mice were immunised with FliC with and without α-GalCer administration (or with α-GalCer alone or PBS). 35 days post immunisation mice received a further dose of FliC and were harvested 4 days later (i.e. day 39).

Analysis of the anti-sFliC IgG response showed that co-immunisation with α-Gal/FliC resulted in a significant increase of IgG titres compare to FliC alone (figure 4.6 A). This was mirrored across several IgG isotypes with the titre of anti-sFliC IgG1 (figure 4.6 B) and IgG2C (figure 4.6 C) also elevated in mice co-immunized with α-Gal/FliC. Interestingly, FliC-specific IgA titres were also significantly higher in mice received α-Gal/FliC compare to FliC at this time (figure 4.6 D).

The frequency of FliC-specific Ab secreting cells was also analysed 39 days after vaccination. The relative frequency of FliC-specific IgG and IgA Ab-secreting cells was significantly increased upon FliC immunisation with iNKT cell activation in the spleen, MLN and BM (figure 4.7 A-C). However, the frequency of FliC-specific IgG and IgA Ab-secreting cells was similar upon FliC immunisation with or without iNKT cell activation in small intestine.

4.1.4 The activation of iNKT cells at the point of vaccination increases germinal centre potential for at least a month post-vaccination.

Assessment of GC B cells in lymphoid tissues harvested 39 days post-vaccination revealed a significantly increase in the frequency of GC B cells in the spleen of mice that received α-Gal/FliC compared to FliC alone (figure 4.8 A). The analysis of number GC B cells revealed a trend higher in spleen with the activation of iNKT cell compare to FliC alone (figure 4.8 A). As expected, immunisation with sFliC only demonstrated a significant increase in the frequency of spleen in compared to controls (figure 4.8 A). Plasma cells at this time demonstrated that the frequency and number of plasma cells was the same in multiple tissue sites with/without the activation of iNKT cell (figure 4.8-10 B). Analysis of memory T-cells in spleen demonstrated significantly increase in the proportion of $CD44^+$ $CD62L^-$ with the activation of iNKT cell compare to FliC alone. Whereas the number of $CD44^+$ $CD62L^-$ did not impact (figure 4.8 C). Interestingly, analysis of Tfh showed significantly increase in the frequency and number of Tfh cells with the activation of iNKT cell compare to FliC alone (figure 4.8 D). As expected, FliC only showed a significant increase in the frequency and number of Tfh cells compare to PBS mice (figure 4.8 D). 35 days post FliC immunisation we found that the proportion and number of $CD4^+$ T cells was the same in multiple tissue sites compared to control animals (figure 4.8-9 E, 4.10 C). Analysis of Treg found that indeed the frequency of Treg was increased after the activation of iNKT cells compare to mice only received FliC in the spleen. However, the number of Treg was the same compared to FliC alone (figure 4.8 F).

Analysis of GC B cells in MLN harvested 39 days post-vaccination revealed a significantly increase in the frequency and number of GC B cells of mice that received α-Gal/FliC compared to FliC alone (figure 4.9 A). As expected, immunisation with sFliC only demonstrated a significant increase in the frequency and number of GC B cells in compare to controls (figure 4.9 A). Memory T cells in MLN did increase upon FliC immunisation in proportion and

number. However, the addition of activation iNKT cells did not alter the effect of FliC in memory T cells (figure 4.9 C). Interestingly, analysis of Tfh showed significantly increase in the frequency of Tfh cells with the activation of iNKT cell compare to FliC alone (figure 4.9 D) but the number was the same. Analysis of GC B cells and plasma cells in the small intestine showed that all groups were the same and no differences on germinal centre formation or plasma cells following FliC immunisation (figure 4.10 A-B).

These data supported that iNKT cells do enhance germinal centre formation at the time of FliC immunisation and supported the expectation of the limit impact of FliC on extrafollicular plasma cell response following FliC administration. In addition, iNKT cells do influences the generation of FliC-specific Tfh cells at the time of FliC immunisation. Moreover, iNKT cells influence the induction/expansion of Treg seen in spleen following FliC immunisation but not in different tissues.

4.1.5 iNKT cell activation at the point of FliC immunisation does not affect the Ab response to FliC.

Thus far we had found that co-administration of an iNKT cell agonist glycolipid (α-GalCer) together with FliC immunisation resulted in a sustained increase to produce FliC-specific Ab and form germinal centres following antigen re-exposure. We next sought to see whether any of these differences were detectable early after vaccination. To this end, mice were immunised with FliC with and without α-GalCer administration (or with α-GalCer alone or PBS) and harvested 7 days post immunisation.

Analysis of serum revealed little by way of an anti-sFliC IgM response however FliC-spcific IgG was detected in vaccinated mice although no difference was seen in mice that also received α-GalCer (figure 4.11).

In addition, IgG Ab secreting cells were not detected in any tissue studied and IgM Ab secreting cells were only detected at low numbers in the spleen (figure 4.12). Again, the small number of Ab-secreting cells in the spleen were unaltered by the presence of α-GalCer during FliC vaccination.

4.1.6 Increased germinal centre formation can be detected early in mice that received α-Gal/FliC.

Given the lack of evidence that iNKT cell activation influences serum Ab secretion or Ab secreting cells we next looked earlier in the germinal centre response by flow cytometry 7 days after vaccination (figure 4.13-15).

Analysis of the spleen revealed that α-Gal/FliC mice harboured increased frequencies and numbers of both GC B cells and Tfh compare to mice that had received FliC alone, α-GalCer alone or control mice (figure 13 A and D). Interestingly, the same analysis of the MLN did not show a difference in either GC B cells or Tfh between α-Gal/FliC mice and mice that had received FliC only (figure 4.14 A and D). Not surprisingly no response was seen amongst multiple cell types in the SI at this early time point (figure 4.15).

4.4 Discussion

This Chapter examined the impact of iNKT cell activation together with FliC administration on subsequent innate and adaptive immune responses to FliC. This was achieved using the agonist glycolipid α-GalCer. We hypothesised that as iNKT cells can co-produce Th1 and Th2 cytokines (such as a subset of NKT1) these cells may act to further propagate the immune response to FliC following activation. However, it has been observed that a Th2 response normally dominates following immunisation with FliC. This finding is supported by studies that found flagellin to be capable of stimulating T and B cell responses with a strong Th2 bias to itself and co-administered antigens (349, 350, 352). These studies confirmed that Th2 responses can develop in vivo likely induced by FliC only in the absence of exogenous adjuvants (349, 350, 353). Taken together, these features suggest that the coordinated use of α-Gal/FliC may enhance long-lived systemic and mucosal humoral immunity (315).

While FliC can lead to T and B cell responses through the generation of Tfh, GC, memory B cells and plasma cells, we first sought to examine whether iNKT cell activation might impact these immune cells (315). Indeed, iNKT cell activation at the time of FliC immunisation increased the induction of Tfh, GC and enhanced antibody production (figure 4.1, 2, 3 A, 4.6, 7, 8, 9, 13 A and D). Additionally, it was found that the activation of iNKT cells also resulted in more pronounced antibody switching to IgG2C (figure 4.1 and 4.6). Our data clearly provides evidence that activating NKT cells, even in a non-targeted manner, can increase the effectiveness of FliC immunisation.

It has been shown that following recognition of flagellin by TLR5, TLR5 acts as endocytic receptor leading to the processing and presentation of flagellin epitopes on MHC class II to $CD4^+$ T cells (331, 354). We found that the additional of NKT cells enhance the Ab production that is specific for flagellin. This is possible because, the optimal production of antibodies requires co-presentation of both CD1d and class II to stimulate sufficient humoral immune

responses (355, 356). Therefore, this was seen in study that try the immunization of α-GalCer with soluble antigen in same time or immunization of α-GalCer 24hr before or after soluble antigen. This study found that immunisation of α-GalCer in the same time with soluble antigen enhance the optimal T cell responses however, this enhancement was lost before and after 24hr. Consistent with this, another study found that immunization of α-GalCer and OVA using MHC class II-deficient mice results in limited induction of OVA-specific Ab, while in WT mice, α-GalCer enhances OVA-specific Ab production (355). The same study found that immunization of α-GalCer and OVA using CD1d-deficient mice results in limited induction of OVA-specific Ab, suggesting that the enhancement of the Ab production that seen in vivo provided by NKT cells. However, NKT cells alone were unable to provide sufficient B-cell help and this is significant as a non-protein, such as *lipoarabinomannan*, is a restricted CD1-binding antigen showed a limited Ab response in vivo (357, 358). Taken together, stimulation of sufficient humoral immune responses resulting in optimal production of antibodies requires co-presentation of both CD1d and class II (355, 356).

Another possibility is that the additional engagement of the activation NKT cells influences DC to maximize T-cell priming (359). This study found that DC loaded with both peptide and α-GalCer on the same DC showed a better immune response compared to both peptide and α-GalCer loaded on separate DC. Therefore, we have found that immunization of α-Gal/FliC results in enhance of FilC-specific Ab production following challenge with FliC only, suggesting that FliC-specific $CD4^+$ Th cells have been primed in response to α-GalCer. This was shown by a study using OVA-expressing tumours that found that co-administration of α-Gal/OVA boosted the CD4 T cell/cytotoxic T cell response against tumours, supporting the idea that the activation of NKT cells can influences the function of class II/class I-restricted T cells post-immunisation (359). Consistent with this, another study supported the idea that DCs rapidly maturated in response to α-GalCer (360). In this study, they found that injection of α-

GalCer alone 24hr up regulated DCs co-stimulation molecules (CD80/86, CD40), suggesting rapid maturation of DCs in vivo as this was also seen in LPS. This led us to examine whether the loss of CD1d affects the maturation of DCs and upregulation of costimulatory molecules 24 hr post-FliC administration.

The data in this chapter also supports the augmentation of Ig in serum and an expanded pool of Ab-secreting cells when Flic is given in consort with iNKT cell activated. Interestingly, after immunisation with α-Gal/FliC serum IgG, IgG1 and IgG2c were all augmented with the compared to that in mice that had received flagellin alone at both 18 days or 39 days post activation (figure 4.1, 6). This correlated with increased numbers/frequency of sFliC-specific IgG-secreting cells in spleen, MLN and BM (figure 4.2, 7). Indeed, the titre of IgA was also markedly increased by 39 days post vaccination (figure 4.6) despite no differences being observed at earlier timepoints. This is suggestive that coincident activation of iNKT cells maintained humoral responses to flagellin in the longer term.

It has been previously demonstrated that isotype switching can be affected by the cytokine environment provided by Th cells and NKT cells (361, 362). The large IgG1 response seems to be affected by IL-4 production by both Th cells and NKT cells, suggesting Th2 biased (363). However, the induction of IgG2c response likely depends on IFNγ provided by both cells (363-365).

DCs have been shown to rapidly mature following cognate interaction with NKT cells in a process that is independent of TLR signalling and MyD88. However, maturation of DCs requires a TLR-dependent pathway, especially via TLR5, but not MyD88 (168, 366, 367). It therefore appears that TLR5-dependent maturation of DC leads to preferential Th2-type responses, whereas NKT cell-mediated maturation leads to a dominant Th1 response even in MyD88 knockout mice (360, 368).

Taken together, our observations led us to examining whether iNKT cell activation can still enhance the response to FliC in the absence of TLR5 signalling. In addition, absence of NKT cells using CD1d-deficient mice whether affect the immune responses following FliC vaccination, as iNKT cells can be activated by proinflammatory cytokines independently of the agonist glycolipid.

In conclusion, we found that iNKT cell activation coincident with FliC vaccination boosts immunity following FliC vaccination by enhancing Ab production, increasing the number of Ab-secreting cells and boosting the GC reaction. Importantly, some of the effects were maintained for at least a month post vaccination.

4.5 Figures

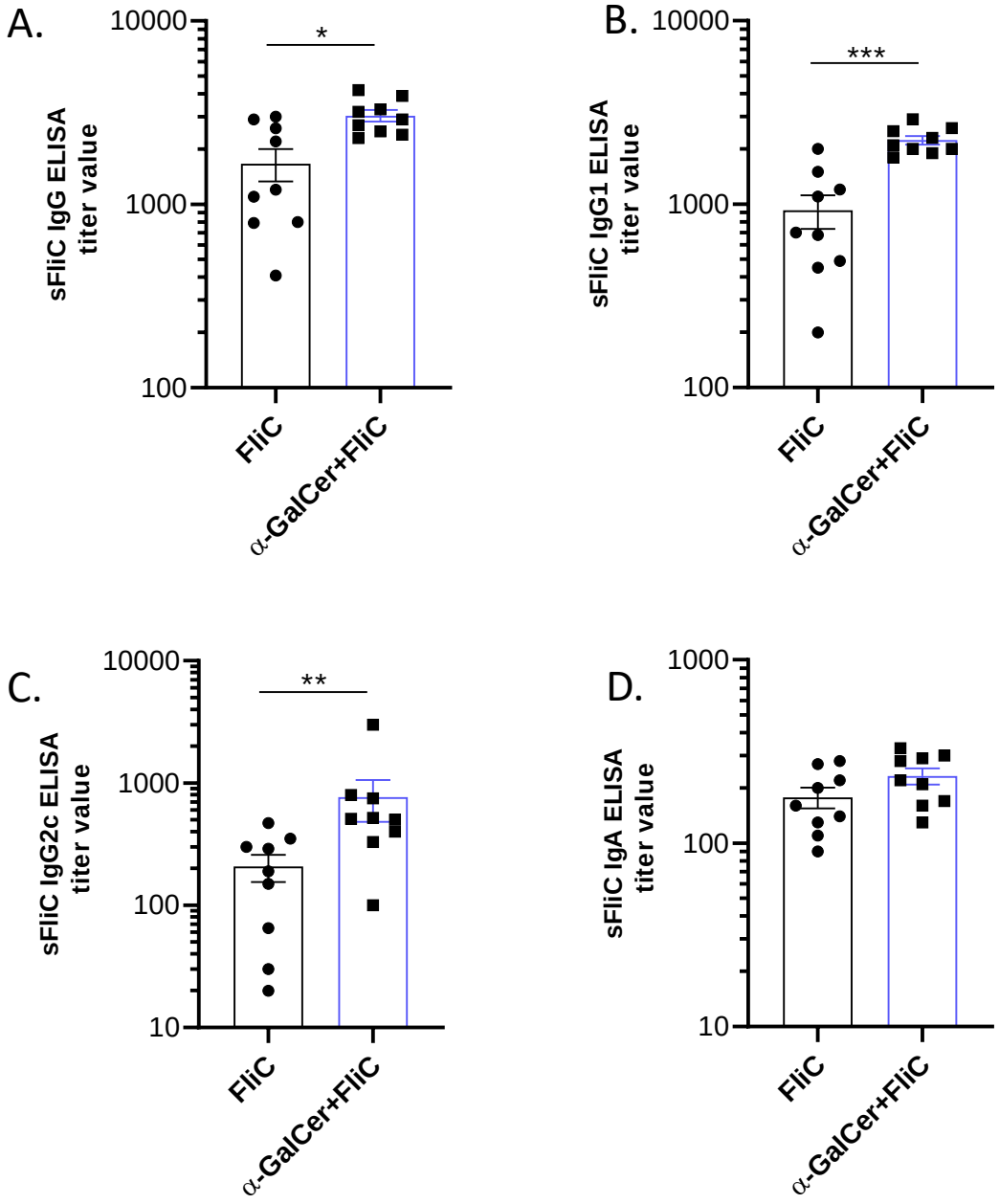


Figure 4.1 Co-immunisation with αGalCer and sFliC increases anti-sFliC antibody in the serum compared to FliC alone

WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS. 14 days later mice received 20μg FliC i.p. (or PBS for PBS control mice). Serum from such mice were collected 4 days later (Day 18). Serum anti-FliC IgG, IgG1, IgG2c and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2C, and D) IgA titre. n=9 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

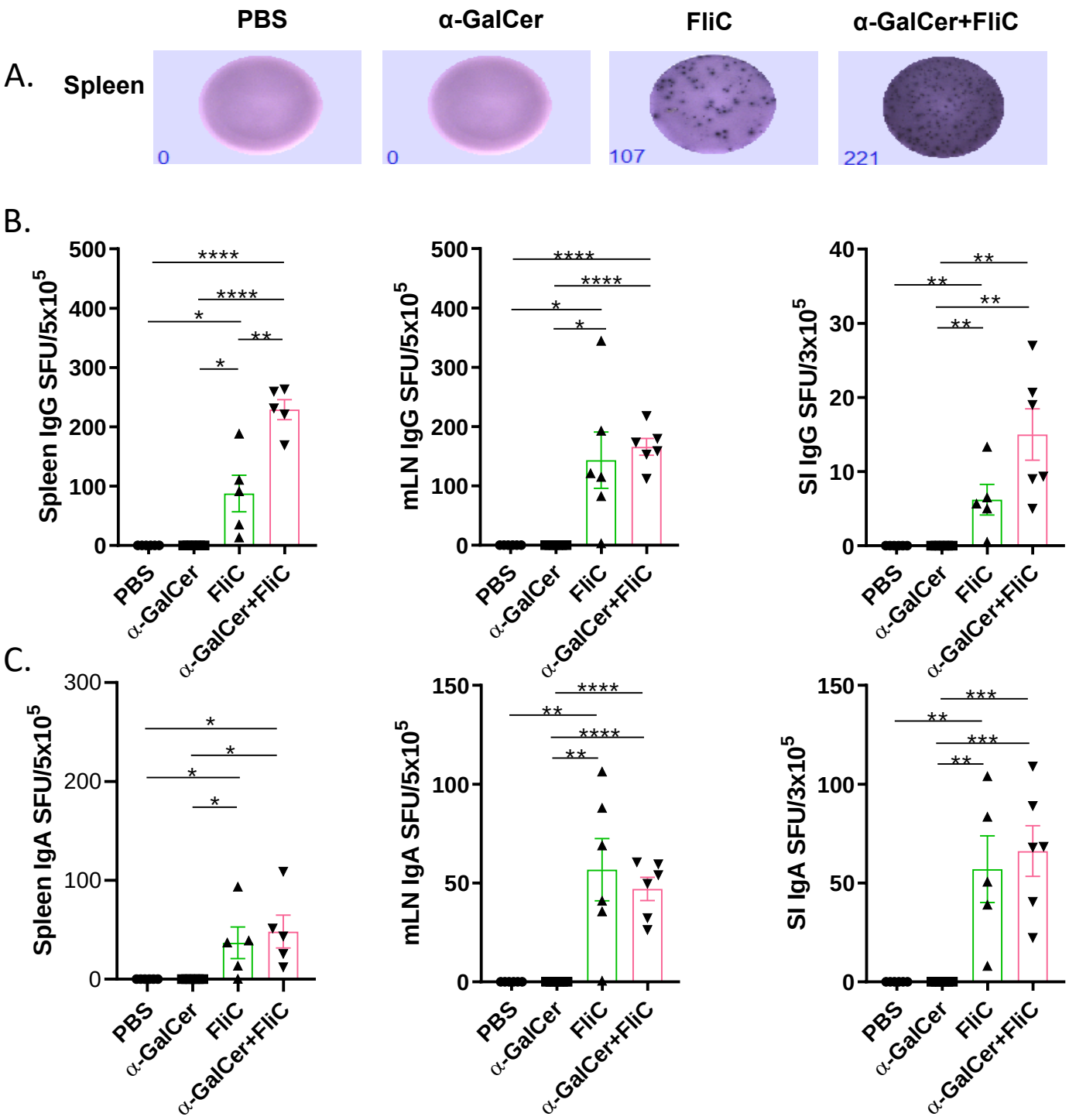


Figure 4.2 The activation of iNKT cells concomitant with FliC immunisation increase the number of IgG Ab-secreting cells in spleen

WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS. 14 days later mice received 20μg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 18). Single cell suspensions were prepared from spleen, mesenteric lymph nodes and small intestine. ELISPOT analysis of sFliC IgG and IgA responses in the spleen, mesenteric lymph nodes and small intestine. A) Representative pictures of wells showing spot-forming units (SFUs) FliC-specific IgG in spleen. B) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgG in spleen, mesenteric lymph nodes, small intestine. C) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgA in spleen, mesenteric lymph nodes and small intestine. n=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

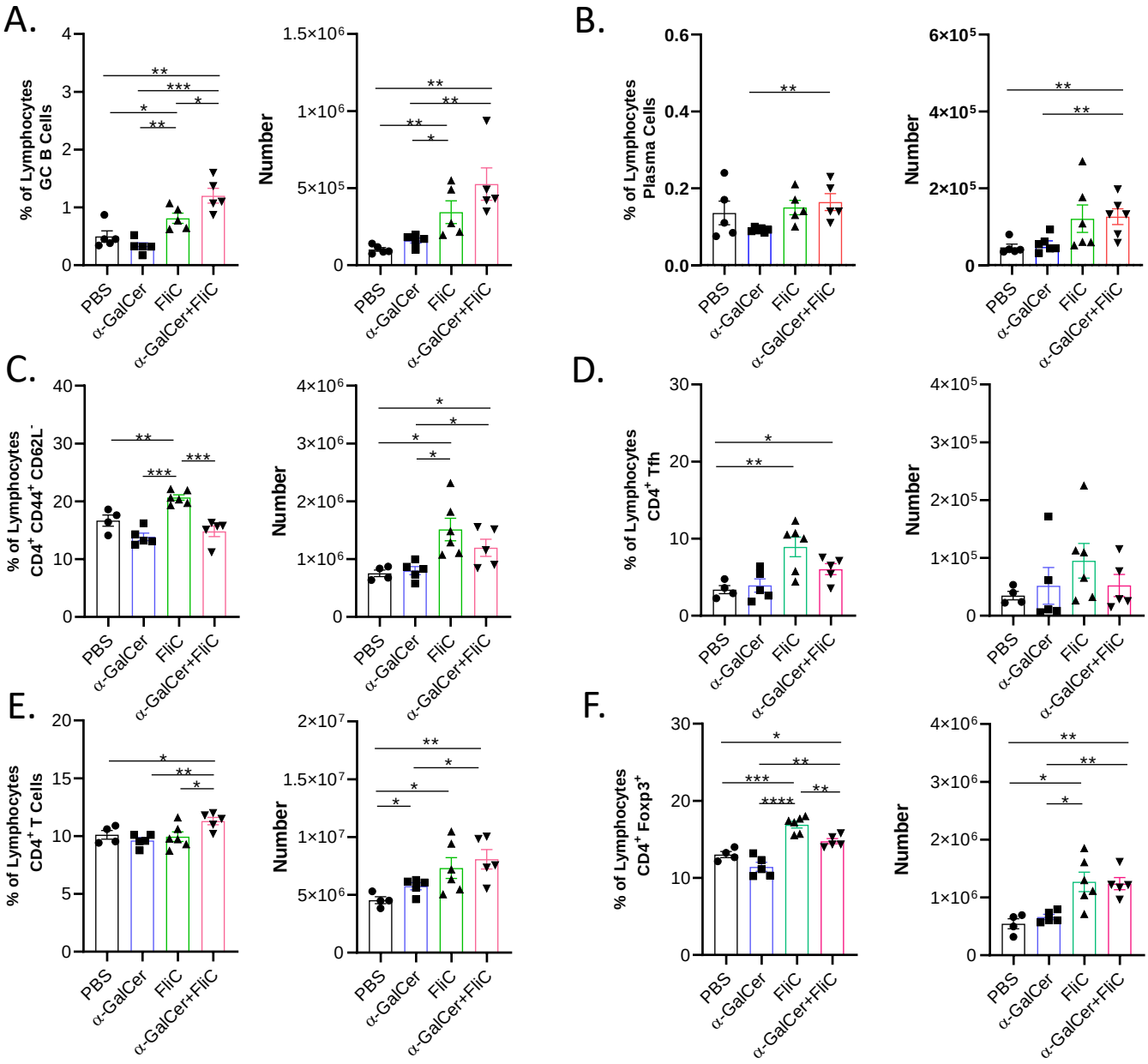


Figure 4.3 Activation of iNKT cells at the time of vaccination results in an expanded pool of GC B cells in the spleen

WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS. 14 days later mice received 20μg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 18). Single cell suspensions were prepared from spleen. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

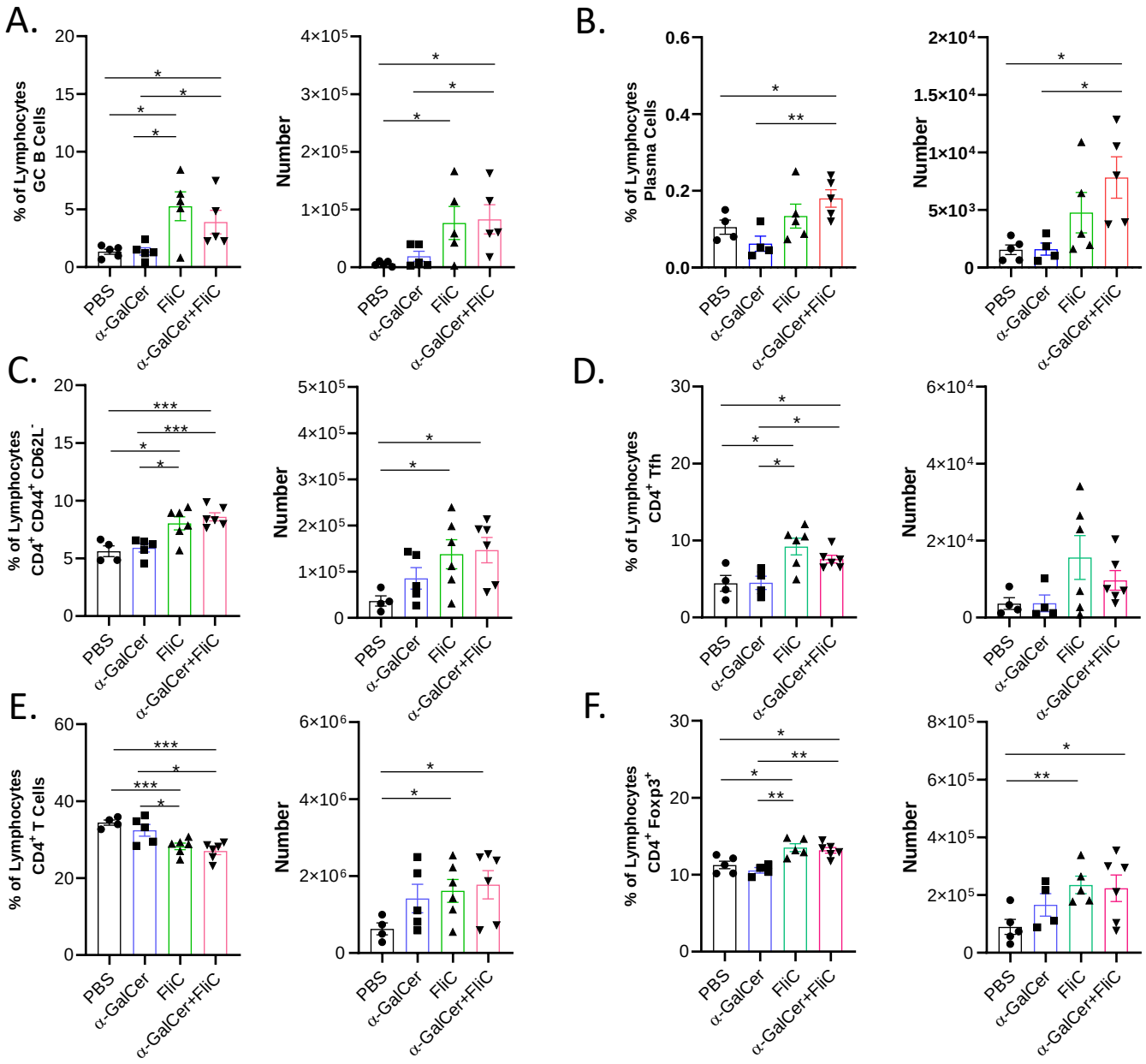


Figure 4.4 iNKT cell activation and FliC vaccination does not alter GC B cell formation in mesenteric lymph nodes

WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS. 14 days later mice received 20µg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 18). Single cell suspensions were prepared from MLN. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.

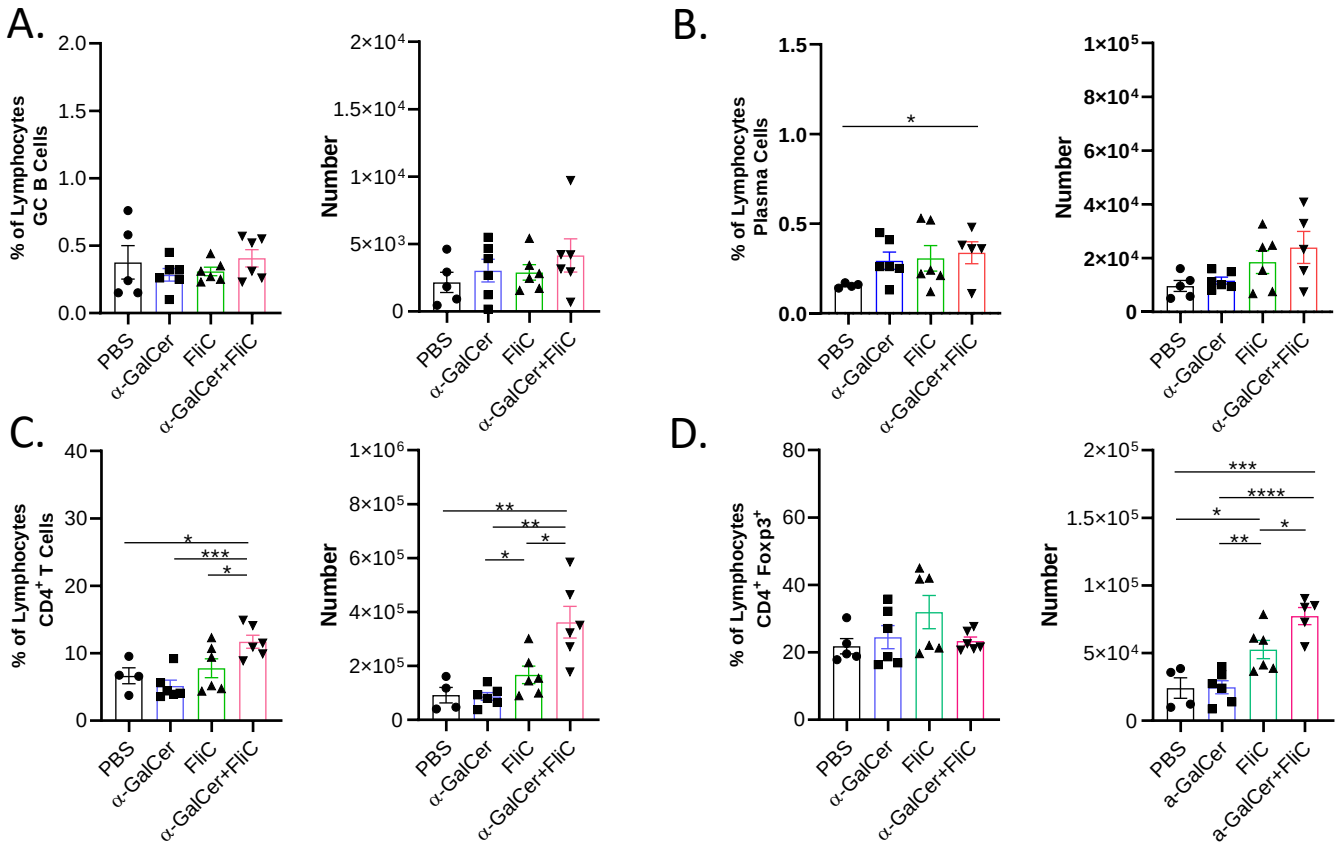


Figure 4.5 The activation of iNKT cells at the time of FliC vaccination does not alter the immune cell make-up of the small intestine compared to that of mice that had received FliC only

WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS. 14 days later mice received 20µg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 18). Single cell suspensions were prepared from small intestine. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD4^+$. D) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

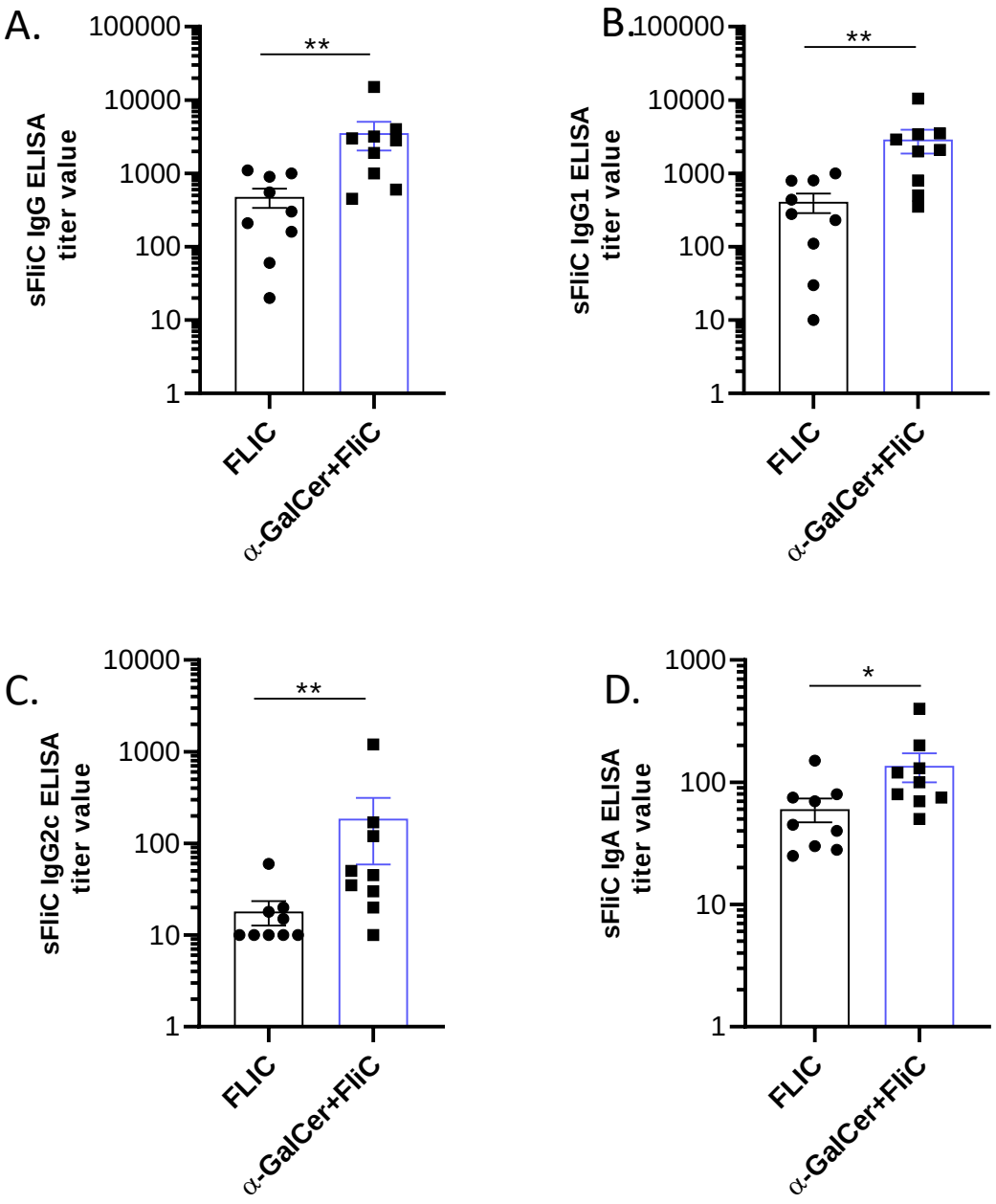


Figure 4.6 Enhanced anti-sFliC antibody induction is sustained following co-immunisation with αGalCer and sFliC

WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS. 35 days later mice received 20µg FliC i.p. (or PBS for PBS control mice). Serum from such mice were collected 4 days later (Day 39). Serum anti-FliC IgG, IgG1, IgG2C and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2c, and D) IgA titre. n=9 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

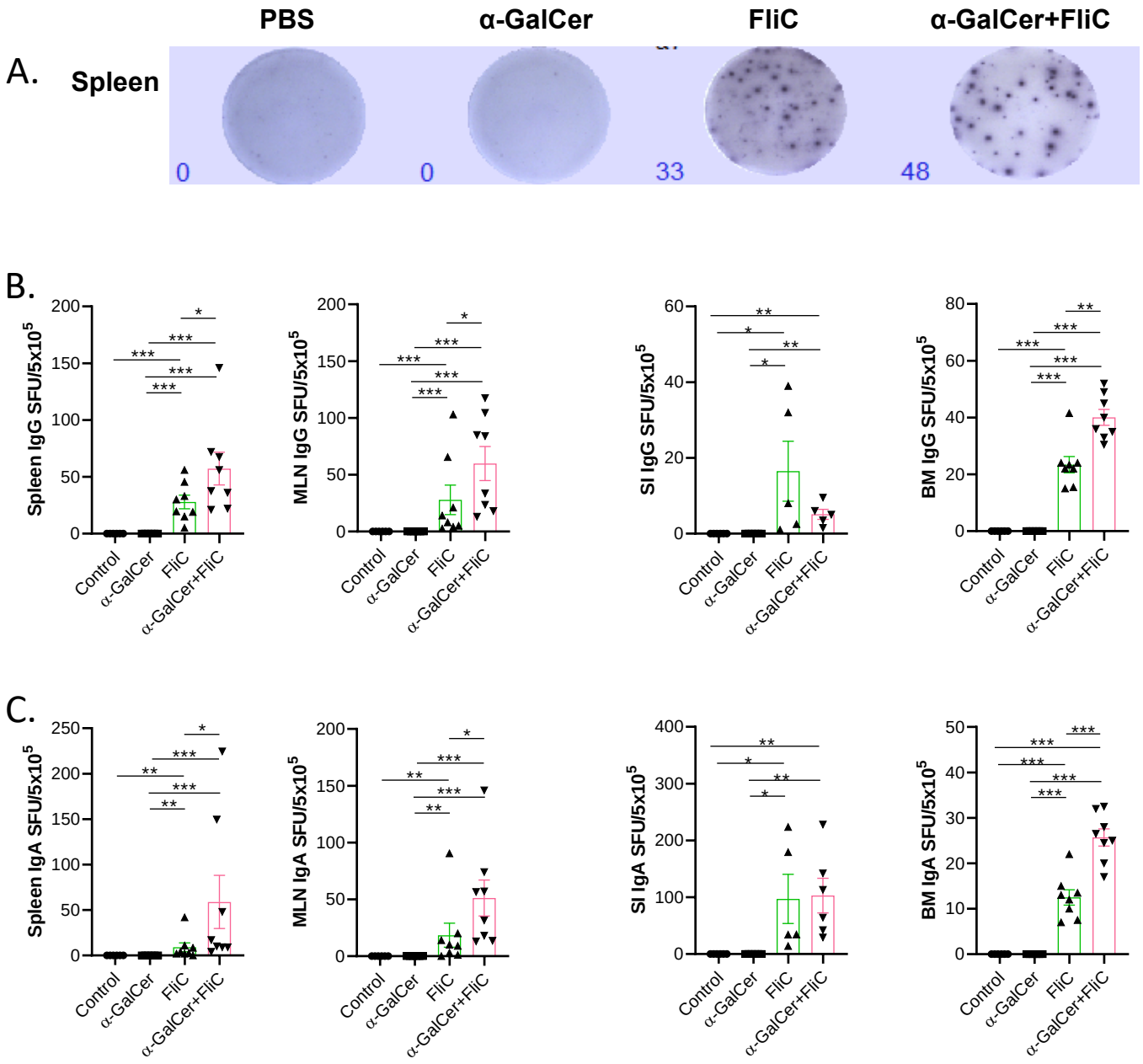


Figure 4.7 α-Gal/FliC co-immunisation results in increased numbers of IgG and IgA Ab-secreting cells in spleen MLN and bone-marrow

WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS. 35 days later mice received 20μg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 39). Single cell suspensions were prepared from spleen, mesenteric lymph nodes, small intestine and bone marrow. ELISPOT analysis of sFliC IgG and IgA responses in the spleen, mesenteric lymph nodes, small intestine and bone marrow. A) Representative pictures of wells showing representative SFUs FliC-specific IgG in spleen. B) Bar-charts showing number of SFUs per 5×10^5 FliC-specific IgG cells in spleen, mesenteric lymph nodes, small intestine and bone marrow. C) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgA in spleen, mesenteric lymph nodes, small intestine and bone marrow. n=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

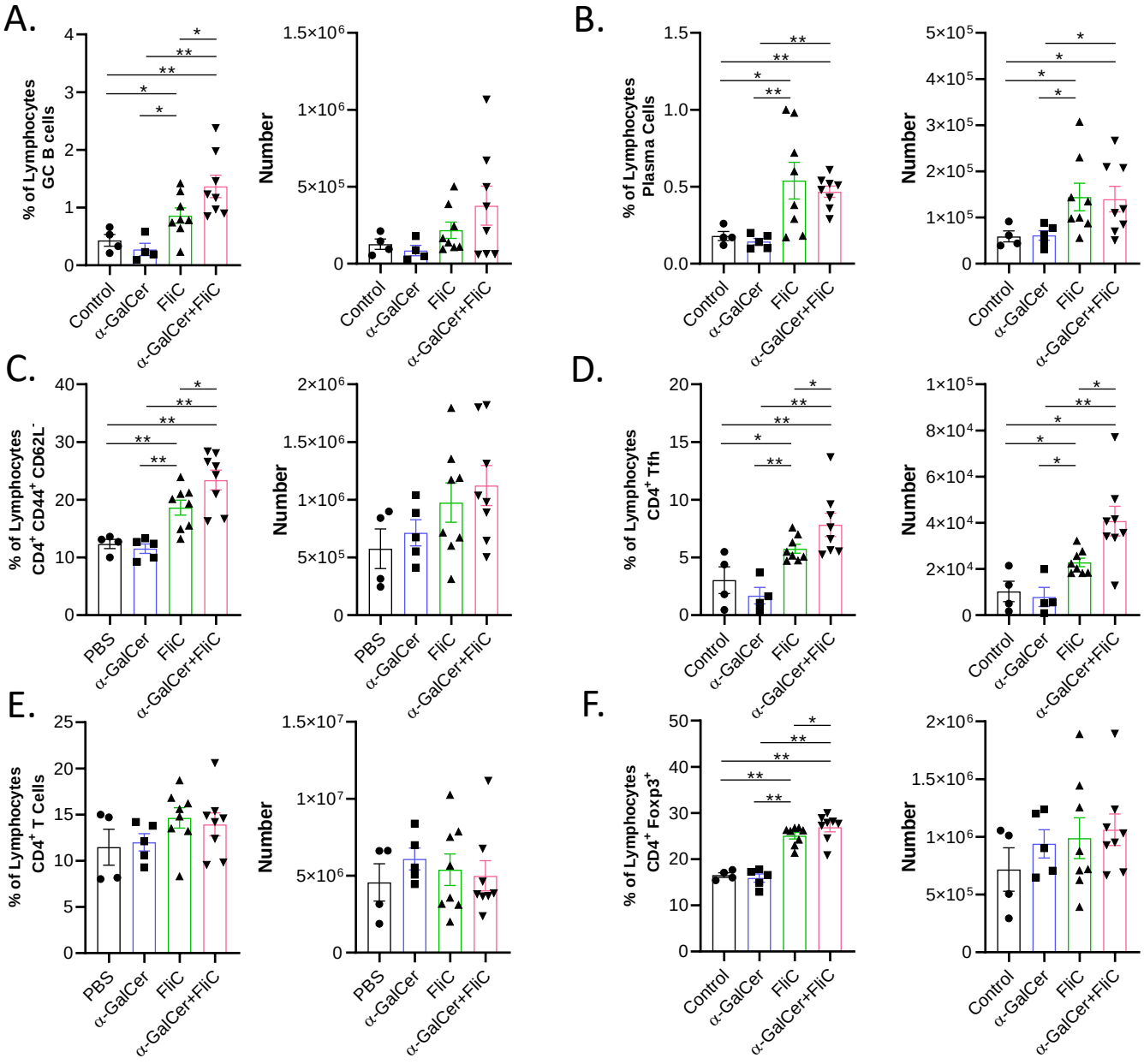


Figure 4.8 Splenic GC B cells and Tfh are increased 39 days post co-immunisation compared to in mice that received FliC alone

WT C57BL/6 (B6) mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS. 35 days later mice received 20µg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 39). Single cell suspensions were prepared from Spleen. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=8-9 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

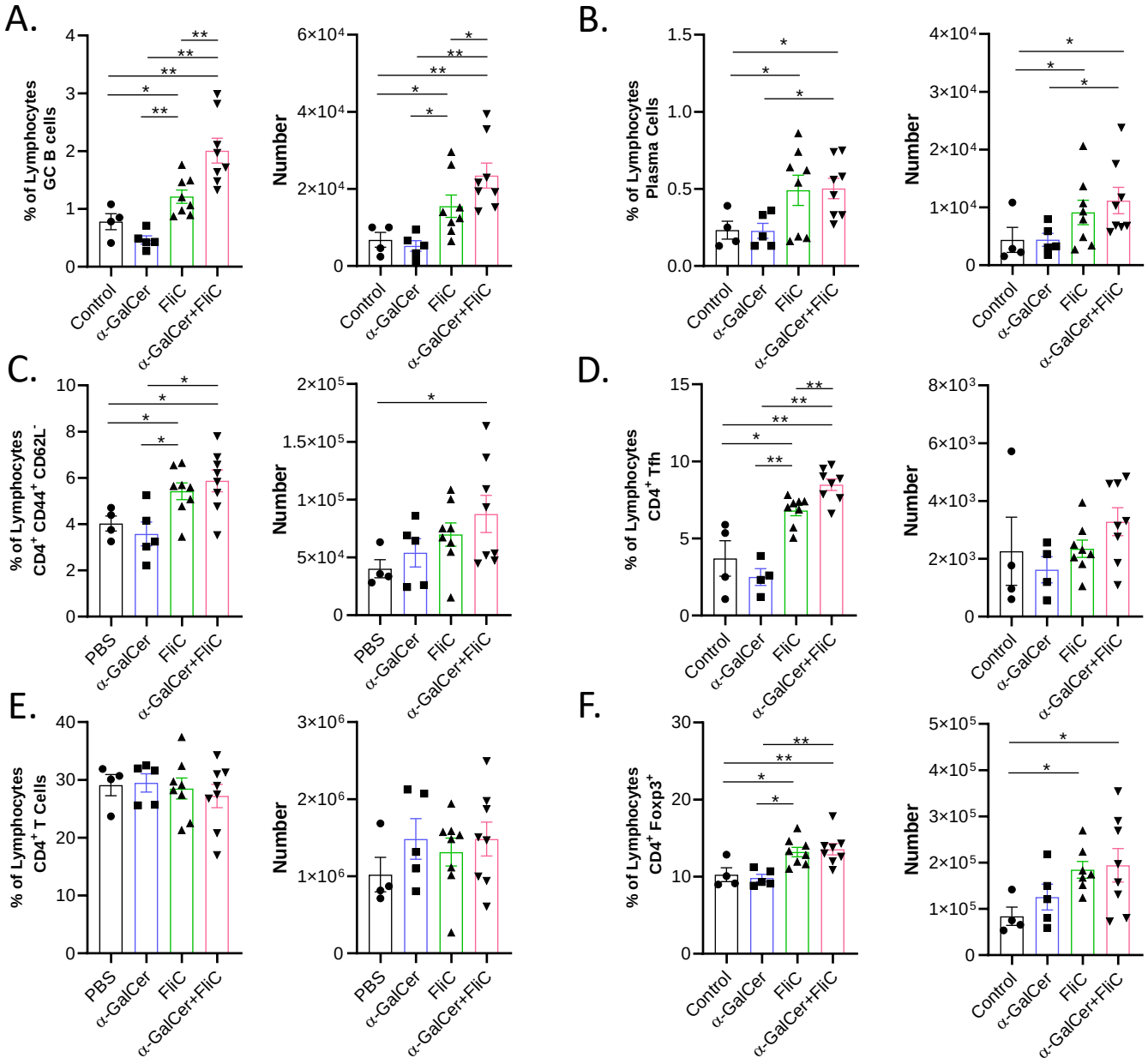


Figure 4.9 GC B cells and Tfh in the MLN are increased 39 days after α-Gal/FliC co-immunisation compared to in mice that received FliC alone

WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS. 35 days later mice received 20μg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 39). Single cell suspensions were prepared from mesenteric lymph nodes. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=8-9 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$.

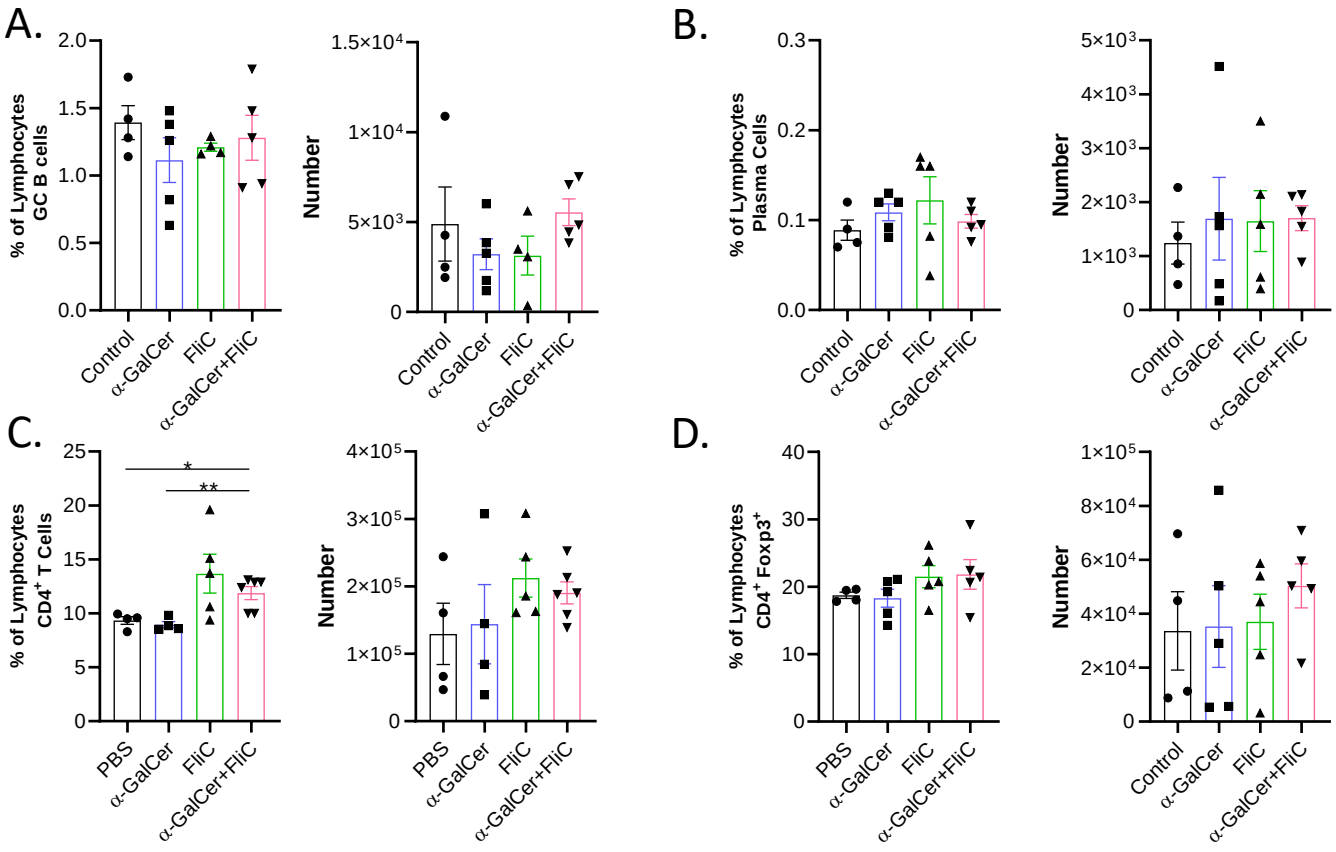


Figure 4.10 The activation of iNKT cells does not influence humoral immunity nor cellular immunity to FliC immunization in the small intestine

WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS. 35 days later mice received 20μg FliC i.p. (or PBS for PBS control mice). Tissues from such mice were harvested 4 days later (Day 39). Single cell suspensions were prepared from small intestine. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD4^+$. D) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$.

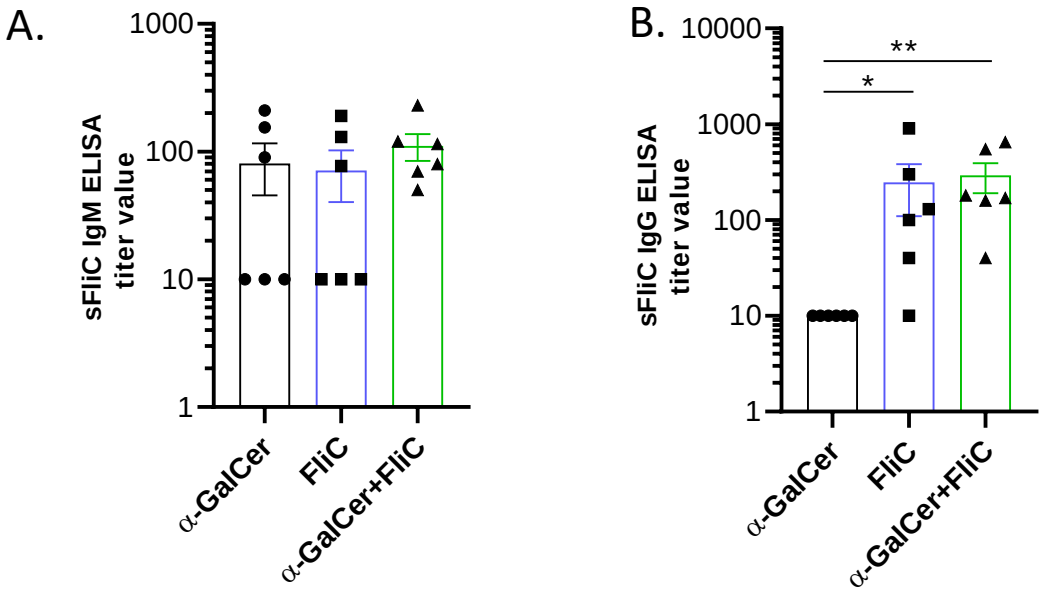


Figure 4.11 Vaccination with FliC induces IgG that is not affected by the activation of iNKT cells in the first 7 days.
WT B6 mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, both α-Gal/FliC or PBS for 7 days. Serum from such mice were collected (Day 7). Serum anti-FliC IgM and IgG titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgM titre, B) Bar-charts showing serum anti-FliC IgG titre. n=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

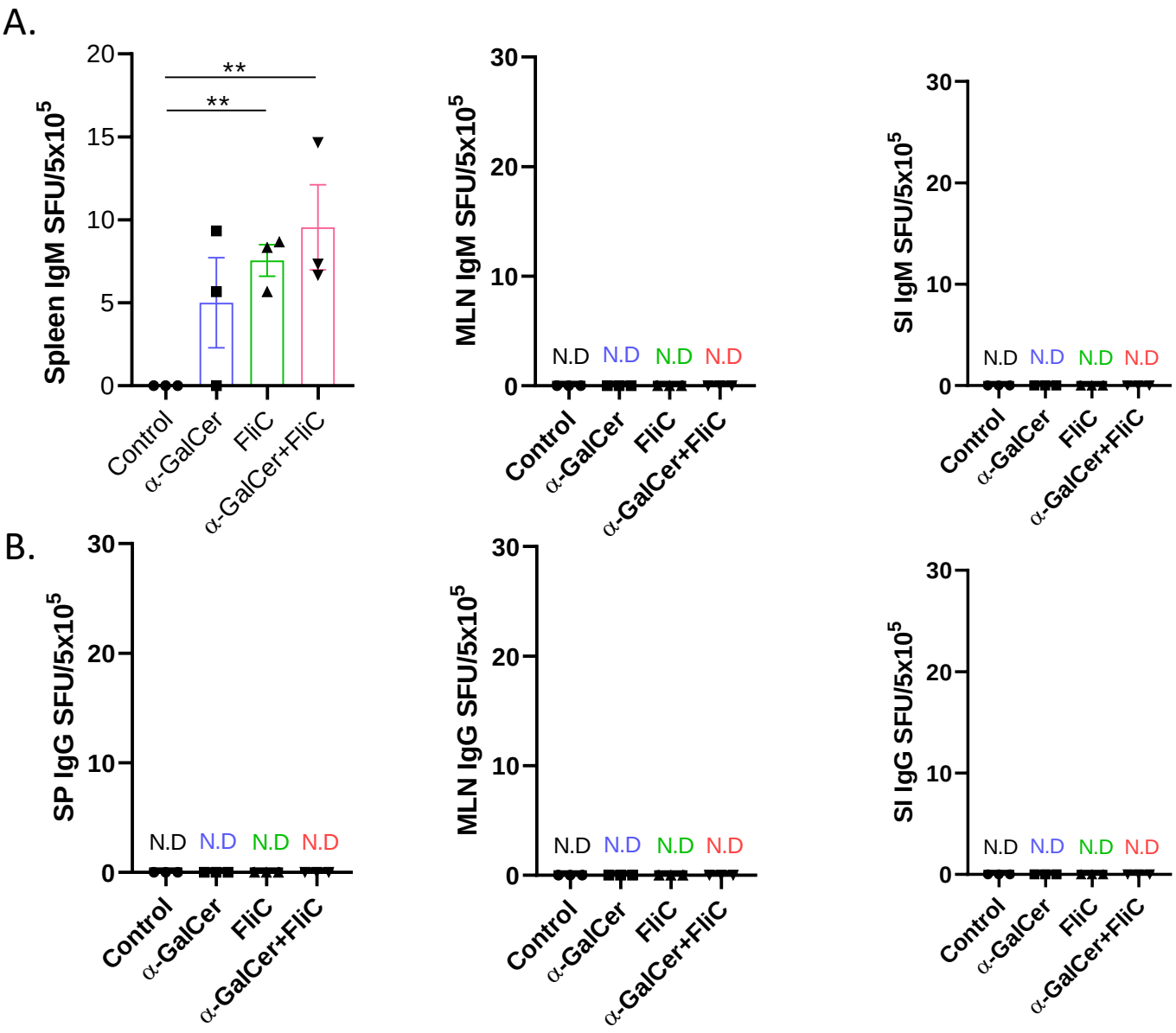


Figure 4.12 Low to no FliC-specific Ab-secreting cells are detectable by 7 days post-vaccination.

WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS for 7 days. Tissues from such mice were harvested (Day 7). Single cell suspensions were prepared from spleen, mesenteric lymph nodes and small intestine. ELISPOT analysis of sFliC IgM and IgG responses in the spleen, mesenteric lymph nodes and small intestine. A) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgM in spleen, mesenteric lymph nodes and small intestine. B) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgG in spleen, mesenteric lymph nodes and small intestine. n=3 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

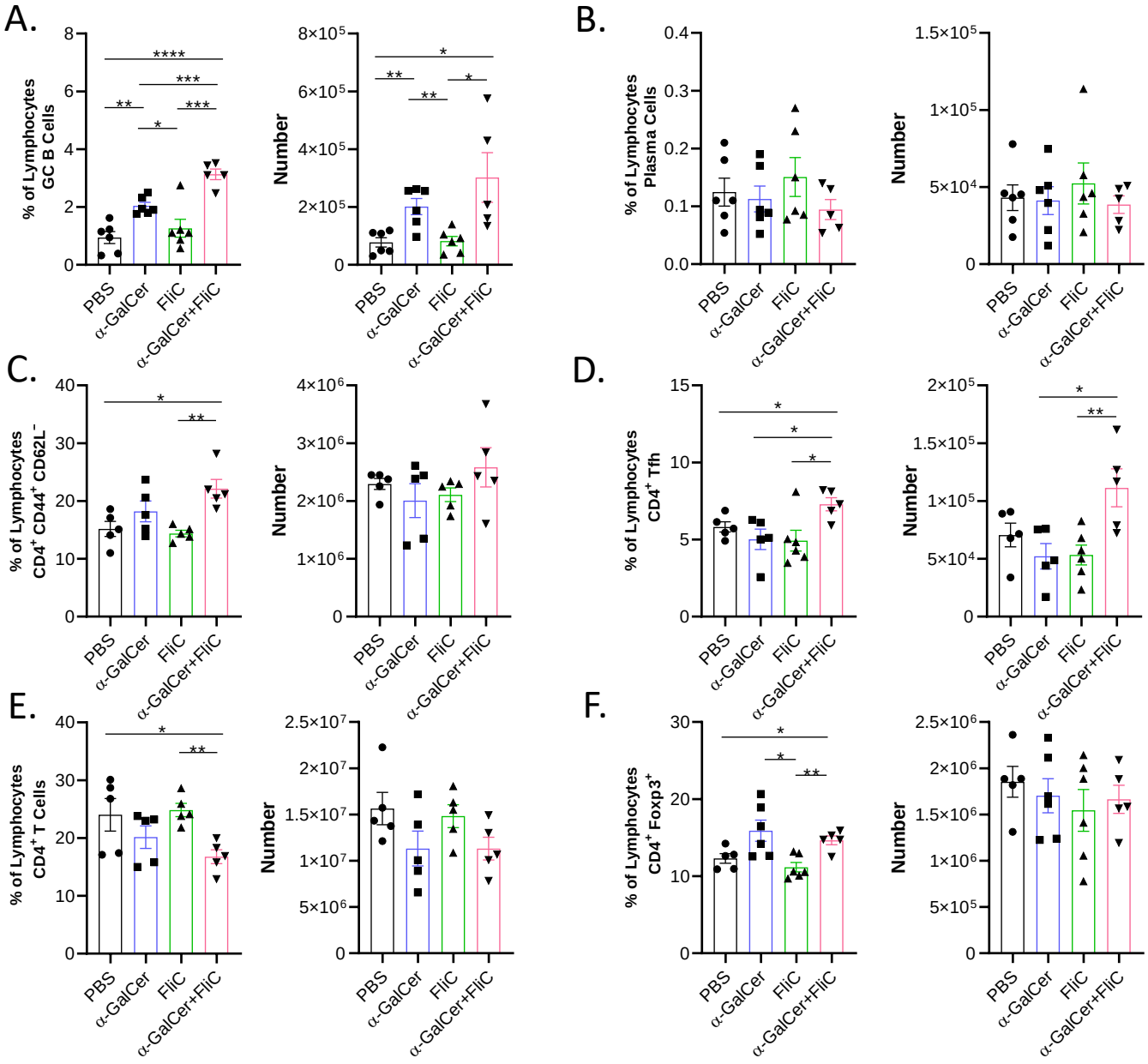


Figure 4.13 The activation of iNKT cells together with FliC rapidly increases germinal centre formation in the spleen as evidenced by increased GC B cell and Tfh.
WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS for 7 days. Tissues from such mice were harvested (Day 7). Single cell suspensions were prepared from Spleen. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

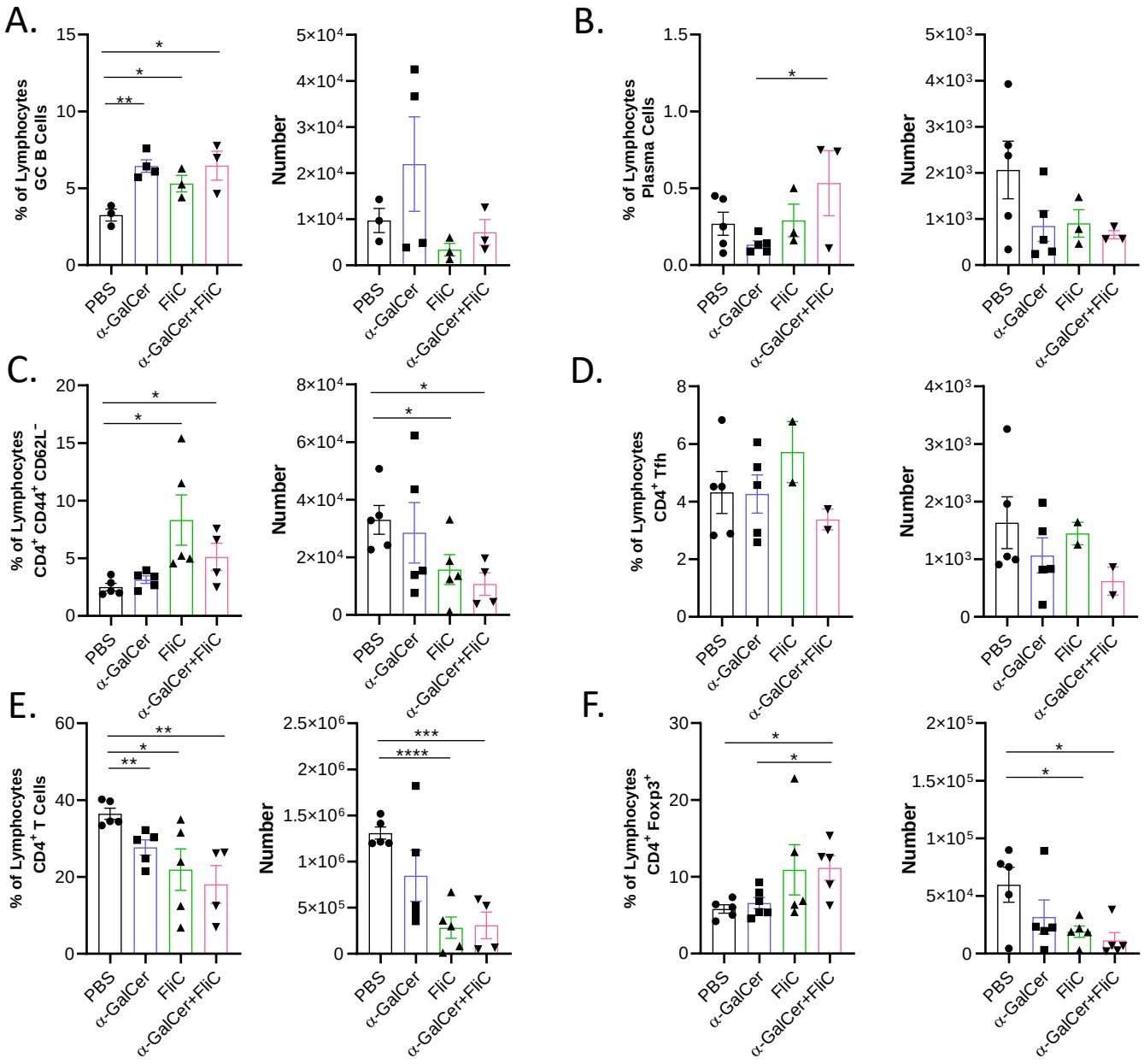


Figure 4.14 iNKT cell activation together with FliC administration minimally impacts immune cell numbers in mesenteric lymph nodes at day 7.

WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS for 7 days. Tissues from such mice were harvested (Day 7). Single cell suspensions were prepared from mesenteric lymph nodes. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=3-5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

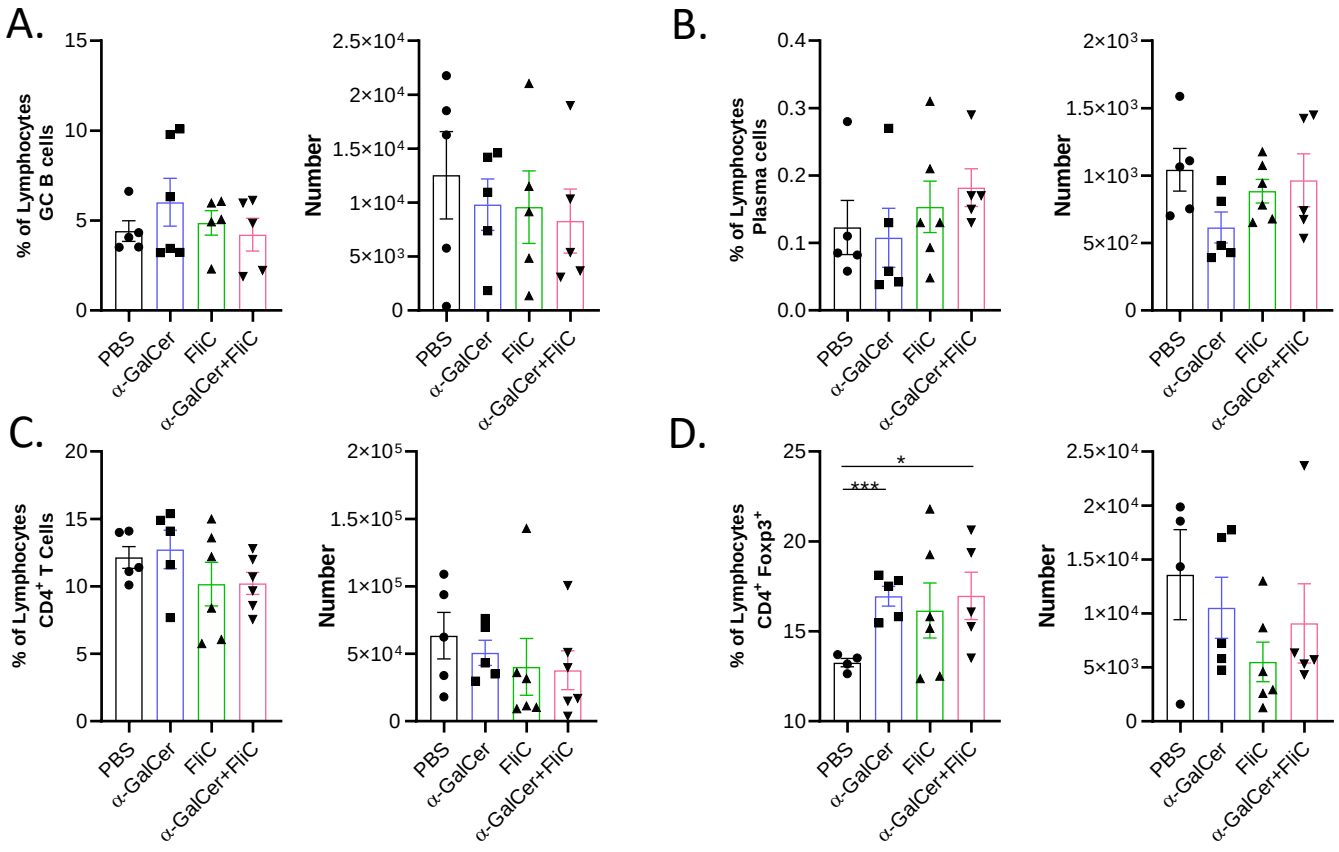


Figure 4.15 FliC immunisation fails to impact adaptive immunity in the small intestine at day 7.

WT B6 mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, both α-Gal/FliC or PBS for 7 days. Tissues from such mice were harvested (Day 7). Single cell suspensions were prepared from small intestine. A) Bar-chart showing the percentage and the total number of GC B cells. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD4^+$. D) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.

CHAPTER 5: THE OPTIMAL IMMUNITY TO FLIC VACCINATION REQUIRES TLR5 LIGATION AND INKT CELL ACTIVATION

5.1 Introduction

iNKT cells play a key role in the modulation of intestinal immunity whereby their function relates to the presence of various populations of APC that can present glycolipid in context of CD1d (295). Commensal microbiota are important in this matter as they are thought to be involved in maintaining homeostasis of mucosal immunity, while perturbation of mucosal homeostasis leads to intestinal inflammation and asthma (3). Therefore, microbiota regulate intestinal immunity through bidirectional interactions where host mucosal immunity shapes the composition of the host microbiota (4, 5). Interestingly, a study on CD1d deficient mice showed defects in commensal microbial growth as well as accelerated microbial colonisation in comparison with WT mice suggesting that NKT cell function impacts the microbiome. In addition, an increased number of commensal intestinal bacteria were found in the $CD1d^{-/-}$ mice compared to WT (6).

The deletion of CD1d expression on $CD11c^{+}$ cells only (i.e. DC) resulted in decreased proportions of NKT17 cells in the mesenteric lymph node and small intestine (295, 321). This was further confirmed following oral administration of αGalCer to WT mice, when it was found that the proliferation of NKT2 and NKT17 was greatly increased two-day post-administration but not in mice with $CD1d^{-/-}$ DC (295). Together these findings provide evidence that $CD11c^{+}$ cells, have the ability to regulate and activate intestinal iNKT cells (1). Consequently, this study highlighted the crosstalk between the DCs and iNKT cells in which they can control the immune cell populations in the intestinal compartment. Therefore, combining the delivery of molecules that activate iNKT cells with PRR agonists may provide a platform for efficient adjuvant vaccines.

iNKT cells has been shown to mobilizing other innate immune response upon activation, especially by its agonist c-glycoside. Upon activation, iNKT cells have been shown to activate NK cells via IFN-γ secretion in a process that can be further enhanced by IL-12 production by

DCs (369, 370). Activated NK cell then in turn secrete high levels of IFN-γ which plays a critical role in the clearance of intracellular pathogens such as STm (371, 372). iNKT cells interacting with DC that present glycolipid can also deliver other signals through molecular interactions such as CD40/CD40L (372). Such interaction stimulates DCs to produce IL-12 and enhances a positive feedback loop between DCs and iNKT cells. This means IL-12 stimulates iNKT cells to secret IFN-γ, and IFN-γ stimulates DCs to increase IL-12 expression (372).

Jα18 or CD1d deficient mice have been reported to suffer spontaneous infections, including mucosal infections, such as *Listeria monocytogenes* and *Salmonella typhimurium*, suggesting that iNKT cells play a non-redundant role in immune response against microbial organisms (223). A study found that following oral *Listeria monocytogenes* infection, iNKT cells upon activation is the major producer of IFN-γ results in induction of NK cells to produce further IFN-γ in order to enhance bacterial clearance (373, 374). The same study transfer iNKT cells into $RAG^{-/-}$ mice then infected mice with *Listeria monocytogenes,* transferred mice with iNKT cells showed a reduction in bacteria burden and death rate compared to un-transferred mice. The same study used CD1d deficient mice and mice showed induction of systemic bacterial burden after oral infection of *Listeria monocytogenes* (373, 374).

In *Salmonella typhimurium* infection, iNKT cells were shown to be activated an to produce IFN-γ (226, 375). Interestingly, the activation of iNKT cells was found to result from the recognition of endogenous glycolipid together with IL-12 secreted from LPS stimulated DC (226). Taken together, these findings support a role for iNKT cells in influencing the immune response against pathogens and regulating the colonisation of bacteria.

As mentioned previously, DCs express TLR5, which can be ligated by flagellin (376-380). Recognition results in the dimerization of TLR5 and signalling via MyD88, MAPK, and NF-kB pathways resulting in the activation of DCs, the upregulation of CD86, MHC II, CD80 and the secretion of various cytokines and chemokines (379, 381, 382). It has been reported that

TLR5-deficient mice fail to induce an adaptive immune response following flagellin administration (313, 383, 384). In addition, it has been found that despite the lack of immunity triggered by TLR5, $TLR5^{-/-}$ mice display resistance to oral Salmonella typhimurium infection as these bacteria use TLR5 and $CD11c^{+}$ intestinal DCs to deliver systemic infection (313). Such mice have also been reported to have an increased basal intestinal inflammatory response and suffer from a metabolic syndrome such as hypertension and hyperphagia (385, 386) . Taken together, all observations suggest that TLR5 is a key contributor to the intestinal homeostasis that is required between the commensal bacteria and intestinal immune cells and the prevention of harmful intestinal inflammation.

5.2 Aims and Hypothesis

Given that iNKT cells have been shown to activate DC we hypothesised that the activation of iNKT cells may circumvent the requirement for TLR5 for effective adaptive immunity upon FliC vaccination. Therefore, we sought to examine the response to FliC in TLR5 deficient mice in the presence or absence of concomitant iNKT cell activation.

We also wanted to assess whether iNKT cells had any involvement in immune responses following FliC vaccination without exogeneous glycolipid activation as iNKT cells can be activated by proinflammatory cytokines independently of agonist glycolipid.

5.3 Results

5.3.1 iNKT cell activation can act as an adjuvant in the absence of TLR5

FliC administration has been shown to be sufficient to generate both T and B cell responses to itself and leads to a degree of protection from STm which has been shown to be TLR5 dependent (9). In this thesis we showed that the use α-GalCer as an additional adjuvant can activate iNKT cells and further enhance the immune response to FliC. Therefore, we sought to examine whether iNKT cell activation can enhance the response to FliC in the absence of TLR5 signalling. To this end, TLR5-deficient mice were immunised with FliC with and without α-GalCer administration (or WT mice with FliC alone). 14 days post immunisation mice received a further dose of FliC and were harvested 4 days later (i.e. day 18).

Assessment of the anti-sFliC IgG response revealed that TLR5-deficient mice co-immunized with α-Gal/FliC demonstrated a significant increase of IgG titres in serum compare to TLR5-deficient immunized with FliC only and was no different to the levels found in WT mice (figure 5.1 A). This was consistent in both the anti-sFliC IgG1 and IgG2C response where the co-immunized TLR5-deficient mice with α-Gal/FliC displayed a significant increase of IgG1 titres in serum compare to TLR5-deficient mice FliC alone which was similar to WT mice immunized with FliC alone (figure 5.1 B and C). Interestingly, FliC-specific IgA titres were not rescued upon α-Gal/FliC administration and remained absent as in TLR5-deficient mice immunized with FliC alone (figure 5.1 D). These data suggest that TLR is required to generate class-switched Ab responses to FliC but that iNKT cell activation could partially rescue this response in the absence of TLR5.

5.3.2 iNKT cell activation influence the production of FliC specific Ab secreting cells in the MLN independently of TLR5

To assess whether iNKT cell activation can influence the generation of Ab-secreting cells in the absence of TLR5 signalling, we assessed the number of IgG and IgA FliC-specific Ab-

secreting cells generated following FliC administration with and without the activation of iNKT cells (figure 5.2). TLR5-deficient mice were primed for 14 days with and without iNKT cell activation and day 14 boost with FliC as before.

The relative frequency of TLR5-deficient mice FliC-specific IgG and IgA Ab-secreting cells was significantly decreased upon FliC immunisation with or without iNKT cell activation in the spleen, MLN and small intestine compared to WT immunized mice with FliC only (figure 5.2 A -C). However, in the MLN, an IgG and IgA responses was detectable in α-Gal/FliC immunised TLR5-deficient mice that was absent from sFliC-immunised TLR5-deficient mice (figure 5.2 B and C). This increase in the IgG and IgA response in MLN was significantly lower than WT mice received FliC. This indicates that the activation of iNKT cells can partially restore the plasma cells secreting IgG and IgA independent of TLR5 in MLN at this time.

5.3.3 GC formation following FliC is independent of TLR5 in MLN

Since the humoral response to FliC is T-dependant, and FliC as adjuvant can generate sFliC-specific GC B cells and plasma cell, we next sought to examine whether concomitant iNKT cell activation in the absence of TLR5 affected the generation of GC B cells and plasma cells following FliC administration (figure 5.3-5). To this end, TLR5-deficient mice were immunised with FliC with and without α-GalCer administration (or WT immunized with FliC alone). 14 days post immunisation mice received a further dose of FliC and were harvested 4 days later (i.e. day 18).

Analysis of GC B cells in the spleen and MLN 18 days post-FliC immunisation showed that the increased frequency and number of GC B cells following immunisation was dependent on TLR5 (figure 5.3 and 4 A). However, iNKT cell activation was only found to restore GC B cell frequency in the MLN but not in the spleen (figure 5.3 and 4 A).

Assessment of plasma cells (figure 5.3-5 B), memory T cells (figure 5.3-5 C) and Treg (figure 5.3-5 F) in spleen, MLN and small intestine 18 days post-FliC immunisation demonstrated that the frequency and number of such cells were the same in all groups.

Analysis of the frequency and number of Tfh in spleen post-FliC immunisation showed that iNKT cell activation did not restore Tfh frequencies or numbers in the spleen in TLR5-deficient (figure 5.3 D). In the MLN there was no significant difference between any of the groups in terms of Tfh frequency or number (figure 5.4).

Taken together, these data demonstrate that iNKT cell activation can augmentation IgG responses following FliC administration in the absence of TLR5 signals. The data further suggest that the impact of iNKT cell activation promotes germinal centre response and IgG and IgA secreting cells in the MLN without a significant impact on splenic responses.

5.3.4 Optimal production of anti-FliC antibodies following vaccination is dependent on iNKT cells.

We and others have shown that intestinal DCs have been reported to express CD1d and the expression of CD1d is higher in cDC2 ($CD103^+$ $CD11b^+$) compared to other subsets (Figure 3.5). cDC2 has been reported to be central to the initiation of both T and B cell responses following FliC where they were found to migrate to the MLN from the small intestine. Given that iNKT cells can be activated by a combination of self-glycolipid recognition and proinflammatory cytokines we sought to examine whether iNKT cells were involved in the generation of immune responses to FliC, even in the absence of exogenous glycolipid administration. To this end, CD1d-deficient mice were immunised with FliC or PBS (or WT mice with FliC). 14 days post immunisation mice received a further dose of FliC or PBS and were harvested 4 days later (i.e. day 18; figure 5.6).

Analysis of the anti-sFliC IgG response following FliC vaccination showed that CD1d-deficient mice immunized with FliC made a poor IgG response compared to WT mice (figure 5.6 A). This was also apparent on examining IgG subclasses with anti-sFliC IgG1 Ab (figure 5.6 B), anti-sFliC IgG2C Ab (figure 5.6 C) and anti-sFliC IgA Ab (figure 5.6 D) titres all significant decreased in CD1d-deficient mice compared to WT mice.

5.3.5 The production of FliC specific Ab secreting cells is dependent on iNKT cells.

To assess whether the absence of the iNKT cell can influence the generation of Ab-secreting cells, we assessed the frequency of IgG and IgA FliC-specific Ab-secreting cells generated in response to FliC administration in the presence or absence of NKT cells (figure 5.7).

In keeping with serum Ab, the relative frequency of FliC-specific IgG and IgA Ab-secreting cells in the spleen, MLN and small intestine of CD1d-deficient mice was significantly lower than immunised WT mice (figure 5.7 A-C). However, a low frequency of both IgG and IgA secreting cells were detected in the spleen and MLN of $CD1d^{-/-}$ mice (figure 5.7 A-C). Taken together these data suggest that NKT cells are critical mediators of the humoral response seen upon FliC immunisation.

5.3.6 GC B cells, Tfh and plasma cells generated following FliC vaccination are dependent on the presence of NKT cells

The humoral response to FliC is T-dependant and as such FliC administration results in the generation of sFliC-specific GC B cells, Tfh and plasma cells. Therefore, we sought to examine whether the absence of iNKT cell activation affected the germinal centre reaction (figure 5.8-10). To this end, CD1d-deficient mice were again immunised with FliC or PBS (or WT immunized with FliC). 14 days post immunisation mice received a further dose of FliC or PBS and were harvested 4 days later (i.e. day 18) and tissue was analysed by flow cytometry.

Analysis of GC B cells in the spleen 18 days post-FliC immunisation revealed that, whilst not absent, the proportion and number of FliC-specific GC B cells in the spleen of CD1d-deficient mice was markedly reduced compared to that seen in WT mice following FliC administration (figure 5.8 A). This difference was also mirrored in a reduced frequency and number of plasma cells (figure 5.8 B) and Tfh (figure 5.8 D) in $CD1d^{-/-}$ compared to WT control mice.

18 days post FliC immunisation in spleen we found that the proportion and number of $CD4^{+}$ T cells in FliC immunised CD1d-deficient mice or FliC immunised WT mice were both significantly increased compared to PBS control mice (figure 5.8 E) whilst the frequency but not number of Treg was reduced in mice deficient in NKT cells (figure 5.8 F).

Analysis of the MLN 18 days post-FliC immunisation was similar to the spleen in that both the frequency and number of GC B cells (figure 5.9 A) and plasma cells (figure 5.9 B) in immunised CD1d-deficient mice was significantly reduced compared to WT control. However, interestingly, the frequency and number of Tfh cells of CD1d-deficient mice or WT mice were unaffected by the loss of NKT cells (figure 5.9 D).

In MLN, the frequency and number of Treg cells of FliC immunised WT mice were significantly increased compared to control animals (figure 5.9 F). Although the number of Treg cells was the same between both groups received FliC, the frequency of CD1d-deficient mice was significantly decrease compared WT mice (figure 5.9 F).

Analysis of the small intestine of immunised $CD1d^{-/-}$ and WT mice failed to detect any discernible difference in the aforementioned populations of cells in this tissue (figure 5.10).

Taken together, these data demonstrate that the loss of NKT cells dramatically impacts the generation of the GC reaction, Ab secreting cells and serum Ab following FliC immunisation.

5.3.7 CD1d-deficient mice harbour an unaltered population of cDC2 in the small intestine

Thus far we have shown that CD1d$^{-/-}$ mice make poor responses to FliC upon immunisation. We next sought to examine whether the observed reduced immunity was simply due disrupted intestinal immunity due to the loss of NKT cells and in particular whether cDC2 were present in CD1d deficient mice. To this end, cDC2 were identified and enumerated in the MLN and small intestine of CD1d$^{-/-}$ mice and compared to those found in naïve WT mice (figure 5.11-12). cDC2 subsets were gated as singlet live leukocytes and were identified as Lin^{-} (CD3, B220, F4/80, Gr1) CD11c^{hi}MHCII^{+}. Mucosal cDC2 were further specified as being CD103^{+} CD11b^{+} (figure 5.11A). The proportion and absolute number of cDC2 was determined in each tissue.

The number and percentage of cDC2 in MLN and small intestines of CD1d-deficient or WT mice were found to be the same (figure 5.11-12 A-B). Moreover, we also assessed the expression of CD1d, CD86 and CD40 on cDC2 subsets in the MLN and small intestine as markers of activation (figure 5.11-12 C). This revealed that CD1d expression, as expected, was not expressed in CD1d-deficient mice (figure 5.11-12 C). The level of expression of CD86 and CD40 by DC2 in the MLN and SI of CD1d$^{-/-}$ and WT was identical (figure 5.11-12 C). These data suggest that the absence of NKT cells does not result in a numeric or phenotypic change in cDC2 in either the MLN or SI.

5.3.8 The loss of NKT cells does not affect the migration of SI cDC2 to MLN 24 hr post-FliC administration

Next, we sought to determine whether the loss of CD1d affects migration of cDC2 to the MLN following FliC administration, as has been previously reported (295, 315, 316). To this end, CD1d-deficient mice or WT mice received 20µg FliC i.p. or PBS and cDC2 derived from the

MLN and small intestine were analysed 24 hours later (figure 5.13-14). The cDC2 were gated as previously mentioned (figure 5.11A).

We found that cDC2 from CD1d-deficient mice increased in frequency and number in MLN 24 hr after immunisation in a manner similar to WT control mice (figure 5.13 A and B). In consort, the cDC2 frequency and number in the small intestine after FliC immunisation was reduced to a similar extent in both CD1d-deficient mice or B6 mice (figure 5.14 A and B). Interestingly, despite the accumulation of cDC2 in the MLN of CD1d-deficient mice following FliC administration, such cells exhibited lower expression of both CD86 and CD40 compare to immunised WT mice (figure 5.13-14 C). Indeed, the expression of CD86 and CD40 in MLN cDC2 from $CD1d^{-/-}$ mice was no different to unimmunised controls (figure 5.13C).

5.4 Discussion

This chapter examined the mechanisms the role of iNKT cells in immune responses generated by FliC immunisation. Flagellin has been shown to be capable of stimulating T and B cell responses with a strong Th2 bias to itself and co-administered antigens in TLR5-dependant manner (349, 350, 352). It has been previously shown that mucosal $CD103^+$ cDC2 preferentially accumulate in the MLN following FliC immunisation, likely due to their high expression of TLR5, which is key to driving immune responses to FliC following immunisation (315, 316, 323). Moreover, in this thesis we found that mucosal $CD103^+$ cDC2 express high levels of CD1d in comparison to other DC subsets found in the small intestine (figure 3.5) (295). Given that iNKT cells have been shown to activate DC we hypothesised that the activation of iNKT cells may circumvent the requirement for TLR5 for effective adaptive immunity upon FliC vaccination.

Interestingly, TLR5-deficient mice after immunisation with α-Gal/FliC generated IgG FliC-specific Ig similar to WT mice immunised with only flagellin 18 days post activation (figure 5.1 A-C). This suggests that α-GalCer presentation by cDC2 results in DC maturation independently of TLR5 and that such DC are able to stimulate humoral immunity to FliC enhance antibody production in vivo, even independent of TLR5 manner. This was further explained by study found that DC following α-GalCer administration in vivo, is the major producer of IL-12 (387). Such production of IL-12 in response to α-GalCer requires CD40/CD40L interaction between NKT cells and DC (387). This also agree with many studies that found DC in response to T cell-associated CD40L or other microbial stimuli acts as a dominant producer of IL-12 (388-390). These observations have been further explained our finding of DC is not affected by the deletion of TLR5 following administration of α-Gal/FliC. Taken together, this is concluded that α-GalCer can provide DC maturation independent of TLR5 however optimal stimulation of humoral immunity is still required TLR5 ligation.

Although we have shown that α-GalCer can trigger DC2 maturation it remained to be seen whether iNKT cells played any role in DC activation following immunisation with FliC. Despite the fact that no exogenous glycolipids were present when FliC was administered alone, iNKT cells can be activated by a combination of self-glycolipid recognition and cytokines such as IL-12 and IL-18 secreted by DCs. Therefore, it remained a possibility that iNKT cells could play a role in the generation of the immune response to FliC. Surprisingly, we found that the FliC-specific antibody response, FliC-specific Ab secreting cells and the germinal centre reaction generated on FliC immunisation was markedly reduced or absent in CD1d-deficient mice (Figure 5.6 A-C). Therefore, unexpectedly, iNKT cells appear to play a key role in facilitating responses to FliC even in the absence of agonist glycolipids.

Due to the unexpected finding that FliC immunisation relied on the presence of iNKT cells to generate immune responses, we initially wanted to rule out the trivial explanation that there was simply disrupted intestinal immunity due to the loss of NKT cells which resulted in a loss of cDC2 from the SI. However, cDC2 in CD1d-deficient mice were present in similar numbers and frequencies in SI and MLN compared to WT B6 mice (figure 5.11-12 A-B). Furthermore, cDC2 were found to migrate as efficiently to the MLN as in WT mice (figure 5.13-14). This raised the possibility of whether cDC2 activated in the absence of iNKT cells were functionally less capable of generating T cell responses to FliC. Interestingly, despite the accumulation of cDC2 in the MLN in the absence of NKT cells following FliC administration, such cells exhibited lower expression of both CD86 and CD40 compared to immunised WT mice (figure 5.13-14 C). Therefore, we would suggest that cDC2 require a two-step process in order to undergo full maturation following FliC immunisation where TLR5 ligation alone is enough to trigger proinflammatory cytokine secretion and migration to the MLN. However, signals from iNKT cells (be they cellular or soluble) are required for cDC2 to achieve full activation, upregulate costimulatory molecules and the optimal stimulation of FliC-reactive T cells.

Interestingly, whilst α-GalCer administration compensated for many aspects of TLR5 triggering, IgA was entirely absent in TLR5-deficient mice even after immunisation with α-Gal/FliC (figure 5.1 D). In addition, both IgA and IgG2c were absent in CD1d-deficient mice after immunisation with α-Gal/FliC (figure 5.6 C-D). In the case of flagellin studies, it has been reported that IgA isotype switching is highly dependent on TLR5 manner (316, 391). In addition to the need for TLR5, IgA switching was selectively dependent on the recruitment and migration of $CD103^+$ DCs to the mesenteric lymph nodes following FliC immunisation (316). The same study found that flagellin-specific IgA switching was also dependent on the presence of T cells, suggesting the importance of TLR5 processing and presenting peptide to MHC class II (316, 331). However, a later study found that type I IFN receptor signalling was required to deliver anti-FliC IgA/IgG2c responses (391). The study found that in IFNAR1-deficient mice, the production of both IgA and IgG2c antibodies was markedly reduced, whereas these responses were normal in response to ovalbumin-specific antibodies (391). The study concluded that type I IFN signalling is required for the production of flagellin-specific IgA/IgG2c Ab responses (391). It has been reported that TLR5 deficiency fails to stimulate IFN-β production through the TLR5 and MyD88 signalling pathways, resulting in the absence of type I IFN signalling (391).

Our experiments using TLR5 deficient mice showed that mice that received α-Gal/FliC had an absence of IgA responses but IgG2c was significantly induced (Figure 5.6 C). Due to the link between the major production of IFN-γ by activated iNKT cells and IgG2c associated to a Th1 response (349), it is possible that the alteration of cytokine environment provided by NKT cells may affect isotype switching of IgG2c.

Our findings in CD1d-deficient mice also raise the possibility that the observed loss of IgA in $TLR5^{-/-}$ and $CD1d^{-/-}$ mice was due to alterations in gut microbial homeostasis (295, 392, 393). TLR5-deficient mice demonstrated reduced levels of mucosal FliC-specific IgA antibodies

compared to WT mice in the steady state (394), which results in damage to the mucosal barrier and causes microbial dysbiosis (386). This is possible that TLR5 is maintained a balance between the host and pathogens. In CD1d-deficient mice, it has been reported these mice had changes in the IgA repertoire that were caused by alterations in the gut microbiota (295). Furthermore, even in the absence of α-Gal, human NKT cells were able to stimulate IgA antibody production by B cells in vitro (395). Taken together, these results indicate that despite the role of TLR5 in promoting intestinal health, the involvement of NKT cells either directly or indirectly enhances this process.

In conclusion, we found that for optimal immunity following immunisation with FliC both TLR5 signalling and NKT cells are required. TLR5 is required to generate IgA class-switched Ab responses to FliC but that iNKT cell activation could partially rescue this response in the absence of TLR5. Our data also demonstrate that the loss of NKT cells dramatically impacts the success of FliC immunisation and that iNKT cells were required for optimal DC activation and the expression of important costimulatory molecules by migrating cDC2.

5.5 Figures

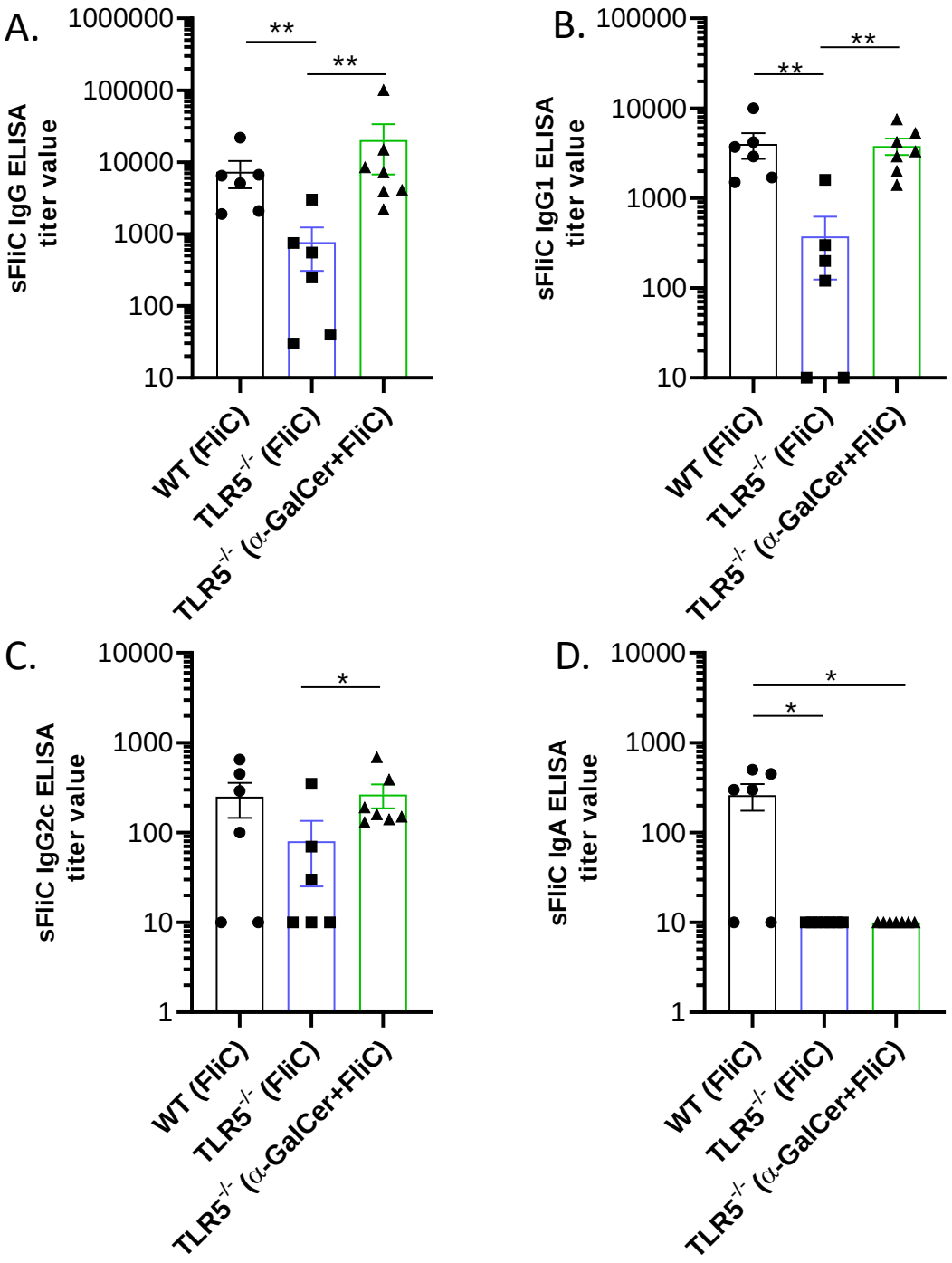


Figure 5.1 The activation of iNKT cells compensates for the loss of TLR5 and enables anti-sFliC IgG antibody production following FliC administration.
WT B6 or TLR5$^{-/-}$ mice received i.p. injection of either 20μg FliC, 2μg α-GalCer, or both α-Gal/FliC. 14 days later mice received 20μg FliC i.p. Serum from such mice were collected 4 days later (Day 18). Serum anti-FliC IgG, IgG1, IgG2c and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2c, and D) IgA titre. n=6-7 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

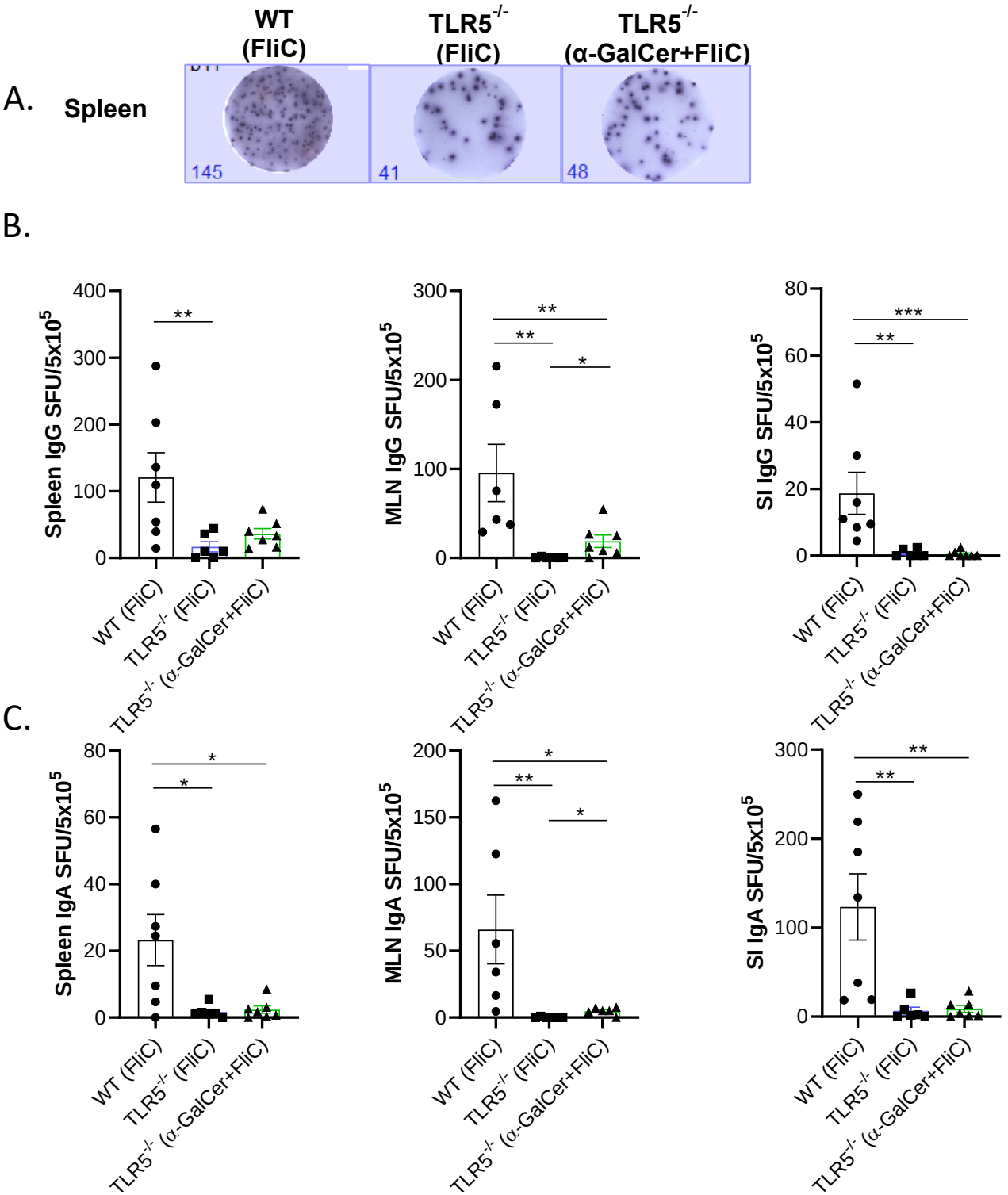


Figure 5.2 iNKT cell activation increases the frequency of FliC specific Ab secreting cells in MLN independently of TLR5.

WT B6 or TLR5$^{-/-}$ mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, or both α-Gal/FliC. 14 days later mice received 20µg FliC i.p. Tissues from such mice were harvested (Day 18). Single cell suspensions were prepared from spleen, mesenteric lymph nodes and small intestine. ELISPOT analysis of FliC IgG and IgA responses in the spleen, mesenteric lymph nodes and small intestine. A) Representative pictures of wells showing SFUs FliC-specific IgG in spleen. B) Bar-charts showing number of SFUs per 5 × 10^5 cells FliC-specific IgG in spleen, mesenteric lymph nodes and small intestine. C) Bar-charts showing number of SFUs per 5 × 10^5 cells FliC-specific IgA in spleen, mesenteric lymph nodes and small intestine. n=6-7 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.

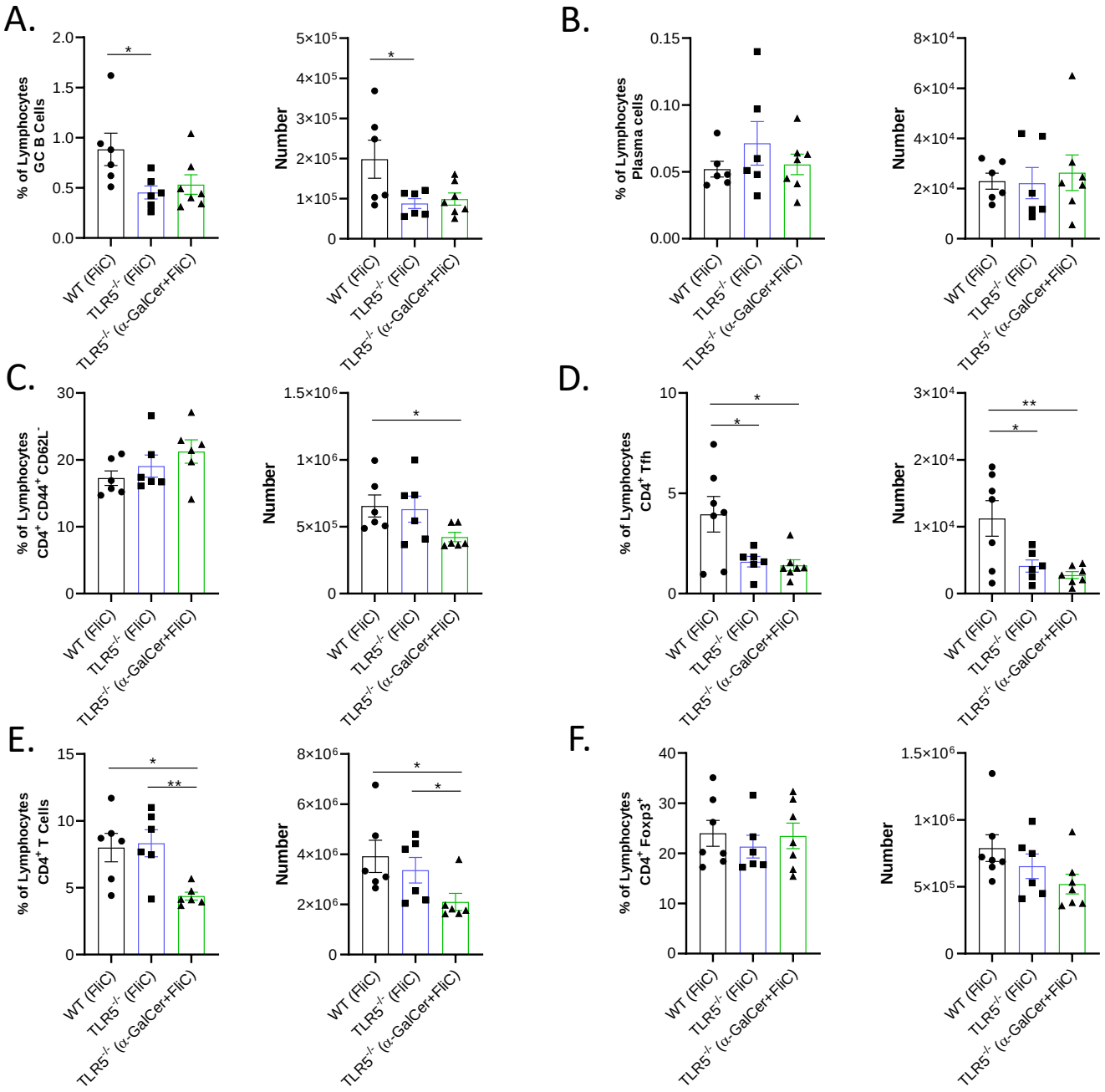


Figure 5.3 The activation of iNKT cells fails to induce GC formation or Tfh generation in the spleen in the absence of TLR5.

WT B6 or TLR5$^{-/-}$ mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, or both α-Gal/FliC. 14 days later mice received 20µg FliC i.p. Single cell suspensions were prepared from Spleen. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of CD44^{+} CD62L^{-}. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of CD4^{+}. F) Bar-chart showing the percentage and the total number of Foxp3^{+} Treg. N=6-7 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

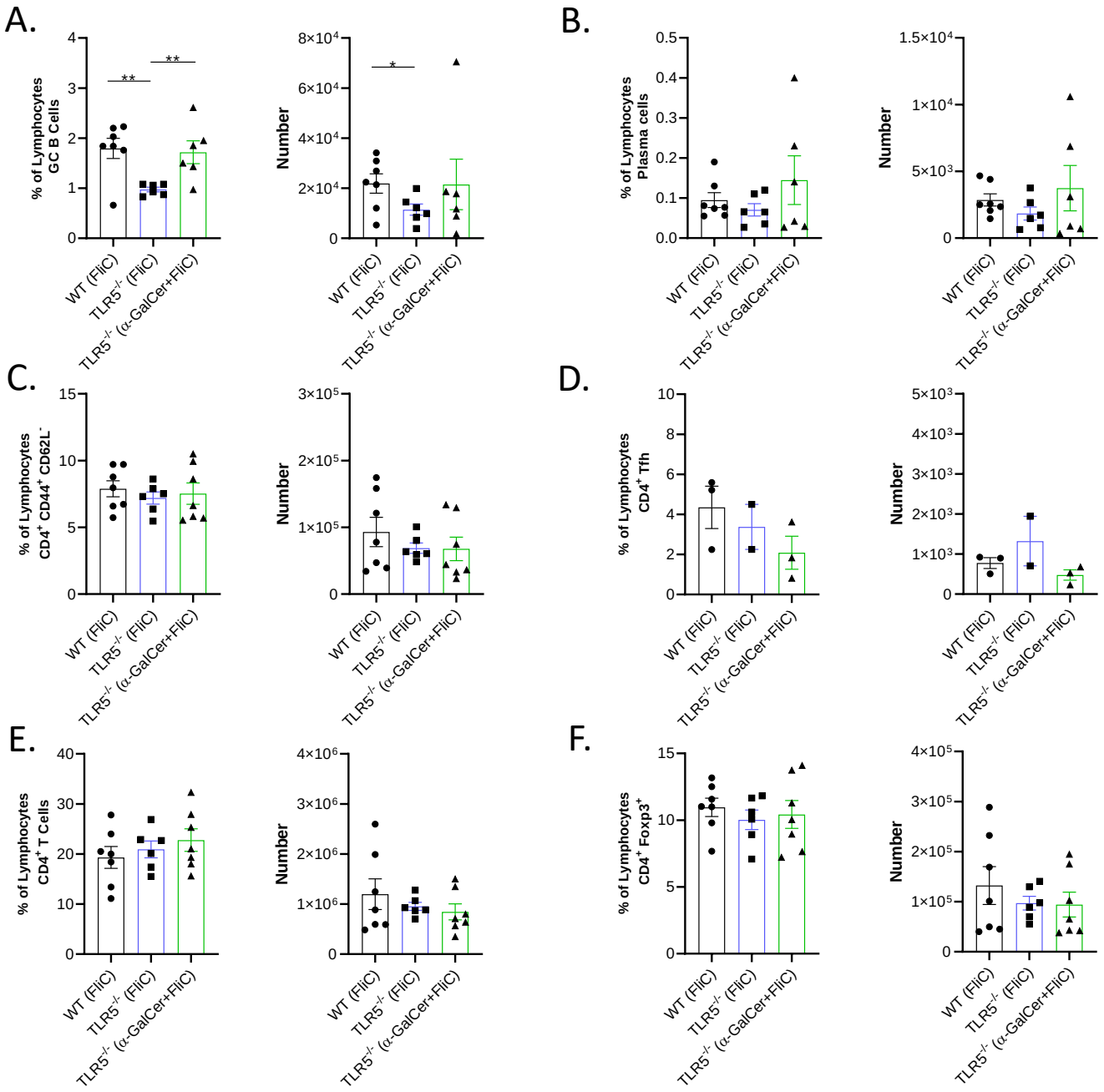


Figure 5.4 GC B cell generation in the MLN following FliC immunisation is restored by iNKT cell activation without a requirement for TLR5.

WT B6 or TLR5$^{-/-}$ mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, or both α-Gal/FliC. 14 days later mice received 20µg FliC i.p. Single cell suspensions were prepared from MLN. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of CD44^{+} CD62L^{-}. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of CD4^{+}. F) Bar-chart showing the percentage and the total number of Foxp3^{+} Treg. N=6-7 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

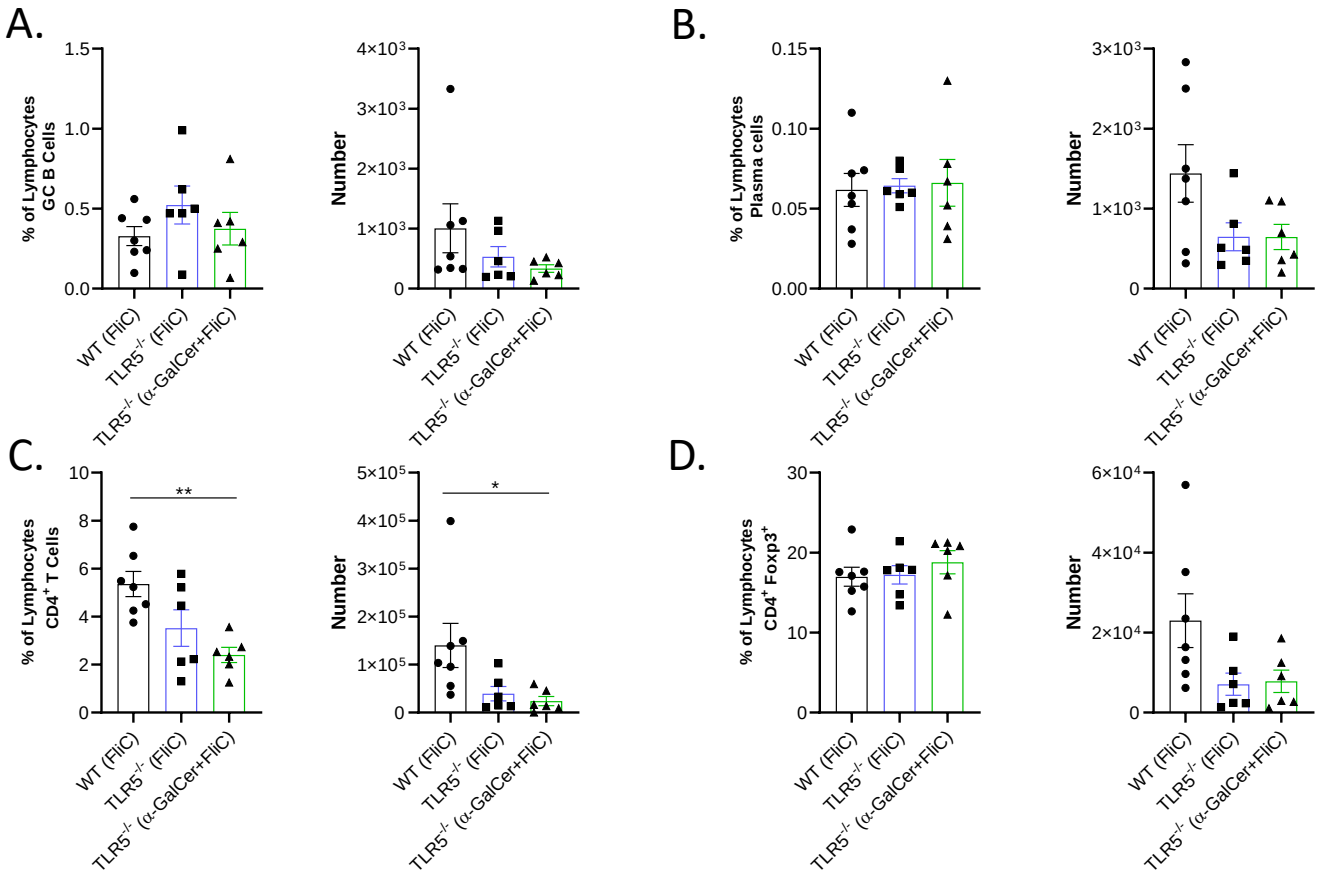


Figure 5.5 The activation of iNKT cells does not impact TLR5-independent humoral immunity in the small intestine.

WT B6 or TLR5$^{-/-}$ mice received i.p. injection of either 20µg FliC, 2µg α-GalCer, or both α-Gal/FliC. 14 days later mice received 20µg FliC i.p. Single cell suspensions were prepared from small intestine. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of CD4^{+}. D) Bar-chart showing the percentage and the total number of Foxp3^{+} Treg. N=6-7 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

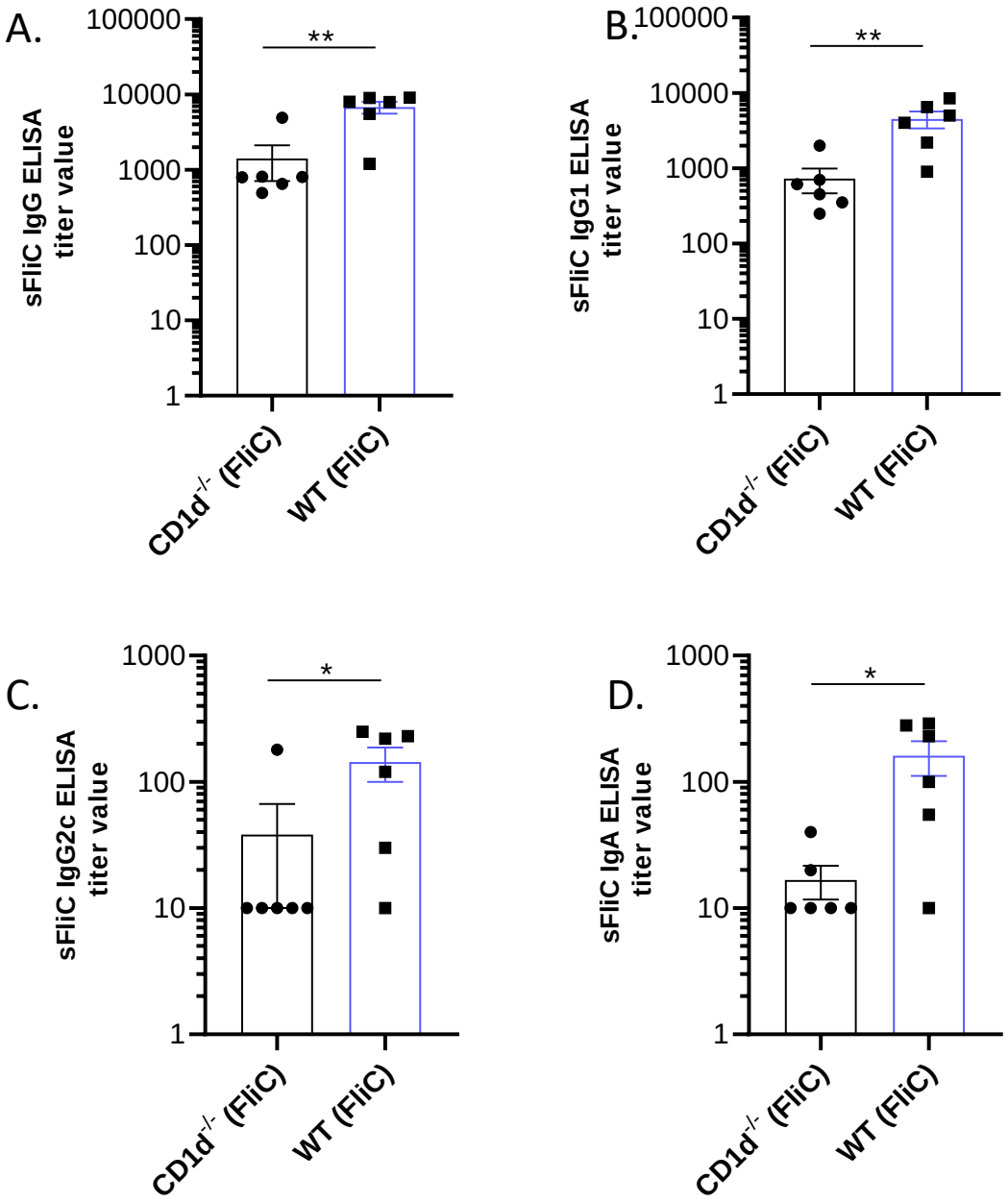


Figure 5.6 Vaccination of CD1d deficient mice markedly reduces the induction of anti-sFliC antibody in the absence of the NKT cells.
WT B6 or CD1d$^{-/-}$ mice received i.p. injection of 20µg FliC. 14 days later mice received 20µg FliC i.p. (Day 14). Serum from such mice were collected 4 days later (Day 18). Serum anti-FliC IgG, IgG1, IgG2c and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2c, and D) IgA titre. n=6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$.

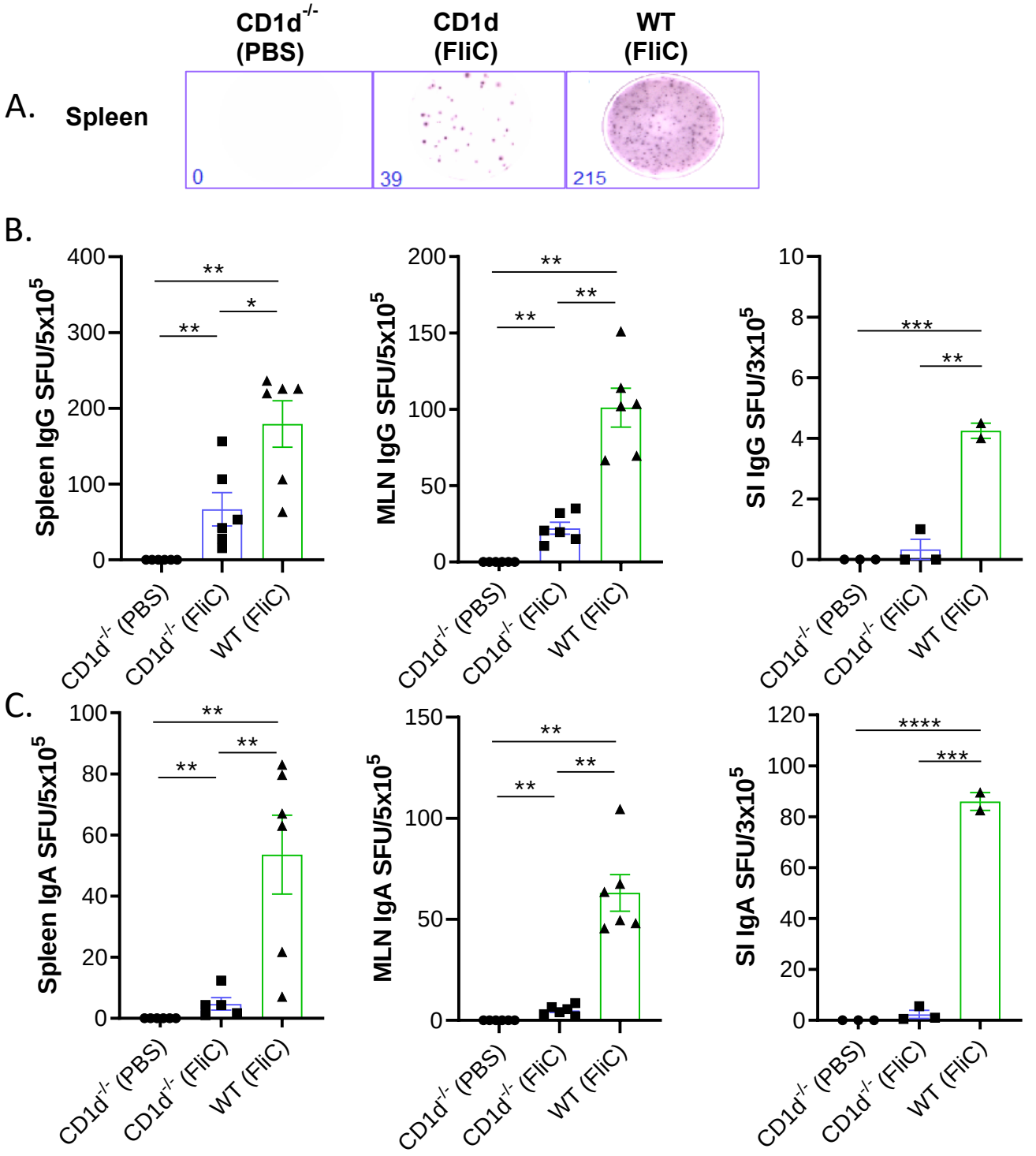


Figure 5.7 The induction of FliC specific Ab secreting cells following immunisation is dependent on iNKT cells.

WT B6 or CD1d$^{-/-}$ mice received i.p. injection of 20µg FliC or PBS. 14 days later mice received 20µg FliC i.p. or PBS. Tissues from such mice were harvested (Day 18). Single cell suspensions were prepared from spleen, mesenteric lymph nodes and small intestine. ELISPOT analysis of sFliC IgG and IgA responses in the spleen, mesenteric lymph nodes and small intestine. A) Representative pictures of wells showing representative SFUs FliC-specific IgG in spleen. B) Bar-charts showing number of SFUs per 5 × 10^5 cells FliC-specific IgG in spleen, mesenteric lymph nodes and small intestine. C) Bar-charts showing number of SFUs per 5 × 10^5 cells FliC-specific IgA in spleen, mesenteric lymph nodes and small intestine. n=6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

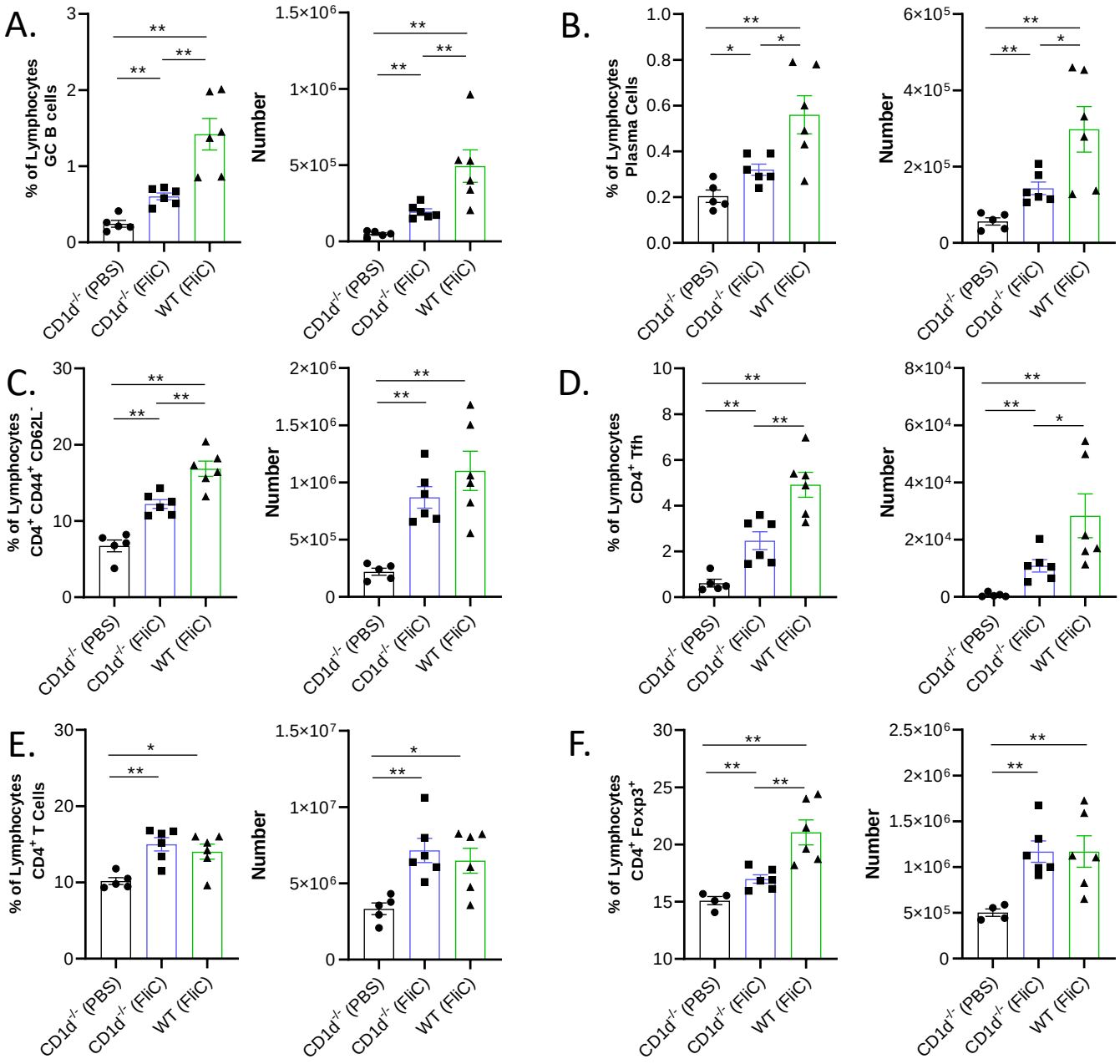


Figure 5.8 The absence of iNKT cells markedly impacts the generation of germinal centres in the spleen following FliC immunisation.

WT B6 or CD1d$^{-/-}$ mice received i.p. injection of 20µg FliC or PBS. 14 days later mice received 20µg FliC i.p. or PBS. Tissues from such mice were harvested (Day 18). Single cell suspensions were prepared from Spleen. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of CD44^{+} CD62L^{-}. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of CD4^{+}. F) Bar-chart showing the percentage and the total number of Foxp3^{+} Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

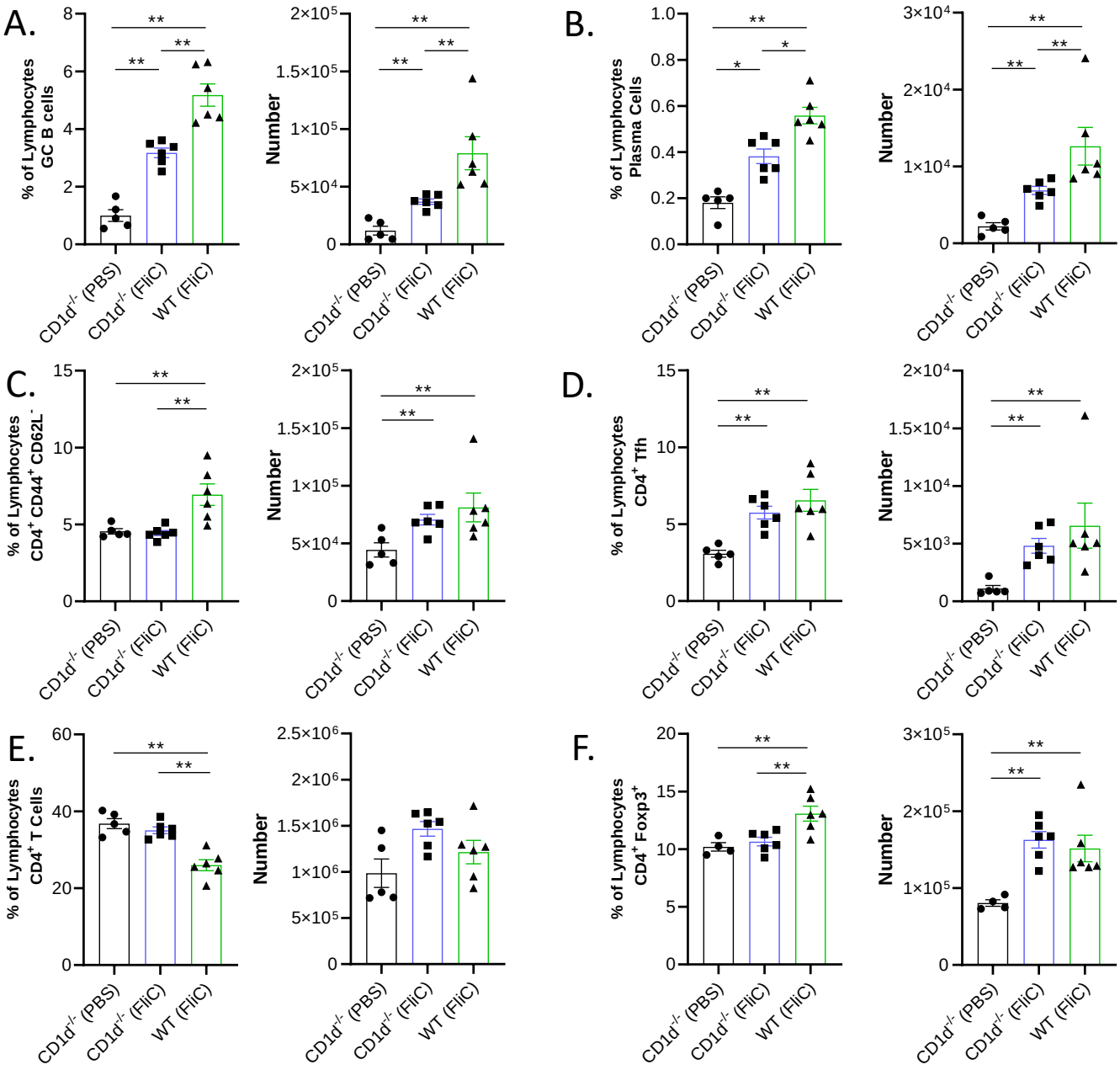


Figure 5.9 FliC vaccination of CD1d-/- mice results in significant perturbation of GC B and plasma cell generation in the MLN following FliC immunisation.
WT B6 or CD1d$^{-/-}$ mice received i.p. injection of 20µg FliC or PBS. 14 days later mice received 20µg FliC i.p. or PBS. Tissues from such mice were harvested (Day 18). Single cell suspensions were prepared from MLN. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of CD44^{+} CD62L^{-}. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of CD4^{+}. F) Bar-chart showing the percentage and the total number of Foxp3+ Treg. N=5-6 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

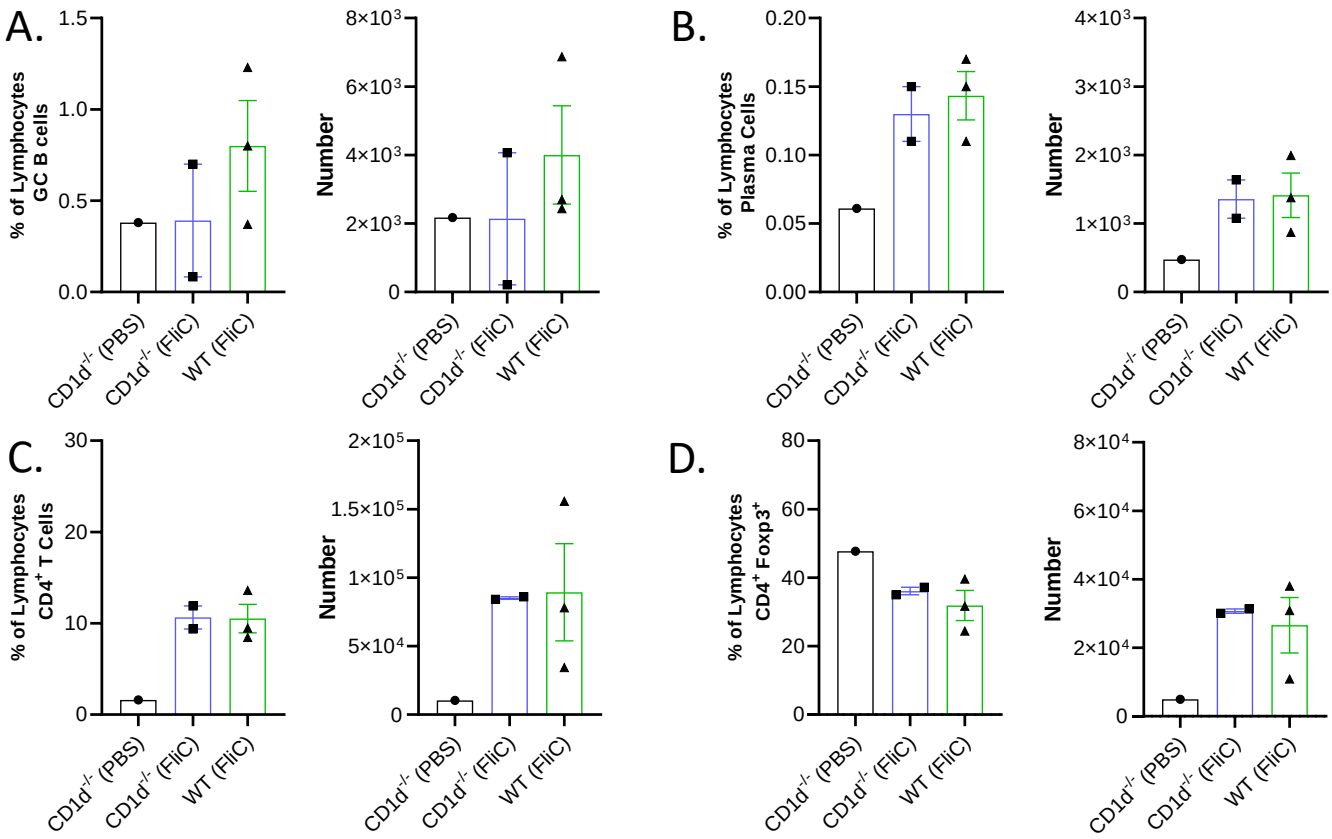


Figure 5.10 The absence of iNKT cells does not alter FliC responses in small intestine. WT B6 or CD1d$^{-/-}$ mice received i.p. injection of 20µg FliC or PBS. 14 days later mice received 20µg FliC i.p. or PBS. Tissues from such mice were harvested (Day 18). Single cell suspensions were prepared from small intestine. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of CD4^{+}. D) Bar-chart showing the percentage and the total number of Foxp3^{+} Treg. N=1-3 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01.

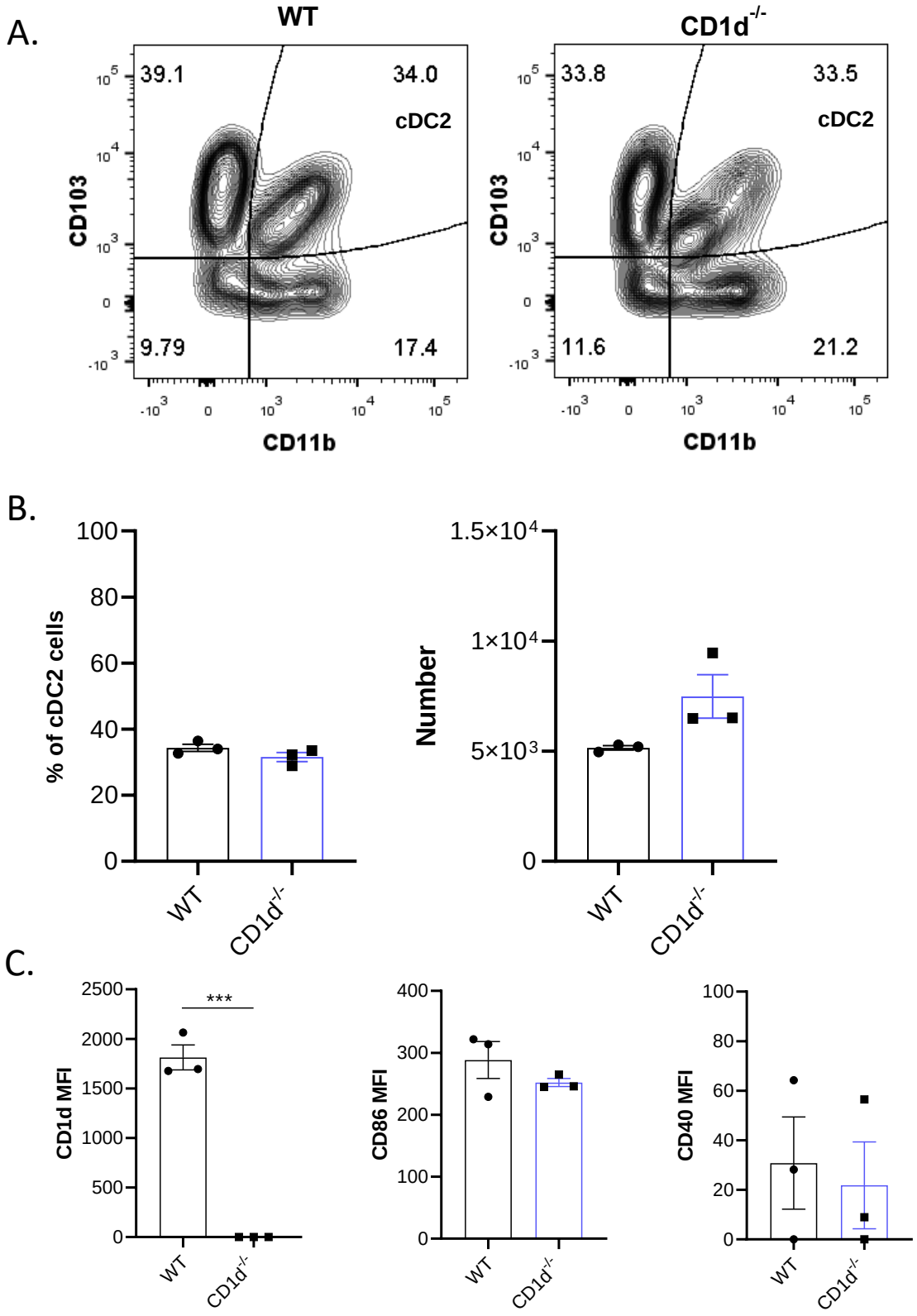


Figure 5.11 CD1d deficient mice harbour similar DC populations to WT mice in the MLN.

cDCs were selected as singlets live cells, cDC were identified in MLN as Lin$^-$ (CD3, B220, F4/80, Gr1) CD11c^{high}. cDC were selected as CD11c^{high} MHCII$^+$ then cDC subsets were shown as mucosal DC2 (CD103$^+$CD11b$^+$), mucosal DC1 (CD103$^+$CD11b$^-$), mucosal CD103$^-$(CD103$^-$ CD11b$^-$). A) Representative plots with percentages for DC2 subsets were shown. B) Percentage and number of DC2 subsets. C) Bar-charts of MFI of CD1d, CD86 and CD40 expression on DC2 subsets. Data are of 3 mice, expressed as mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P < 0.01, ***P < 0.001.

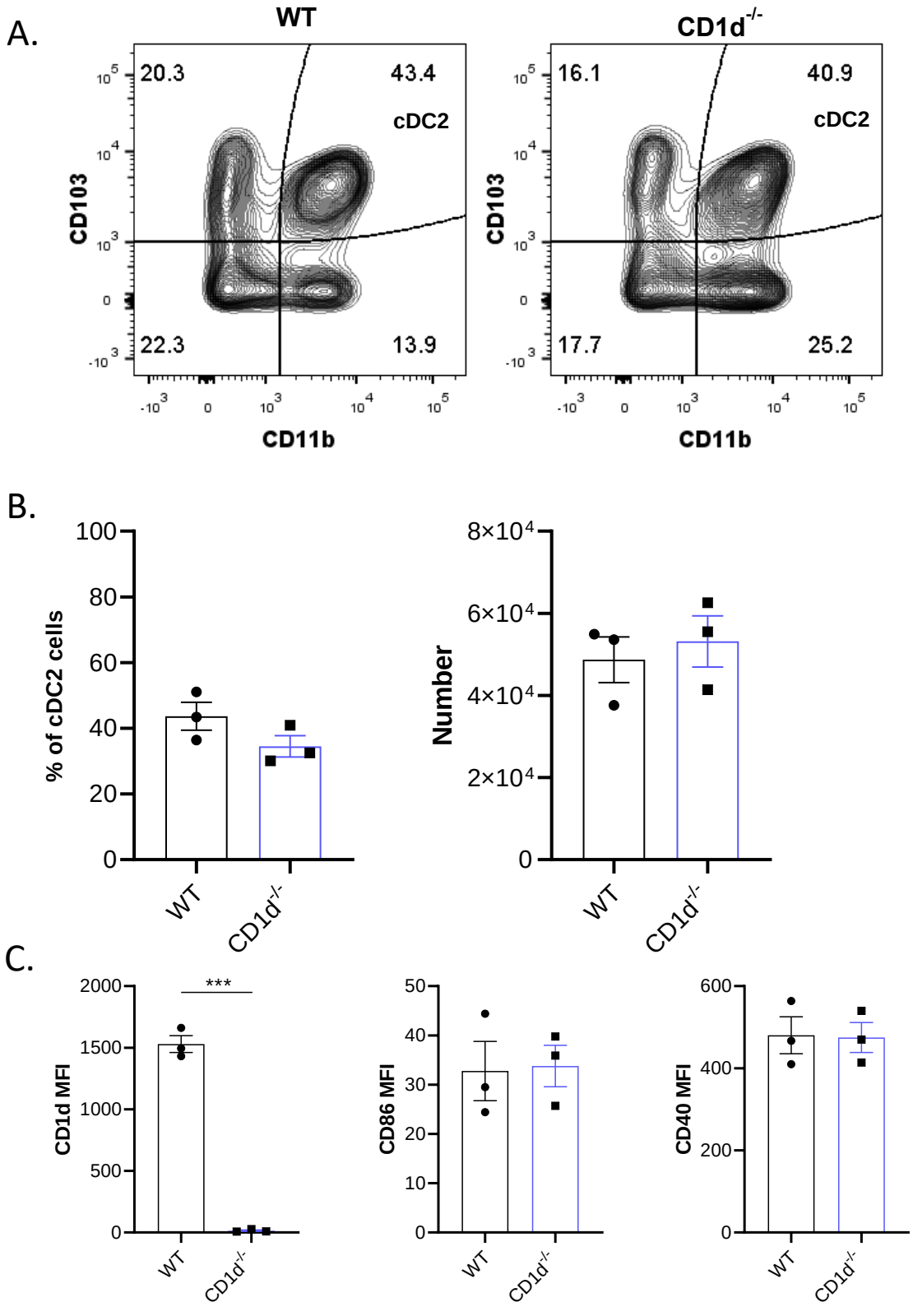


Figure 5.12 Similar DC subsets are found in the small intestine of both CD1d-/- and WT mice.

A) Representative plots with percentages for DC2 subsets were shown. B) Percentage and number of DC2 subsets. C) Bar-charts of MFI of CD1d, CD86 and CD40 expression on DC2 subsets. Data are of 3 mice, expressed as mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P < 0.01, ***P < 0.001.

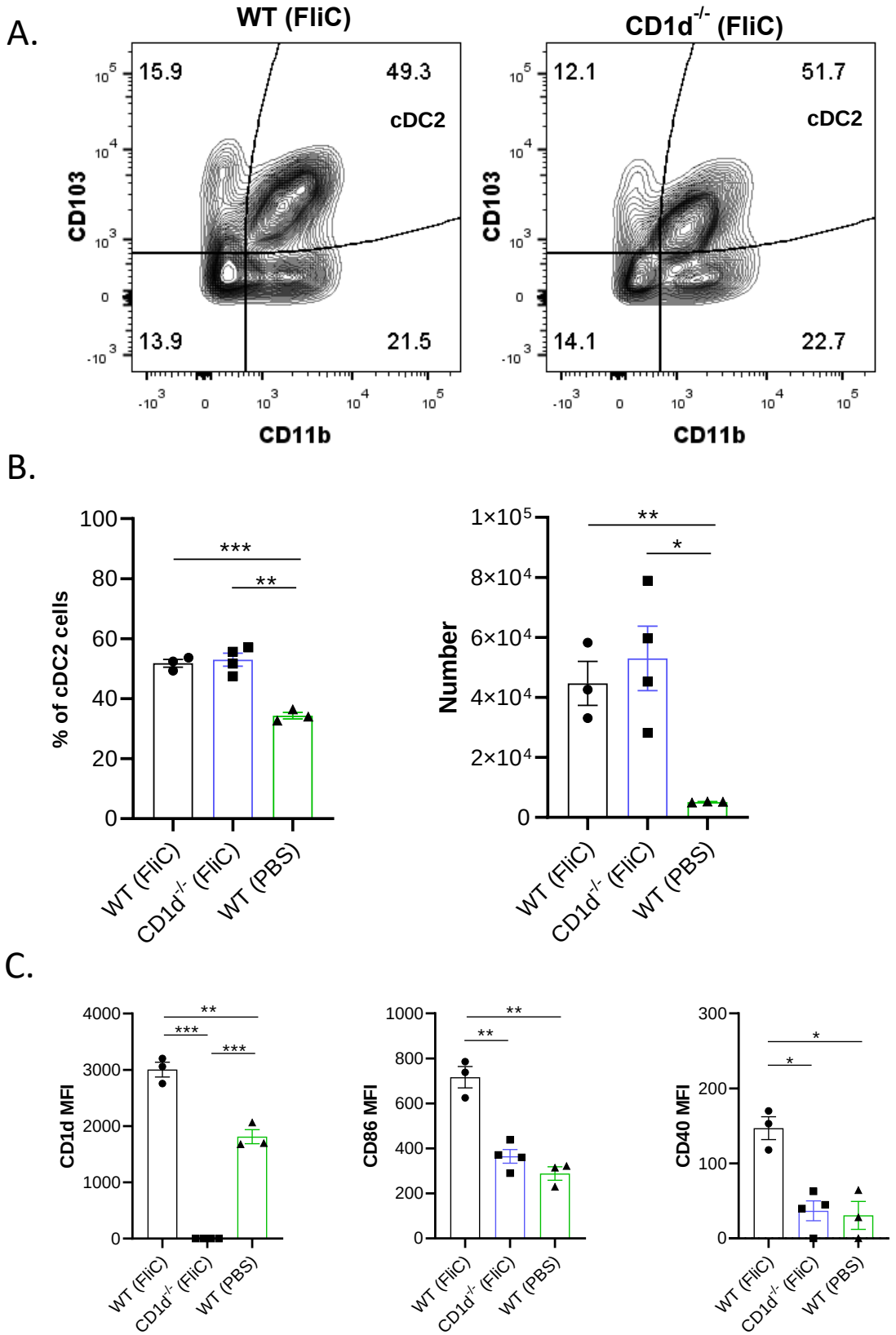


Figure 5.13 present in the MLN 24 hr post-FliC administration fail to upregulate costimulatory molecules in the absence of NKT cells.

A) Representative plots with percentages for DC2 subsets were shown. B) Percentage and number of DC2 subsets. C) Bar-charts of MFI of CD1d, CD86 and CD40 expression on DC2 subsets. Data are representative of mice (n=3-4), expressed as mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P < 0.01, ***P < 0.001.

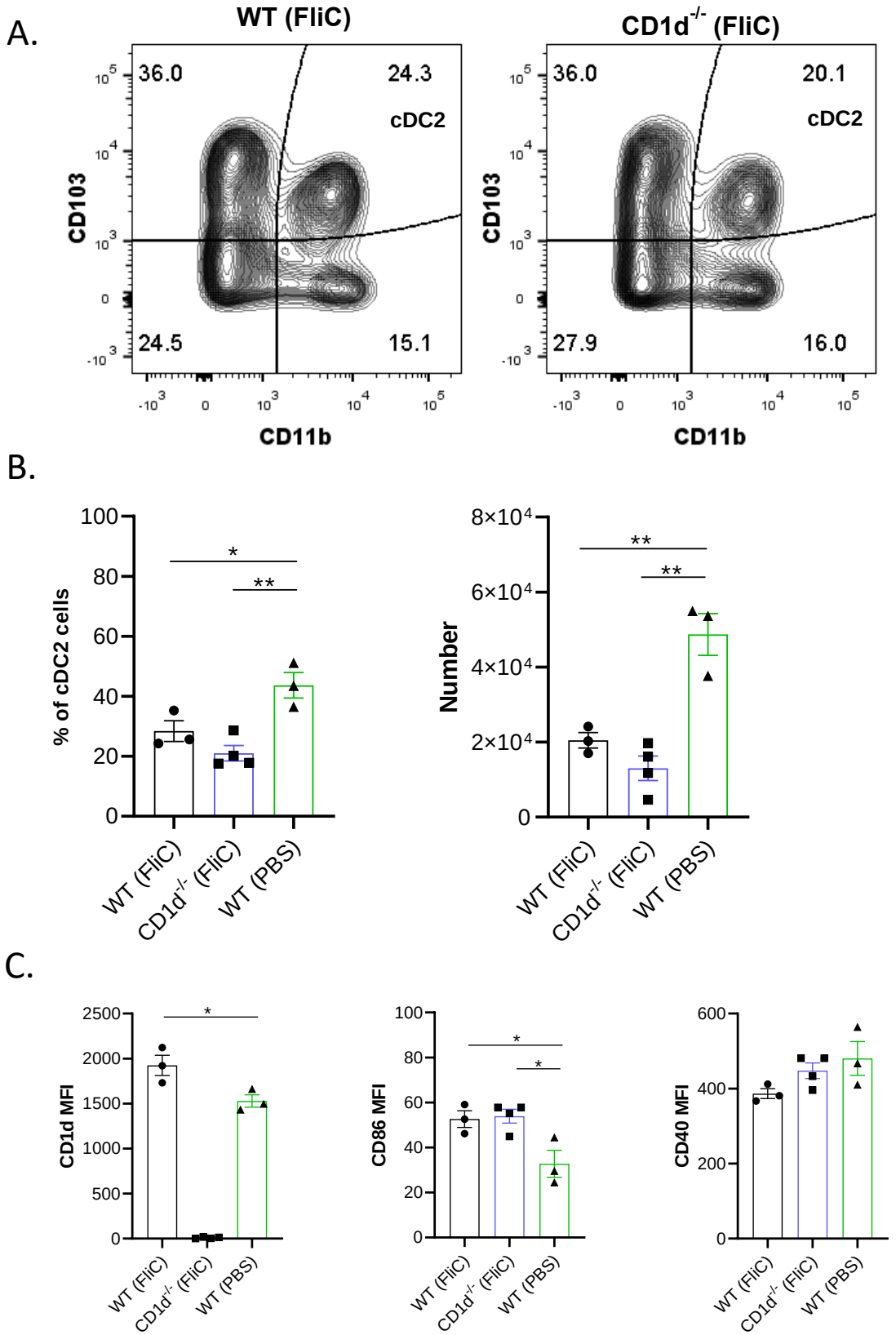


Figure 5.14 CD1d deficient mice display no differences in cDC2 in comparison to WT mice 24 hr post-FliC administration in small intestine.

A) Representative plots with percentages for DC2 subsets were shown. B) Percentage and number of DC2 subsets. C) Bar-charts of MFI of CD1d, CD86 and CD40 expression on DC2 subsets. Data are of 3 mice, expressed as mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P < 0.01, ***P < 0.001.

CHAPTER 6: C-GLYCOSIDE BOOSTS IMMUNITY TO FLIC VACCINATION BETTER THAN α-GALCER

6.1 Introduction

Primary infection of virulent and attenuated strains of STm require $CD4^+$ T cells for their recovery and later protection against Salmonella infection (199, 396-399). As such, αβ T cell-deficient mice have been shown to be unable to control STm infection, which resulted in the development of chronic disease (188, 208). Therefore, the differentiation of Th1 cells results in express T-bet and produce IFN-γ are required for the clearance of primary infections; however, B cells and Ab can both help with protection against reinfection (95, 188, 194). The depletion of $CD4^+$ T cells fails both to control infection and protect against reinfection, but the transfer of $CD4^+$ T cells from vaccinated to WT mice leads to a higher level of protection against STm (396, 397, 400). Moreover, class II-deficient mice confirmed the need for $CD4^+$ for infection clearance (188).

Many studies have revealed that infection with Salmonella induces a Th1 response resulting in the production of IFN-γ (400-402). The importance of IFN-γ has been seen in two experiments using mice that lack an IFN-γ receptor or where the IFN-γ receptor has been blocked where both sets of mice displayed impaired resistance and were susceptible to attenuated Salmonella (396, 400, 403). In support of this, many studies have concluded that the neutralisation of the IFN-γ receptor has been shown to affect primary infections, but not the later stages of infection (400, 404). This indicates that IFN-γ is required in the early stage of an STm infection, and that Salmonella-specific memory T cells are responsible for protection against re-infection. In contrast, IL-$4^{-/-}$ mice showed greater resistance and survival rates, compared to WT mice following STm infection suggesting that IL-4 fails to control and protect against STm infection (405).

In addition, the importance of Th responses has been also observed in T-bet deficient mice. These mice still mount T cell responses against STm but fail to clear *Salmonella* due to an impairment in the differentiation of Th1 (189-191, 318, 406). Therefore, strategies that boost

and enhance Th1 responses may be beneficial as it is the Th1 response that is associated with the control and eradication of infection.

It has been shown that infection with STm results in recognition of flagellin and the development of Th1 responses with a robust induction of T-bet and secretion of IFN-γ. However, upon immunisation with its purified form (soluble FliC) Th2 responses prevail with T cell expression of GATA-3 and the secretion of IL-4. In keep with this dichotomy flagellin from live bacteria produces Th1-reflecting IgG2a/c, while soluble FliC produces Th2-reflecting IgG1 (318, 350).

Thus far, we had examined the impact of α-GalCer-mediated iNKT cell activation on immune responses to FliC following immunisation. α-GalCer-CD1d complexes activate iNKT cells and result in rapid, robust Th1 (IFN-γ) and Th2 (IL-4) cytokine secretion. Subsequently, iNKT cell cytokine have been recently used to deliver the therapeutic efficacy based in various disease models. However, α-GalCer may counter the effects of IFN-γ, lowering its adjuvant activity, as a result of the concomitant secretion of IL-4 by (407, 408). In addition, multiple, frequent administrations of α-GalCer has been shown to result in iNKT cell anergy, an undesirable effect when attempting to manipulate iNKT cells to boost immune responses to antigen (409, 410) .

With this in mind, numerous analogues of α-GalCer have been synthesised with a view to allow repeated dosing and importantly to attempt to manipulate the activity of iNKT cells once activated (273, 411, 412). This has led to a number of analogues, such as OCH and C-glycoside (α-C-GalCer) which may show improved adjuvant activity depending on the context. For example, the use of OCH to activate iNKT cells *in vivo* elicits a dominant Th2-biased response (218), while C-glycoside preferentially induces a Th1-biased response (219-221).

It has recently been reported that using C-glycoside induces much higher levels of IFN-γ and IL-12 and lower levels of IL-4 *in vivo* than in mice that received α-GalCer (219-221). Recent studies have revealed that C-glycoside exhibits very potent activity against tumour cells and

malaria parasites because of the production of high levels of IFN-γ biased by C-glycoside (221). In addition, using C-glycoside as an iNKT cell ligand *in vivo* induces enhanced DC activation compared to α-GalCer alone (219, 413). It has been established that DC are fundamental to drive the response to FliC therefore C-glycoside may further activate DC and induce a Th1-biased response that results in enhanced secretion of IFN-γ (219, 413).

6.2 Aims and Hypothesis

We hypothesise that C-glycoside would be a superior adjuvant to α-GalCer and will further improve immune responses to FliC following vaccination. We therefore aimed to co-immunise mice with FliC and α-GalCer or analogues, namely C-glycoside (α-C-GalCer) or OCH, and determine the impact on the B cell/antibody response following FliC immunisation. Moreover, we sought to examine the importance of IFN-γ to the enhanced response to FliC seen when concomitantly activating iNKT cells.

6.3 Results

6.3.1 The Th1-biased glycolipid, C-glycoside, demonstrates increased adjuvanticity compared to the Th1/Th2 inducing α-GalCer.

We have previously shown that co-administration of α-Gal/FliC can boost the immune response to FliC following immunisation. However, α-GalCer activation of iNKT cells results in the co-production of Th1 and Th2 cytokines although it is typically a Th1 response that is required to control and clear many intracellular infections. This led us to examine different α-GalCer analogues that might preferentially generate a more Th1 or Th2 biased response to determine the impact on humoral responses to FliC. To this end, mice were immunised with FliC in combination with C-glycoside, OCH or α-GalCer administration. 14 days post immunisation mice received a further dose of FliC and were harvested 4 days later (i.e. day 18).

Assessment of the anti-sFliC IgG response revealed that mice co-immunized with C-glycoside and sFliC demonstrated a significant increase in IgG titres in serum compare to both OCH or α-GalCer with FliC (figure 6.1 A). This was also true on examining the titre of anti-sFliC IgG1 Ab (figure 6.1 B) and anti-sFliC IgG2C Ab (figure 6.1 C) with increased serum Ab in mice co-immunized with C-glycoside and sFliC compared to mice co-immunized with OCH or α-GalCer together with FliC. However, FliC-specific IgA titres were similar in all groups at this time (figure 6.1 D). These data demonstrate that the activation of iNKT cells by C-glycoside (and therefore a dominant Th1-like pattern of cytokine secretion) results in increased adjuvanticity compared to the use of the Th1/Th2 inducing α-GalCer.

6.3.2 C-glycoside is superior to α-GalCer at boosting the frequency of FliC specific IgG secreting cells in the spleen.

To assess whether iNKT cell activation by C-glycoside also increased the generation of Ab-secreting cells we assessed the frequency of such cells following FliC administration combined

with different α-GalCer analogues (figure 6.2). Mice were primed with FliC and C-glycoside, OCH or α-GalCer and received a FliC boost on day 14 before tissue was harvested 4 days later.

The relative frequency of FliC-specific IgG and IgA Ab-secreting cells in spleen was significantly increased in mice co-immunized with C-glycoside and FliC compared to α-GalCer/FliC (figure 6.2 A-C). However, all glycolipid analogues were found to evoke a similar number of FliC-specific IgG and IgA antibody secreting cells in the MLN (figure 6.2 B-C).

6.3.3 C-glycoside promotes the generation of plasma cells in spleen.

To assess whether C-glycoside activation of iNKT cells resulted a more pronounced GC reaction and resultant plasma cells, the frequency and number of GC B cells, Tfh and plasma cells was assessed following FliC administration together with the different glycolipids (figure 6.3-4).

Analysis of GC B cells and Tfh cells in spleen 18 days post-FliC immunisation showed that the frequency and number of GC B cells and Tfh cells were similar between all groups (figure 6.3 A). However, the frequency and number of plasma cells in the spleen was significantly elevated in mice co-immunized with C-glycoside and FliC compared to both OCH or α-GalCer with FliC (figure 6.3 B). Interestingly, C-glycoside and FliC was found to decrease the frequency of antigen-experienced T cells and Treg cells compared to mice co-immunized with either α-Gal/FliC or OCH/FliC (figure 6.3 C and F), however the absolute numbers of such cells was the same in all groups.

Analysis of GC B cells and plasma cells in MLN 18 days post-FliC immunisation showed that the frequency and number of GC B cells and plasma cells were similar between all groups (figure 6.4 A-B). However, co-administration of C-glycoside and FliC resulted in a significant decrease in the frequency of GC B cells compared to that found in mice that had received α-Gal/FliC (figure 6.4 A). Similarly, the frequency and number of Tfh cells in the MLN of mice

that were co-immunized with C-glycoside and FliC was also diminished compared to mice co-immunized with α-Gal/FliC (figure 6.4 D).

6.3.4 The beneficial impact of C-glycoside administration on Ab production is lost upon IFN-γ receptor blockade.

Thus far it was evident that using a glycolipid that promoted a preferential Th1 pattern of cytokine release by iNKT cells resulted in an increase in humoral immunity to concomitant FliC administration. Given that IFN-γ plays a key immunomodulatory role in Th1 responses we next sought to determine whether IFN-γ secretion was responsible for the adjuvant effect of C-glycoside (figure 6.5). To this end, mice received i.p. injection of either 500μg RatIgG, or 500μg αIFNγRα (Day-1). Mice were then immunised with either FliC only or C-glycoside/FliC the following day (Day 0). 14 days post immunisation mice received a further dose of FliC and were harvested 4 days later (i.e. day 18).

Analysis of total serum anti-sFliC IgG and IgG1 revealed that blockade of IFN-γ signalling did not impact generation of anti-sFliC IgG and IgG1 Ab in mice that had received C-glycoside + FliC (figure 6.5 A-B). Interestingly, IFNγRα blockade in mice co-immunized with C-glycoside and sFliC resulted in a significant abrogation of the anti-FliC IgG2C titre (figure 6.5 C). This was also true of FliC-specific IgA titres which were also decreased in mice co-immunized with C-glycoside and sFliC in the presence of anti-IFNγRα mAb (figure 6.5 D). These data demonstrate that the ability of C-glycoside to increase IgG2C and IgA Ab production to FliC upon immunisation is dependent on IFN-γ receptor signalling.

6.3.5 The frequency of IgG FliC-specific Ab secreting cells in the spleen following c-glycoside/FliC immunisation is markedly reduced in the absence of IFNγ receptor signalling.

Next, we sought to assess whether blocking the IFN-γ receptor would impact the generation of Ab-secreting cells following immunisation. The frequency of IgG and IgA FliC-specific Ab-secreting cells generated following FliC administration with and without the activation of iNKT cells or IFNγ receptor signalling was determined (figure 6.6).

IFNγ receptor blockade was found to nullify the increased frequency of FliC-specific IgG Ab-secreting cells in spleen seen following co-immunisation with C-glycoside/FliC (figure 6.6 A). In contrast, splenic anti-sFliC IgA secreting cells and MLN-derived IgG and IgA secreting cells were not significantly affected by IFNγRα blockade although there was a trend for these cells to be reduced in such mice (figure 6.6 A-B).

6.3.6 GC B cells, Tfh and plasma cells generated following C-glycoside/FliC immunisation are not dependent on IFNγ receptor signalling.

The frequency and number of GC B cells and plasma cells in spleen after C-glycoside and FliC immunisation were similar with and without IFNγ signalling (figure 6.7 A-B). In addition, memory T cell and Tfh in spleen showed that the frequency and number of both populations were also unaffected in mice that received the anti-IFNγRα mAb (figure 6.7 C-D). The same was true for $CD4^+$ T cells and Treg cells (figure 6.7 E-F). Analysis of MLN again failed to detect any difference in any cell population studied following immunisation in the presence or absence of IFNγ signals (figure 6.8).

Taken together, these data demonstrate that IFN-γ signalling is required to promote IgG2C and IgA responses in mice co-immunized with C-glycoside/FliC. However, whilst IFN-γ was

required for the enhanced IgG2C and IgA response seen on C-glycolipid administration, IFN-γ was dispensable for the enhanced GC reaction seen in such mice.

6.3.7 The Th1-biased glycolipid maintains the induction of IgG2C for over month post vaccination.

Thus far we have shown that the co-administration of C-glycoside/FliC results in enhanced anti-FliC IgG in serum and an increased frequency of Ab secreting cells and plasma cells in the spleen (figure 6.1-3). This led us to examine the longevity of serum anti-FliC Ab following C-glycoside/FliC administration (figure 6.9). To this end, mice were immunised of C-glycoside/FliC or FliC only. 14 days post immunisation mice received a further dose of FliC and serum was harvested 14 days later (i.e. day 28).

Analysis of the anti-sFliC IgG/IgG1 response revealed that co-immunized mice had a similar IgG/IgG1 titre in serum compare to mice that received FliC only (figure 6.9 A-B). However, analysis of the anti-sFliC IgG2C response revealed that mice co-immunized mice with C-glycoside and sFliC had a sustained increase in IgG2C titre in the serum compared to the serum of mice that had received FliC alone (figure 6.9 C). FliC-specific IgA titres were similar in between both groups (figure 6.9 D).

6.3.8 The early increase in GC B cells, Tfh, Ab secreting cells and plasma cells promoted by C-glycoside is lost with time.

Next, we assessed whether iNKT cell activation by C-glycoside maintained Ab-secreting cells following FliC administration in the long term (figure 6.10). Mice were primed for 14 days of C-glycoside/FliC or FliC alone and day 14 boost with FliC as before.

The relative frequency of FliC-specific IgG and IgA Ab-secreting cells in spleen, MLN and bone marrow was found to be similar in mice that did or did not receive co-administration of C-glycoside (figure 6.10 A-B).

Finally, the frequency and number of adaptive immune cells found in the spleen and MLN 28 days after FliC immunisation with and without C-glycoside was assessed (figure 6.11-12).

28 days post-FliC immunisation the frequency and number of most of the cell populations analysed in the spleen was found to be similar between both groups (figure 6.11 A-F). However, Tfh were decreased in mice that had received co-immunisation compared to mice that had been injected with FliC only (figure 6.11 D). There was no difference in the number or frequency of any cell type analysed in the MLN by 28 days post-FliC immunisation (figure 6.12) apart from a decrease in the number of plasma cells in the co-immunised group (figure 12 B).

6.4 Discussion

This chapter examined the impact of using a Th1 biasing glycolipid C-glycoside on the immune response seen after FliC immunisation. We and others have reported that the dominant population of the intestinal iNKT cells are NKT1 cells which can produce IFN-γ following activation (295, 301, 320, 321). We have also shown that NKT1 cells are the major population in spleen and small intestine and that the use of α-GalCer as an adjuvant can enhance the response to FliC.

However, α-GalCer activated iNKT cells co-produce Th1 and Th2 cytokines and the concomitant production of Th2 associated cytokines have been postulated to counter the impact of Th1-like cytokines which may limit its adjuvant activity (407, 408). This led us to investigate C-glycoside as an alternative activator of iNKT cells as it has been reported that injection of C-glycoside *in vivo* induces higher levels of IFN-γ and IL-12 and is associated with lower levels of IL-4 than in mice that received α-GalCer (219-221). Moreover, Th1 responses are required for the control of many intracellular infections as they are already observed as infections clearance (406). Therefore, we hypothesised that the use of C-glycoside may act as a better adjuvant to FliC immunisation.

We first sought to examine whether iNKT cell activation using C-glycoside together with FliC can further provide a better response than α-Gal/FliC following FliC immunisation and impact the generation of antibody response following FliC immunisation. Indeed, using C-glycoside to activate iNKT cell at the time of FliC immunisation increased serum IgG, including IgG2c, and the induction of Ab secreting cells and plasma cells in spleen (figure 6.1-3). These data support that using an analogue of α-GalCer to direct a Th1-dominant response by iNKT cells may be beneficial to boost humoral immunity to FliC.

The reason why C-glycoside is a more potent adjuvant than α-GalCer in this setting may due to its use increasing the production of IFNγ from activated iNKT cells, which in turn leads to

prolonged IL-12 secretion from DC which further propagated IFNγ release by NKT and NK cells (219, 221). Indeed it has been that C-glycoside failed to show activity against either malaria or melanoma metastases, or an ability to prolong IFN-γ production, when compared to α-GalCer in IL-$12^{-/-}$ mice (221). The same study found that IL-4 production was unaffected by IL-12.

Another possibility for the increased adjuvanticity of C-glycoside in the reported experiments is that C-glycoside is more stably complexed with CD1d on DCs, which has been shown to result in enhanced DC maturation and a prolonged IFN-γ-biased response (219, 319, 414). In addition, C-glycoside was shown to upregulate CD40L on iNKT and enhance the clonal expansion of NKT cells compared to α-GalCer (415). This suggests that C-glycoside administered *in vivo* results in a greater degree of activation and proliferation of iNKT cells than α-GalCer.

Our previous data led us to examine whether IFN-γ played a major role in the observed increase in humoral immunity seen with C-glycoside (figure 6.1-3). Indeed, mAb-mediated blockade of the IFN-γ receptor diminished the increased numbers/frequency of sFliC-specific IgG-secreting cells in spleen and antibody switching to IgG2c and IgA seen with C-glycoside and FliC administration (figure 6.5 C-D, 6.6 A). These findings suggest that the enhanced antibody switching to IgG2c/IgA requires IFN-γ secreted by NKT1 after activation with C-glycoside. This was supported by studies that found that IgA/IgG2c isotype switching is highly dependent on TLR5 and MyD88 signalling pathway whereas both were dispensable for IgG1 isotype switching (391, 416). Despite the above, a later study found that type I IFN receptor signalling is required to deliver anti-FliC IgA/IgG2c responses (391). Our previous data, however, suggest that the addition of iNKT cells has an effect on isotype switching in IgG2c after the addition of α-GalCer (Figure 5.1 C). It may be possible that this is due to the alteration of the cytokine

environment provided by iNKT cells. This suggests that the Th1 response provided by iNKT cells may affect the isotype switching of IgA/IgG2c.

Our previous data led us to examine the longevity of serum anti-FliC Ab following C-glycoside/FliC administration (figure 6.1-3). Interestingly, after immunisation with C-glycoside/FliC in the serum, IgG2c was augmented with the addition of the Th1 biased response compared to FliC even 28 days post activation (figure 6.9). Other studies have also shown that high levels of IgG2c are dependent on Th1 responses (417). Our data illustrate that a sustained increase in IgG2c titre in the serum, suggesting a hypothesis that our immunisation of C-glycoside+FliC would provide a protection against STm infection. Indeed, when WT mice were infected with *Salmonella*, the dominant isotype produced during the sterilising response was IgG2c (418). Unfortunately, experiments to determine whether C-glycoside+FliC vaccination leads to complete protect were not within the scope of this project. However, in follow-up experiments mice will be immunised with C-glycoside/FliC or FliC only and boosted 14 days later before being infected with STm at day 28 and the infection monitored.

In conclusion, we have found that the activation of iNKT cells by C-glycoside (and therefore a dominant Th1-like pattern of cytokine secretion) results in increased adjuvanticity compared to the use of the Th1/Th2 inducing α-GalCer. Strikingly, C-glycoside/FliC further increased the titre and longevity of serum IgG2C and IgA Ab over and above that seen with α-Gal/FliC. We showed that IFN-γ was required for the enhanced IgG2C and IgA response seen on C-glycolipid administration but that IFN-γ was dispensable for the enhanced GC reaction seen in such mice.

6.5 Figures

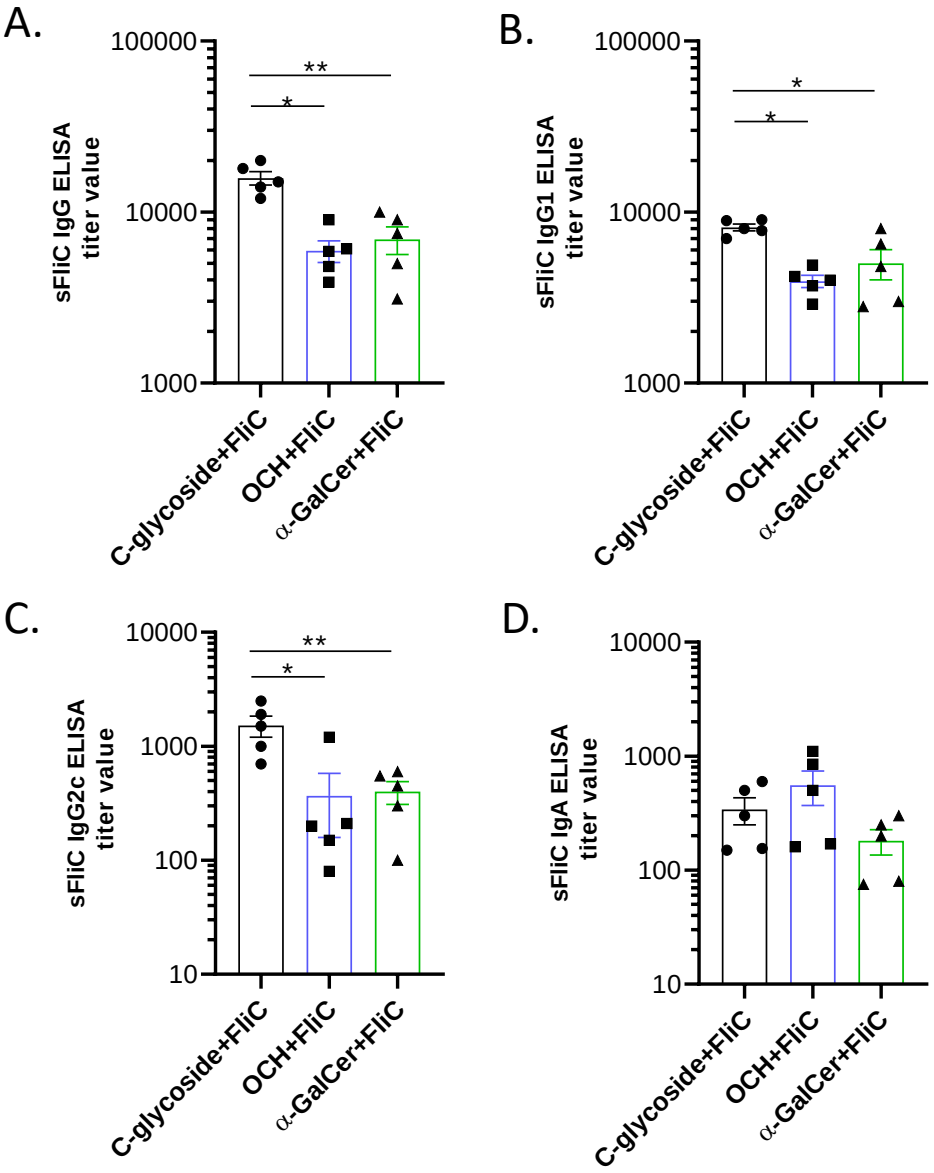


Figure 6.1 C-glycoside is superior to α-GalCer in increasing IgG antibody titre following FliC immunisation.
WT B6 mice received i.p. injection of 10μg C-glycoside/FliC, 10μg OCH/FliC or 2μg αGal/FliC. 14 days later mice received 20μg FliC i.p. Serum from such mice were collected 4 days later (Day 18). Serum anti-FliC IgG, IgG1, IgG2c and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2c, and D) IgA titre. n=5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$.

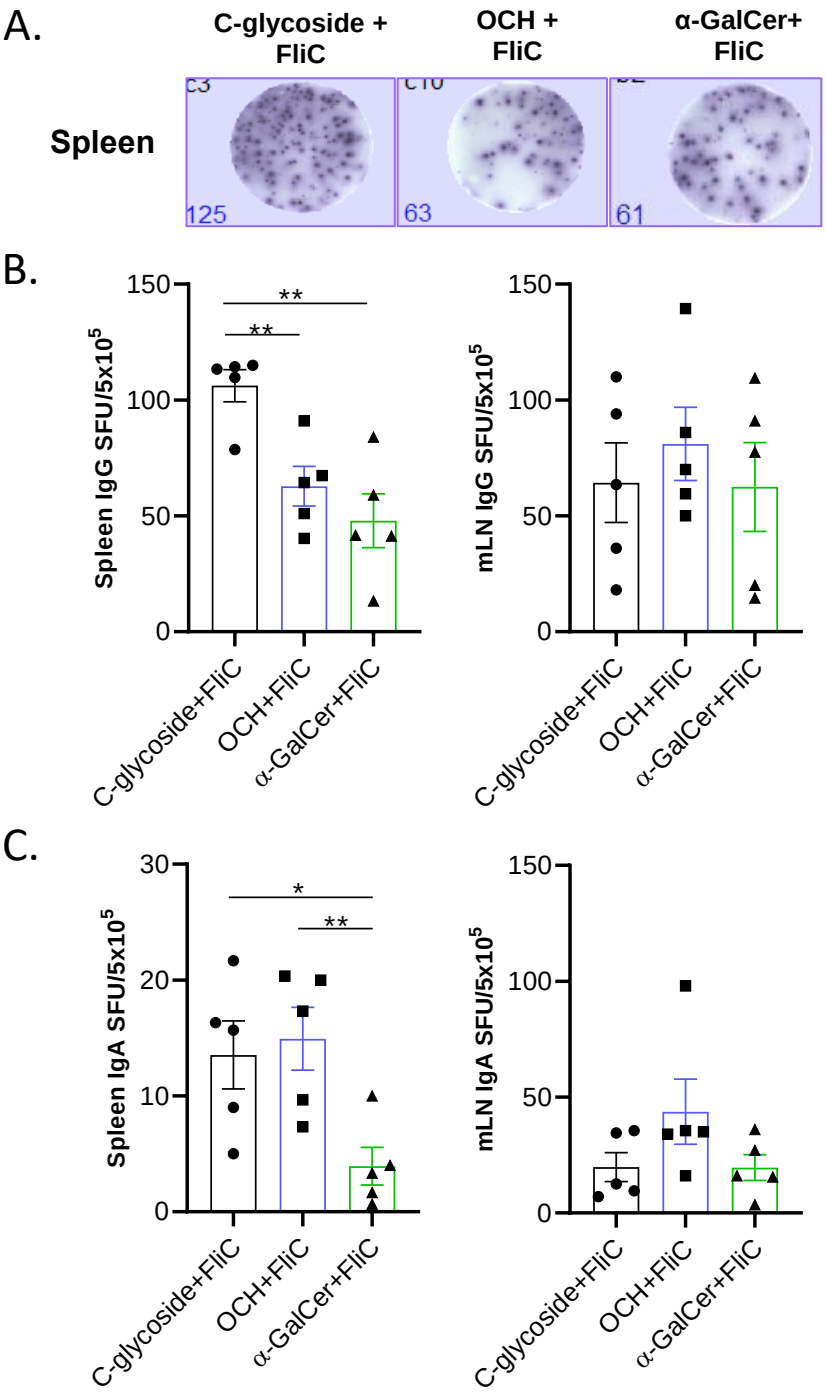


Figure 6.2 C-glycoside increases the frequency of IgG FliC specific Ab-secreting cells in the spleen.

WT B6 mice received i.p. injection of 10µg C-glycoside/FliC, 10µg OCH/FliC or 2µg αGal/FliC. 14 days later mice received 20µg FliC i.p. Tissues from such mice were harvested (Day 18). Single cell suspensions were prepared from spleen and mesenteric lymph nodes. ELISPOT analysis of sFliC IgG and IgA responses in the spleen and mesenteric lymph nodes. A) Representative pictures of wells showing representative SFUs FliC-specific IgG in spleen. B) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgG in spleen and mesenteric lymph nodes. C) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgA in spleen and mesenteric lymph nodes. n=5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$.

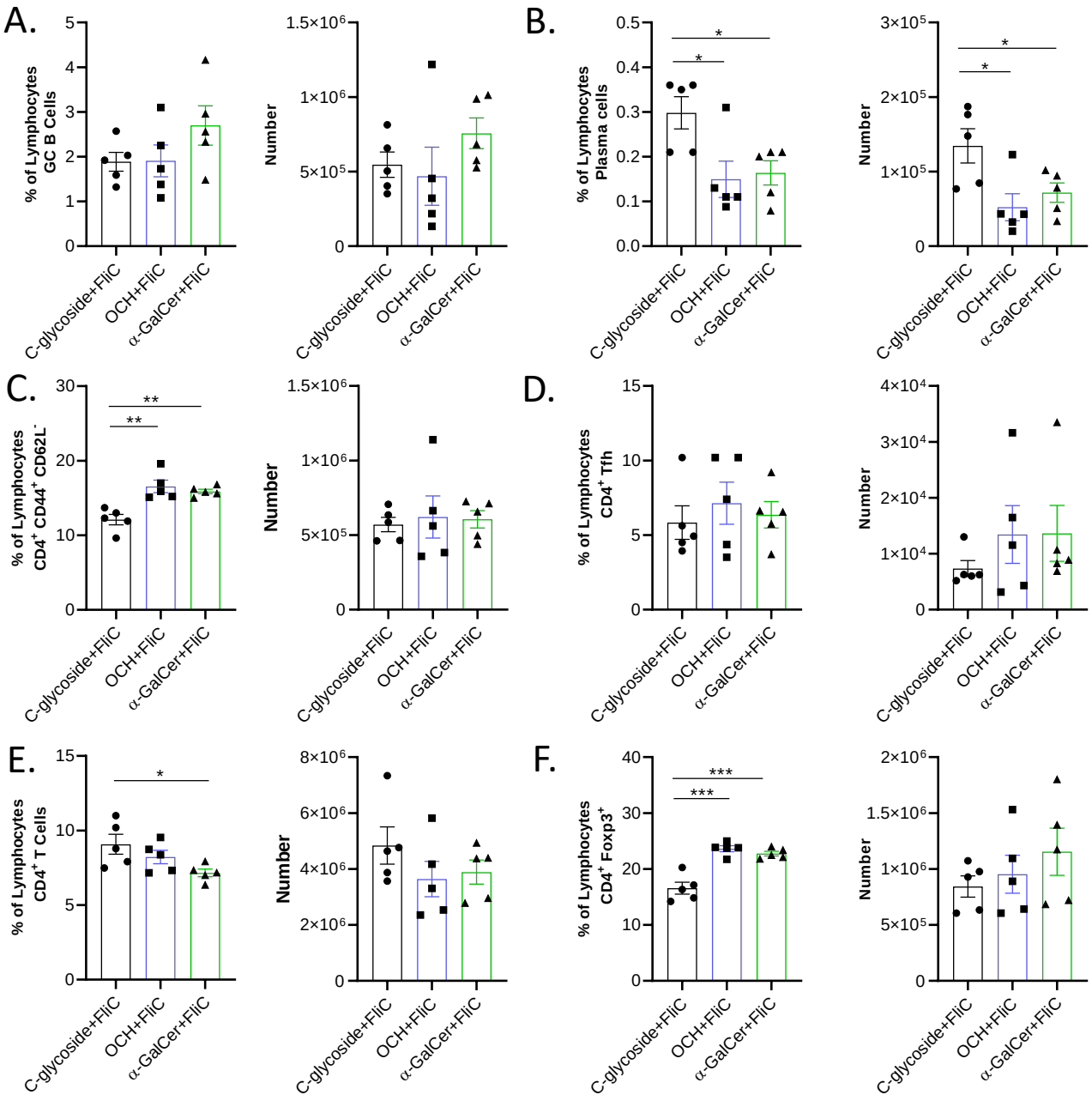


Figure 6.3 C-glycoside together with FliC administration results in increased numbers of plasma cells in the spleen.

WT B6 mice received i.p. injection of 10μg C-glycoside/FliC, 10μg OCH/FliC or 2μg αGal/FliC. 14 days later mice received 20μg FliC i.p. Single cell suspensions were prepared from Spleen. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001.

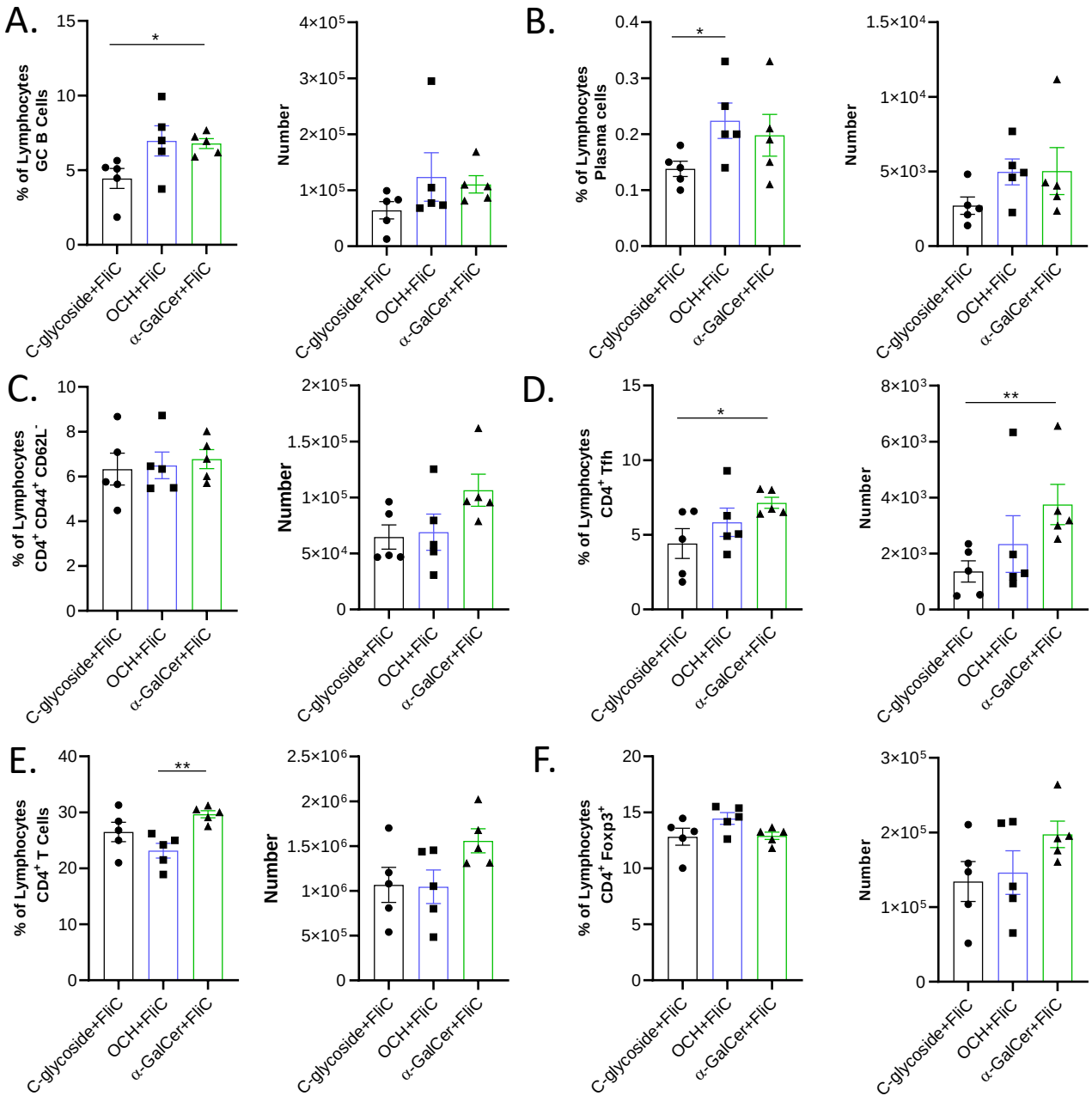


Figure 6.4 C-glycoside activation of iNKT cells diminishes the number of GC B cells and Tfh in the MLN compared to αGalCer.

WT B6 mice received i.p. injection of 10μg C-glycoside/FliC, 10μg OCH/FliC or 2μg αGal/FliC. 14 days later mice received 20μg FliC i.p. Single cell suspensions were prepared from MLN. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *$P < 0.05$, **$P \leq 0.01$.

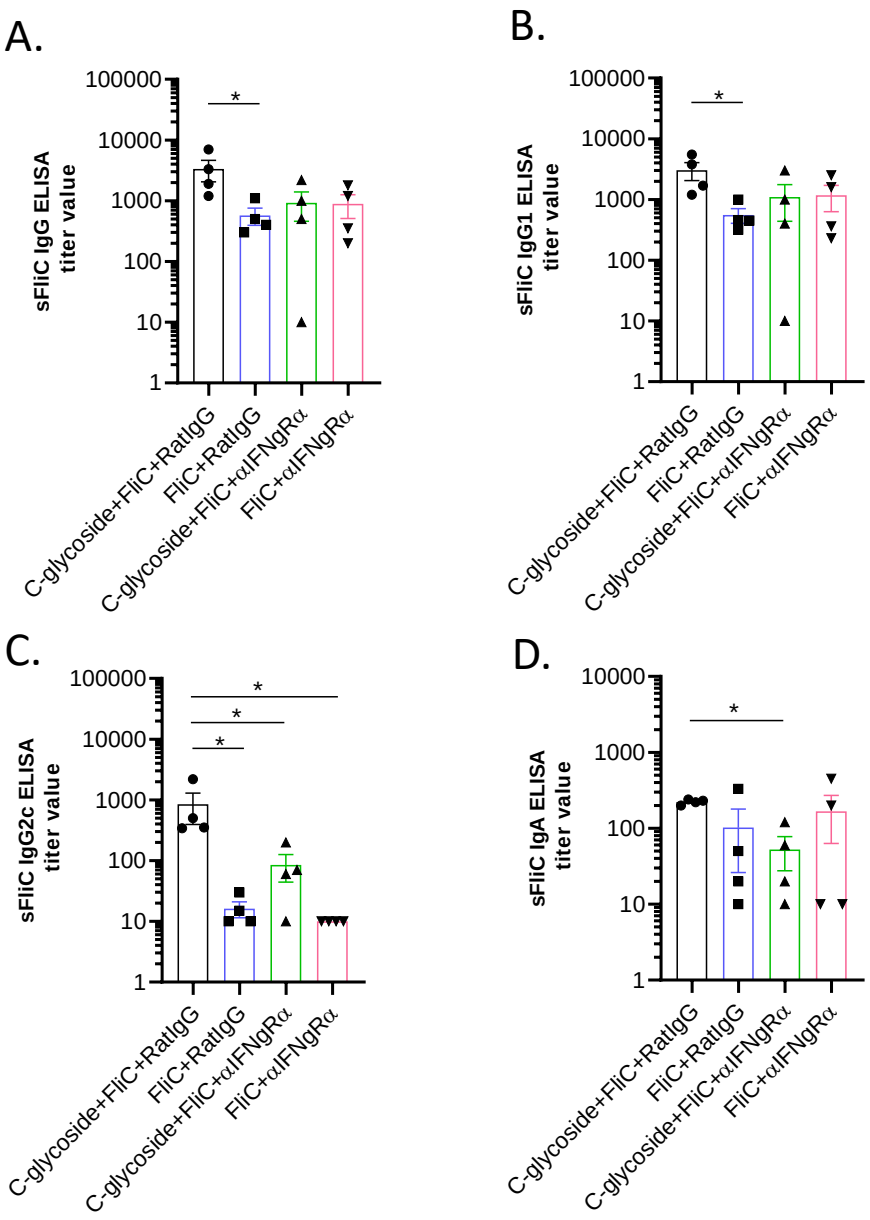


Figure 6.5 IFNγ receptor signalling is required for the enhanced titre of IgG2C and IgA in serum following C-glycoside and FliC administration.

WT B6 mice received i.p. injection of either 500µg RatIgG, or 500µg αIFNγRα (Day-1). Then i.p. injection at day 1 of either 10µg C-glycoside/FliC, or 20µg FliC. 14 days later mice received 20µg FliC i.p. Serum from such mice were collected 4 days later (Day 18). Serum anti-FliC IgG, IgG1, IgG2c and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2c, and D) IgA titre. n=4 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

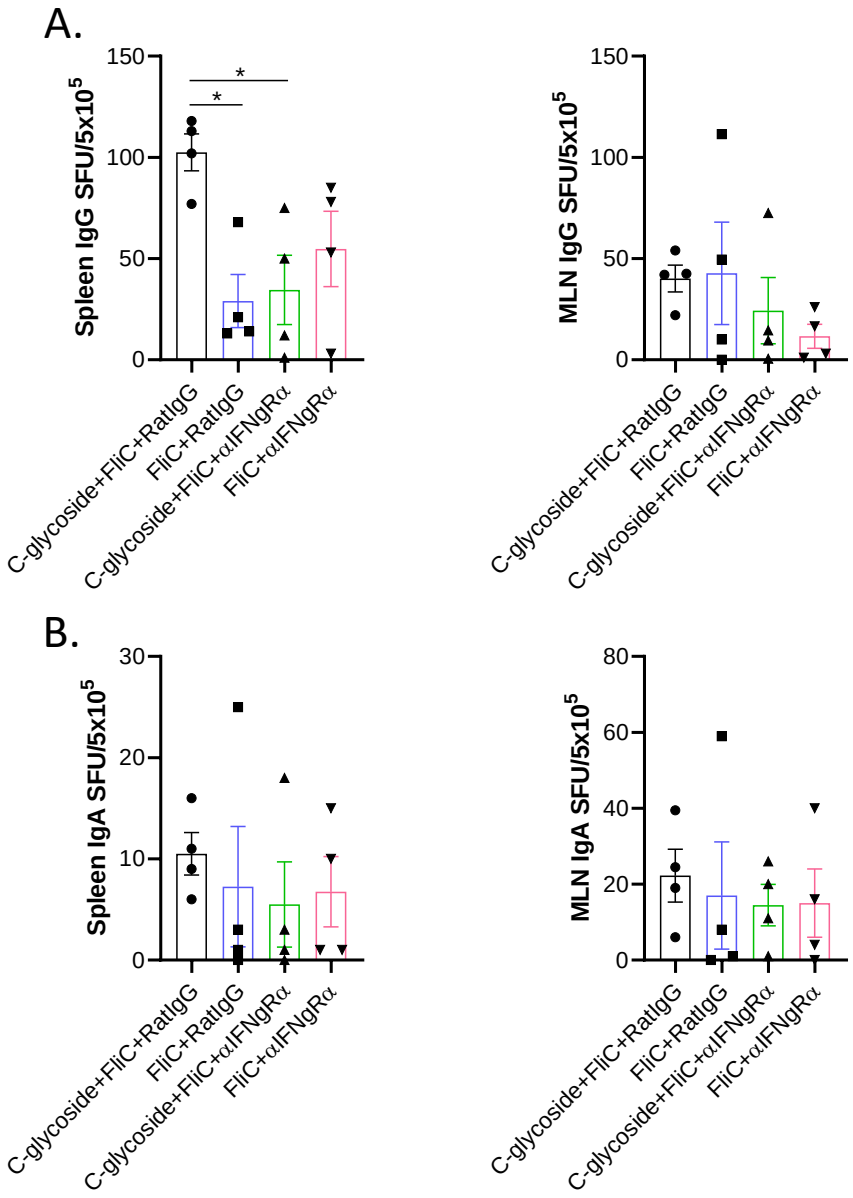


Figure 6.6 The boosted generation of FliC-specific, IgG secreting cells in the spleen by C-glycoside is dependent on IFNγ receptor signalling.
WT B6 mice received i.p. injection of either 500µg RatIgG, or 500µg αIFNγRα (Day-1). Then i.p. injection at day 1 of either 10µg C-glycoside/FliC, or 20µg FliC. 14 days later mice received 20µg FliC i.p. Single cell suspensions were prepared from spleen and mesenteric lymph nodes. ELISPOT analysis of sFliC IgG and IgA responses in the spleen and mesenteric lymph nodes. A) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgG in spleen and mesenteric lymph nodes. B) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgA in spleen and mesenteric lymph nodes. n=4 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

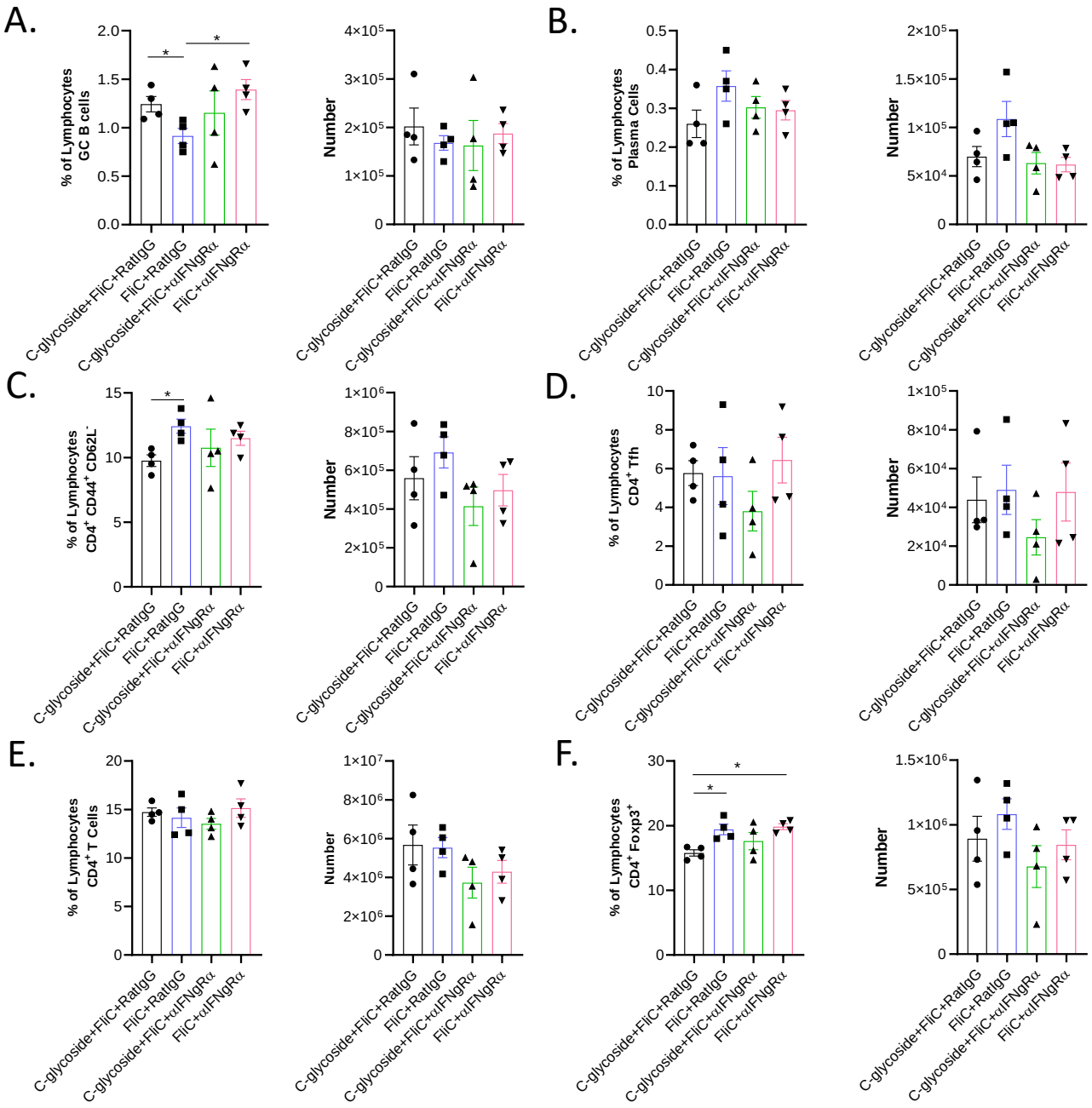


Figure 6.7 The induction of splenic GC B cells and Tfh following C-glycoside/FliC immunisation does not require IFNγ receptor signalling.

WT B6 mice received i.p. injection of either 500µg RatIgG, or 500µg αIFNγRα (Day-1). Then i.p. injection at day 1 of either 10µg C-glycoside/FliC, or 20µg FliC. 14 days later mice received 20µg FliC i.p. Single cell suspensions were prepared from Spleen. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=4 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

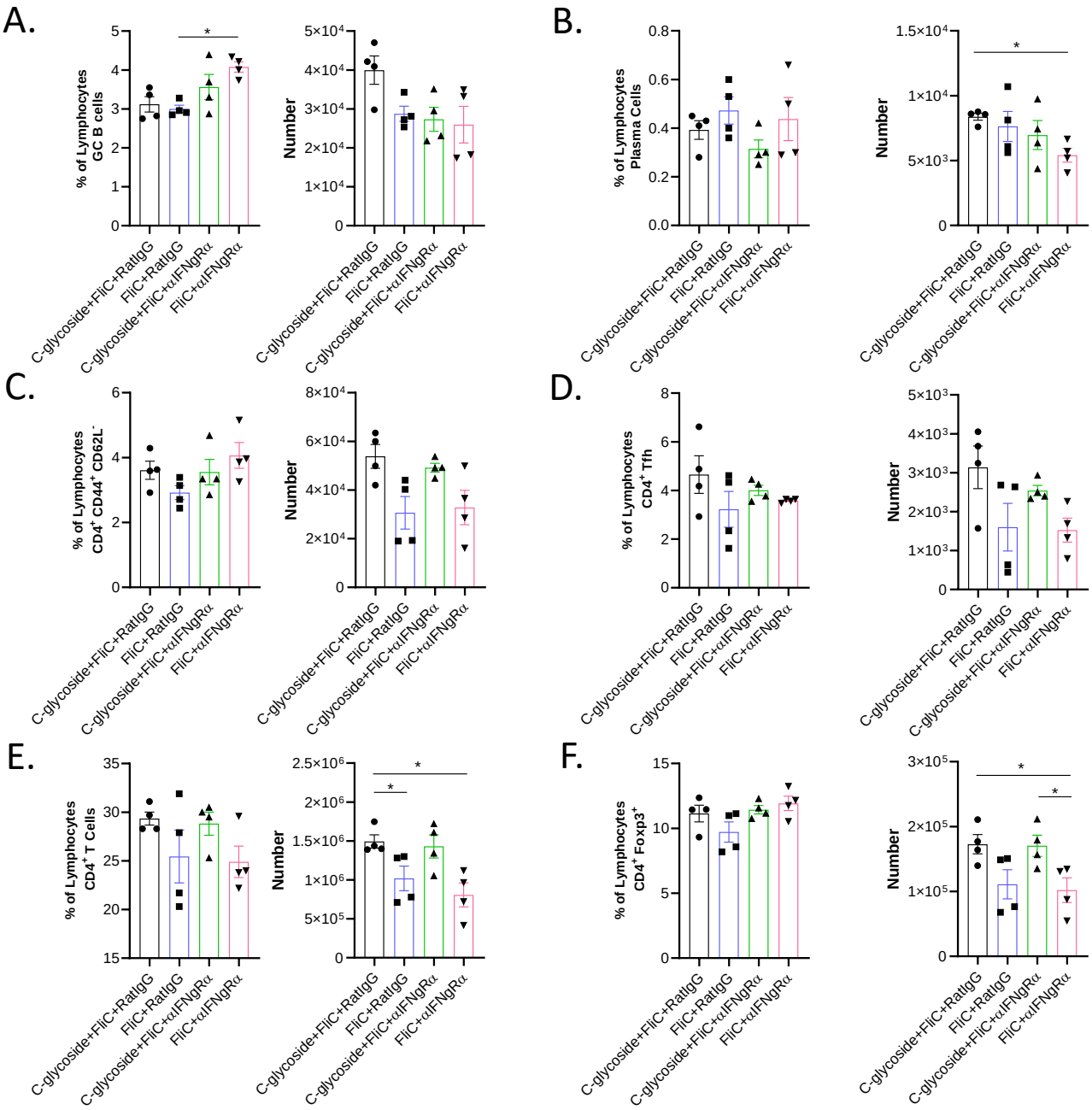


Figure 6.8 Blockade of IFNγ receptor signalling following FliC immunisation has no impact on GC generation in the MLN.

WT B6 mice received i.p. injection of either 500µg RatIgG, or 500µg αIFNγRα (Day-1). Then i.p. injection at day 1 of either 10µg C-glycoside/FliC, or 20µg FliC. 14 days later mice received 20µg FliC i.p. Single cell suspensions were prepared from MLN. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=4 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

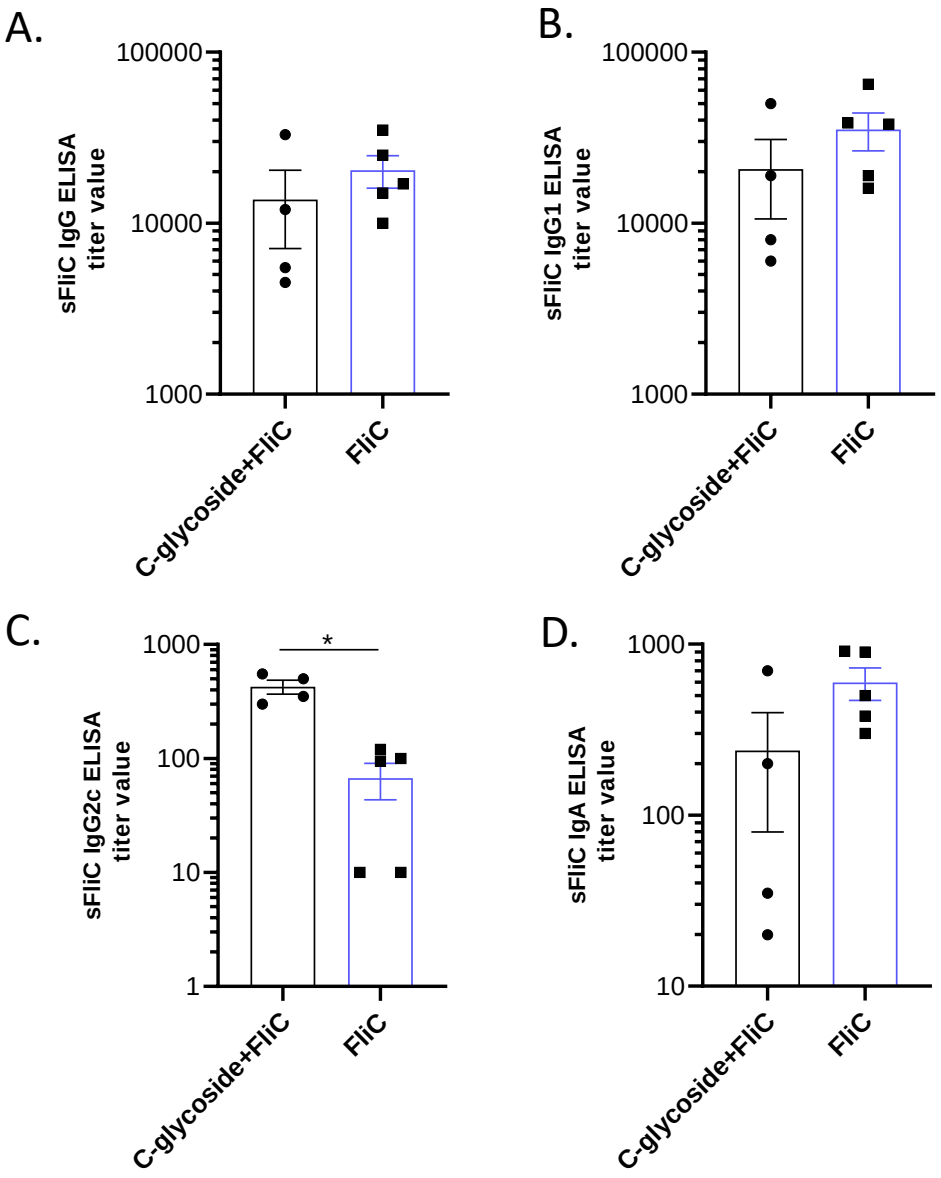


Figure 6.9 Co-immunisation of C-glycoside together with FliC maintains elevated IgG2c serum titre for over month post vaccination.
WT B6 mice received i.p. injection of either 20µg FliC or both 10µg C-glycoside and 20µg FliC. 14 days later mice received 20µg FliC i.p. Serum from such mice were collected 14 days later (Day 28). Serum anti-FliC IgG, IgG1, IgG2c and IgA titres were evaluated by ELISA. A) Bar-charts showing serum anti-FliC IgG, B) IgG1, C) IgG2c, and D) IgA titre. n=4-5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

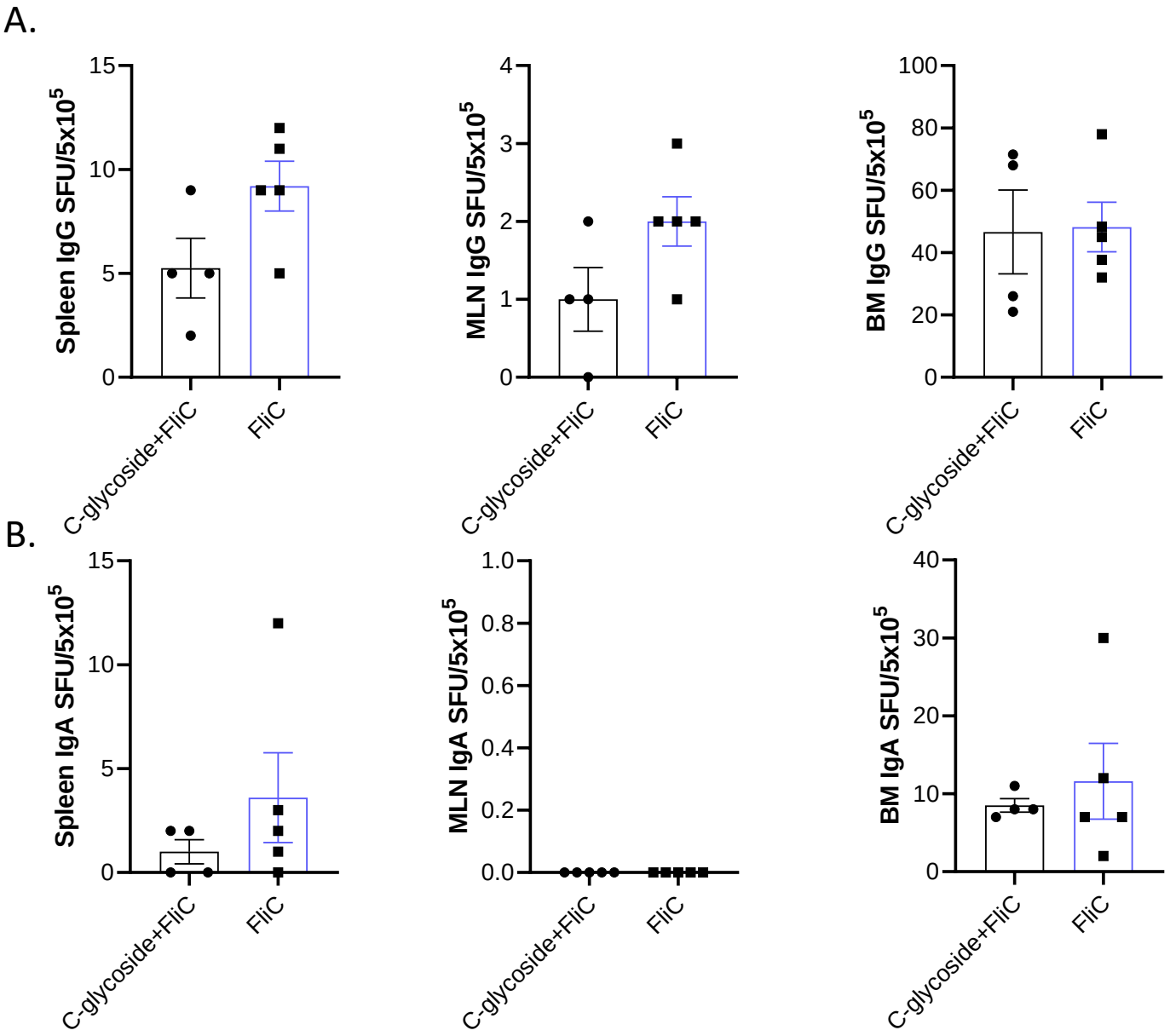


Figure 6.10 C-glycoside does not impact the frequency of FliC-specific Ab secreting cells in the long term.

WT B6 mice received i.p. injection of either 20µg FliC or both 10µg C-glycoside/FliC. 14 days later mice received 20µg FliC i.p. Tissues from such mice were harvested (Day 28). Single cell suspensions were prepared from spleen, mesenteric lymph nodes and bone marrow. ELISPOT analysis of sFliC IgG and IgA responses in the spleen, mesenteric lymph nodes and bone marrow. A) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgG in spleen, mesenteric lymph nodes and bone marrow. B) Bar-charts showing number of SFUs per 5×10^5 cells FliC-specific IgA in spleen, mesenteric lymph nodes and bone marrow. n=4-5 mice per group.

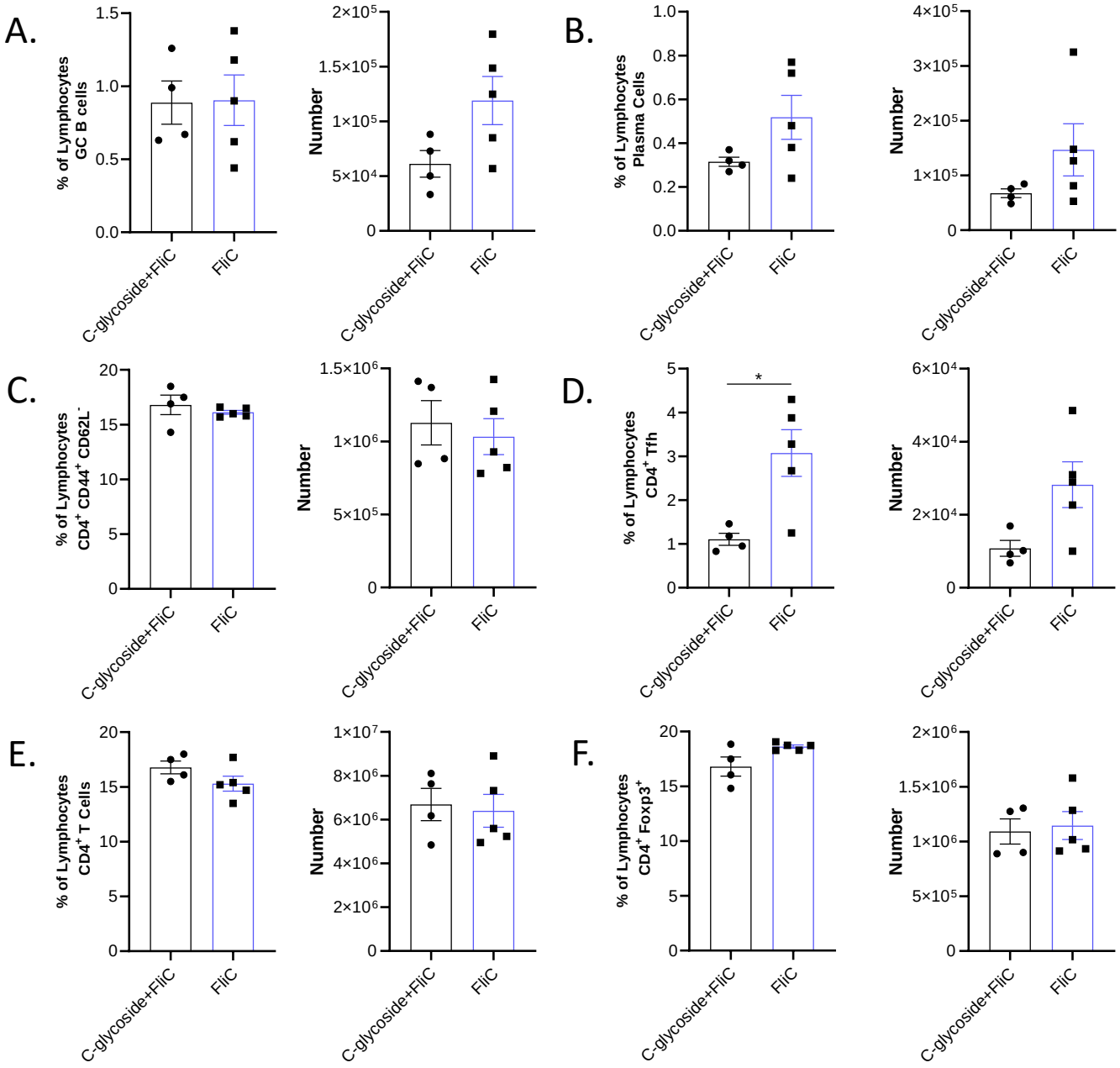


Figure 6.11 C-glycoside fails to affect GC B cells, Tfh or plasma cells over month post vaccination in spleen when administered coincident with FliC.
WT B6 mice received i.p. injection of either 20µg FliC or both 10µg C-glycoside/FliC. 14 days later mice received 20µg FliC i.p. Tissues from such mice were harvested (Day 28). Single cell suspensions were prepared from spleen. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=4-5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

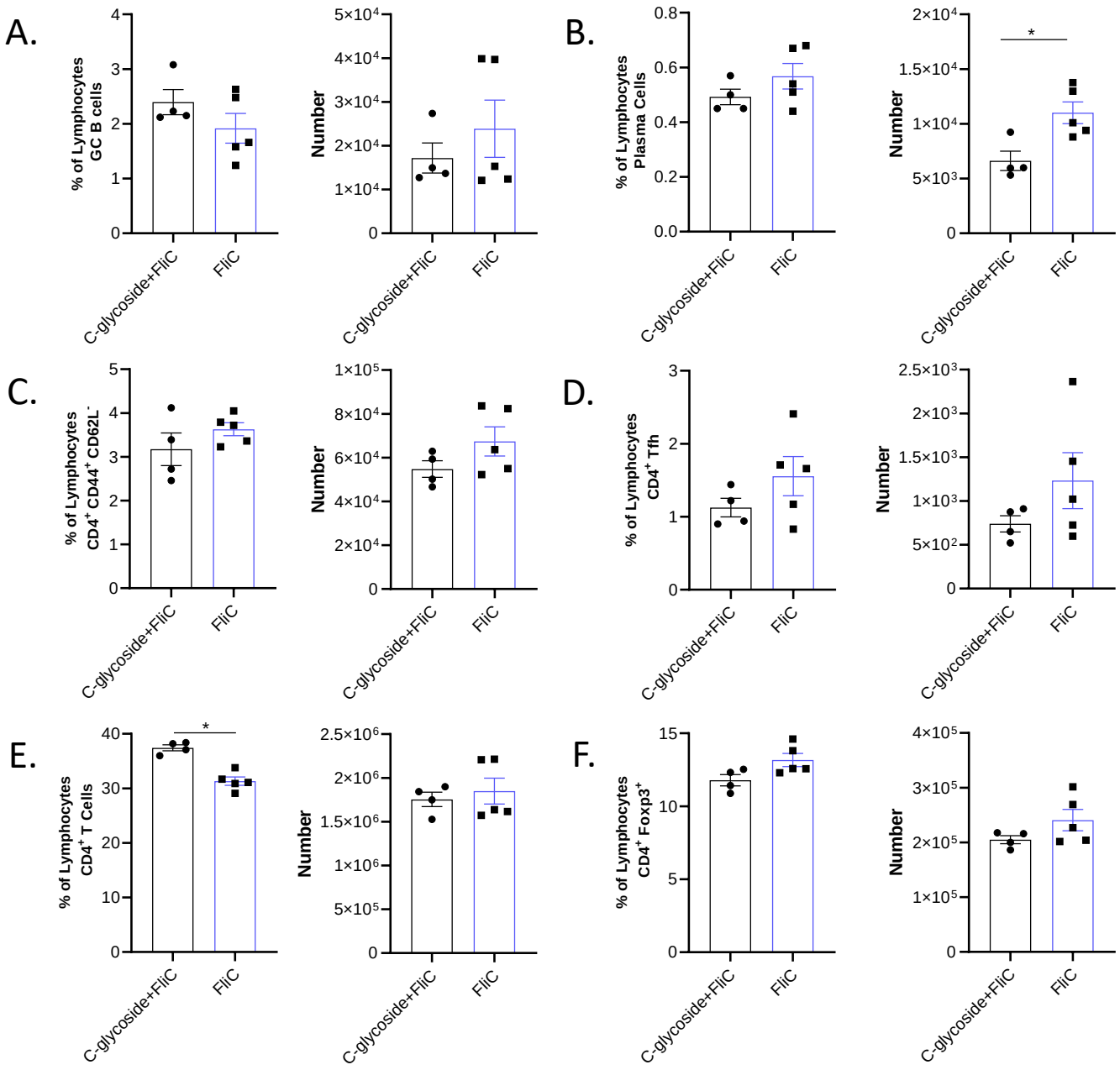


Figure 6.12 C-glycoside fails to promote enhanced frequencies and numbers of GC B cells, Tfh or plasma cells by a month post vaccination in MLN.

WT B6 mice received i.p. injection of either 20µg FliC or both 10µg C-glycoside/FliC. 14 days later mice received 20µg FliC i.p. Tissues from such mice were harvested (Day 28). Single cell suspensions were prepared from MLN. A) Bar-chart showing the percentage and the total number of GC. B) Bar-chart showing the percentage and the total number of plasma cells. C) Bar-chart showing the percentage and the total number of $CD44^+$ $CD62L^-$. D) Bar-chart showing the percentage and the total number of Tfh. E) Bar-chart showing the percentage and the total number of $CD4^+$. F) Bar-chart showing the percentage and the total number of $Foxp3^+$ Treg. N=4-5 mice per group. Data shown are mean ± SEM (error bars; unpaired t-test, two-tailed test). *P < 0.05.

CHAPTER 7: GENERAL DISCUSSION

7.1. Summary of data

7.1.1. Chapter 3

In chapter 3 we sought to confirm the presence of iNKT cells in the SI as well as defining their functional characteristics and subset distribution. iNKT cells were confirmed to be present in the small intestine representing around 0.2% of all small intestine lymphocytes (Figure 3.1 A and B). The majority of intestinal iNKT cells expressed T-bet (65-75%) with a small population expressing RORγt expressing cells (Figure 3.2 A and B). Upon activation, the majority of intestinal iNKT cells (NKT1) produced IFN-γ (~65-70%) with small 10-15% populations producing either IL-17A (NKT17) or IL-4 (NKT2) (Figure 3.2 C and D). Using IFNγ reporter mice confirmed NKT1 as the major population in the small intestine.

The activation of intestinal iNKT cells following i.p. injection of α-GalCer was also assessed. 48 hrs after injection with α-GalCer, the number and percentage of intestinal iNKT cells in α-GalCer mice were dramatically decreased compare to control mice which was likely due to TCR downregulation following activation (Figure 3.4 A and B). Moreover, following α-GalCer administration intestinal iNKT cells were found to have upregulated both PD-1 and CD25 compared to PBS injected mice (Figure 3.4 C and D).

We also sought to determine the level of CD1d expression on mucosal cDC2, known to be important to drive the immune response to FliC. We found that 95% of cDC2 ($CD11b^+$ $CD103^+$) expressed CD1d (Figure 3.5 A and B).

Finally, we sought to determine the impact of iNKT cell activation on mucosal cDC2 following the i.p. administration of α-GalCer 24hr and 48hr. 24hr after α-GalCer i.p. injection, there was a selective accumulation of cDC2 in MLN (Figure 3.6 A and C), which was followed by increased numbers of cDC2 subsets in small intestine by 48hr post α-GalCer administration.

In summary, we showed that the dominant population of iNKT cells in the small intestine are NKT1 cells which can be activated following the administration of α-GalCer i.p. The activation

of intestinal iNKT cells is likely to occur in situ as cDC (in particular cDC2 that are also responsible for the presentation of flagellin) isolated from the small intestine express high levels of CD1d. Taken together the data suggest that iNKT cells activated in the small intestine are likely to influence adaptive mucosal immune responses to flagellin and therefore glycolipids that activate iNKT cells may be a useful adjunct strategy to boost immune responses after vaccination.

7.1.2. Chapter 4

In this chapter, we sought to determine whether iNKT cell activation at the time of FliC administration impacted subsequent FliC-specific adaptive immunity.

In analyses conducted 18 days following FliC and α-GalCer administration, it was found that the activation of iNKT cells markedly improved the antibody response to FliC (figure 4.1 A-D, 4.2 A-B). In addition, additional iNKT cell activation increased germinal centre formation in spleen (figure 4.3 A) albeit without impacting plasma cell and Tfh numbers or frequencies (figure 4.3-5 B-D).

39 days after immunisation, mice that received FliC/α-GalCer showed markedly enhanced IgG, IgG1, IgG2c and IgA antibody levels in serum compared to mice that received FliC alone (figure 4.6 A-D). This correlated with increased numbers/frequency of sFliC-specific IgG/IgA-secreting cells in spleen, MLN and BM (figure 4.7 A-C). The concomitant activation of iNKT cells also enhanced germinal centre formation and Tfh cells in spleen and MLN (figure 4.8 A, D and 4.9 A, D) at this time.

Finally, the response to FliC (+/- α-GalCer) was analysed 7 days post immunisation. However, the analysed revealed no differences between mice that had or had not received α-GalCer.

Collectively, we found that iNKT cell activation coincident with FliC vaccination boosts immunity following FliC vaccination by enhancing Ab production, increasing the number of

Ab-secreting cells and boosting the GC reaction. Importantly, many of the positive effects seen with α-GalCer were maintained for at least a month post vaccination.

7.1.3. Chapter 5

In this chapter, we examined whether α-GalCer could act as a substitute adjuvant for TLR5 using TLR5 deficient mice. In addition, we wanted to determine whether iNKT cells were necessary for the response to FliC as even without exogeneous glycolipid iNKT cells can be activated by proinflammatory cytokines.

In TLR5-deficient mice, iNKT cell activation partially rescued FliC-specific IgG, IgG1 and IgG2c responses but it appeared that TLR5 was a prerequisite for IgA production (figure 5.1 A-D). However, in the MLN, IgG and IgA secreting cells were detectable in $TLR5^{-/-}$ mice immunised with FliC/α-GalCer albeit at reduced levels compared to FliC immunised WT mice (figure 5.2 B-C). In addition, iNKT cell activation promoted germinal centre formation in the MLN but not in spleen in $TLR5^{-/-}$ mice (figure 5.3-4 A).

In CD1d-deficient mice, the loss of NKT cells surprisingly resulted in poor FliC-specific IgG, IgG1, IgG2c and IgA responses following FliC vaccination (figure 5.6 A-D) which correlated with markedly reduced FliC-specific IgG and IgA Ab-secreting cells in all tissues (figure 5.7 A-C). However, in the spleen and MLN, a low frequency of both IgG and IgA secreting cells were detected (figure 5.7 A-C). The loss of NKT cells also dramatically impacted the generation of the GC B cells, Tfh and plasma cells in spleen and MLN following FliC immunisation (figure 5.8-9 A-B, D).

To provide some mechanistic explanation for the lack of response to FliC in $CD1d^{-/-}$ mice we focussed on the necessity for NKT cell activation of DC. We showed that the absence of NKT cells did not result in a numeric or phenotypic change in cDC2 in either the MLN or SI (figure 5.11-12) and that the absence of iNKT cells did not affect the migration of SI cDC2 to MLN 24

hr post-FliC administration (figure 5.13). However, migrated cDC2 in the MLN exhibited lower expression of both CD86 and CD40 in $CD1d^{-/-}$ mice compared to cDC2 in the MLN of FliC-immunised WT mice (figure 5.13 C).

Taken together, we found that for optimal immunity following immunisation with FliC both TLR5 signalling and iNKT cells are required. TLR5 is required to generate IgA class-switched Ab responses to FliC but iNKT cell activation could partially rescue the IgG response in the absence of TLR5. The loss of iNKT cells dramatically impacts the success of FliC immunisation and iNKT cells are required for optimal DC activation and the expression of important costimulatory molecules by migrating cDC2.

7.1.4. Chapter 6

We had previously shown that co-administration of α-Gal/FliC can boost the immune response to FliC following immunisation. In this chapter we examined different α-GalCer analogues that might preferentially generate a more Th1 or Th2 biased response to determine the impact on humoral responses to FliC as opposed to the mixed Th1/2 response seen on α-GalCer administration. We found that the activation of iNKT cells by C-glycoside resulted in increased titres of FliC-specific IgG, IgG1 and IgG2c Ab in serum compared to α-GalCer (figure 6.1 A-C). Moreover, the activation of iNKT cells by C-glycoside resulted in increased FliC-specific IgG/IgA Ab-secreting cells in spleen compared to α-GalCer (figure 6.2 A-C). This was further seen in the numbers/frequency of plasma cells in spleen (figure 6.3 B).

Given that IFN-γ plays a key immunomodulatory role in Th1 responses we next sought to determine whether IFN-γ secretion was responsible for the adjuvant effect of C-glycoside (figure 6.5). Our data suggest that the ability of C-glycoside to exacerbate IgG2C and IgA Ab production to FliC upon immunisation is dependent on IFN-γ signals (6.5 C-D). The relative frequency of FliC-specific IgG Ab-secreting cells in spleen was also significantly reduced in the presence of anti-IFNγRα mAb (figure 6.6 A). However, whilst IFN-γ was required for the

enhanced IgG2C and IgA response seen on C-glycolipid administration, IFN-γ was dispensable for the enhanced GC reaction seen in such mice (figure 6.7 A).

In conclusion, the activation of iNKT cells by C-glycoside results in increased adjuvanticity compared to α-GalCer. Specifically, the ability of C-glycoside enhanced immunity is dependent on IFN-γ signals. The activation of iNKT cells by C-glycoside results in a sustained increase in IgG2c Ab for over month post vaccination.

7.2. General discussion

7.2.1. Challenges of using α-GalCer as vaccine adjuvants

The greatest challenge with using α-GalCer as a vaccine adjuvant is that the immune response and protective efficacy depends on the context of vaccine. For instance, in a study on parasite vaccines, α-GalCer when combined with a number of malaria vaccines showed an efficient enhancement of the immune response against plasmodia parasites (341). However, in contrast, mice co-immunised α-GalCer with a Trypanosoma cruzi DNA vaccine showed less protective immunity and demonstrated a diminished induction of epitope-specific $CD8^+$ T cells against Trypanosoma cruzi (419).

It is well documented that α-GalCer results in the co-production of both Th1 and Th2 cytokines that may lead to an unpredictable effect on the response to a vaccine. Indeed, it has been shown that the effective therapeutic application of iNKT cell activation is dictated by whether the glycolipids trigger selective Th1 or Th2 cytokines (420).

Another outstanding challenge is preventing the induction of iNKT cell non-responsiveness or anergy through repeated dosing of glycolipids (421). Sullivan and Kronenberg have suggested that targeting DCs with a glycolipid agonist may help avoid excessive presentation by B cells, which is thought to result in iNKT cell anergy (319, 409, 421). In addition, it is important to avoid toxicities arising from the activation of iNKT cells, as it has been reported that such

toxicities can cause liver damage and increase the severity of septic shock in mouse models (422). These outstanding challenges make the safe use of glycolipid adjuvants a concern and clearly more work needs to be done to resolve such issues.

7.2.2. Challenges of using Th1-biasing C-glycoside as vaccine adjuvants

C-glycoside is a well-characterised Th1-biasing glycolipid, which was first studied in a mouse malaria model (220, 221, 281, 423). In this mouse model, C-glycoside evoked a remarkably improved response which was 1,000-fold superior than α-GalCer in its anti-malaria efficacy (221, 423). However, for humans, its use is severely limited, as it must be given before sporozoite exposure, an early liver stage of malaria (221, 423).

Further studies have been conducted on the effects of iNKT glycolipid adjuvants in vaccines. In support of this, one study used a live attenuated influenza vaccine combined with α-GalCer (424). This study found that immunised mice showed a reduction in morbidity and mortality after boosting compared to mice that received the vaccine alone. α-GalCer coadministration resulted in an increased number of IFN-γ-secreting T cells and an enhancement of specific influenza virus IgG, IgG1, and IgG2a antibodies. The limitation of this study was that it did not show a direct comparison between C-glycoside and α-GalCer (424). That said these studies support our findings as we showed that a combination of C-glycoside and FliC results in further enhancement of humoral immunity through augmentation of IgG, IgG1 and specifically IgG2c following FliC vaccination.

In humans, C-glycoside failed to stimulate human iNKT cells making C-glycoside unsuitable for human clinical use. However, C-glycoside analogues have been reported to restore recognition either by introducing C6″ substituents or by restricting its conformation through the introduction of an E-alkene linker between the ceramide and the galactose sugar (222, 425). Overall, the activity observed in C-glycoside that makes it a potential candidate adjuvant is its

stability of presentation in CD1d, leading to a sustained in vivo response that results in a Th1 response (426, 427). However, there are a number of other glycolipids that have also been found to drive preferential Th1 responses without the induction of anergy that may be as good if not better than C-glycoside when used as an adjuvant (428, 429).

7.2.3. The use of α-GalCer or its analogues combined with other TLRs as vaccine adjuvants

iNKT cells and TLR ligands can act together to target DCs and modulate their functions potentially promoting vaccine responses (430).

α-GalCer with the TLR9 agonist CpG oligodeoxynucleotide (CpG-ODN) combined with gp140 (clade C gp140 HIV-1 envelope protein antigen) was shown to enhance both $CD4^+$ and $CD8^+$ T cell responses alongside memory T cell responses (431). This was seen in comparison with either α-GalCer + gp140 or CpG-ODN+gp140. This combination resulted in increased production of anti-gp140-specific IgG/IgA in serum. However, antibody production was reduced in mice treated with either α-GalCer +gp140 alone or CpG-ODN+gp140 alone.

Another combination of TLR ligands and a glycolipid adjuvant was used in a study employing the delivery of the malaria antigenic peptide (PyCSP) as a vaccine platform (432). A combination of MPLA as a TLR4 agonist and 7DW8-5 as a glycolipid adjuvant was investigated. MPLA plus 7DW8-5 provided strong induction of memory $CD4^+$ and $CD8^+$ T cell responses in comparison with either adjuvant alone (432). The combination of MPLA/7DW8-5 proved remarkably effective when mice received three doses of immunisation. Following repeated immunisation, the adjuvant enhanced Th1 memory-like effector NKT cells, suggesting that the cells became activated without the induction of anergy. These findings were consistent with another study which found that MPLA and αGalCer enhanced IgG responses as a result of adjuvant combination (344).

Taken together, the modulation of DC activation and cytokine production are emphasised in these studies. This supports the importance of the initial influence on DCs and their cytokine production. We have provided evidence to support the hypothesis that C-glycoside may work through aiding the maturation of cDC2 in the SI. However, the laboratory is looking to refine this approach by making glycolipid-FliC linked molecules in order to target both glycolipid and FliC to the same DC.

7.2.4. The use of α-GalCer or its analogues as vaccine adjuvants against infectious diseases

The life cycle of malaria parasites is characterised by two rapid stages: the liver stage and the blood erythrocyte stage. This complicated life cycle provides parasites with an advantage in terms of immune evasion due to antigenic variation. However, it has been recognised that IFN-γ and $CD8^+$ T cells play a significant role in fighting malaria parasites (433). It has been reported that viral vectors expressing malaria antigens are able to protect against the liver stages (434, 435). However, these still need to be improved to provide an effective vaccine with long-term protection against this parasite.

Many studies have investigated the use of α-GalCer as an adjuvant to improve parasite vaccines. A study found that immunisation with α-GalCer and γ-irradiated sporozoites (γ-spz), known as a parasite vaccine, showed an improvement in protective anti-malaria immunity compared to γ-spz immunised alone (341). Immunised mice with the combination of α-GalCer/γ-spz showed a 10-fold reduction in parasite load compared to γ-spz only.

The effect of α-GalCer has also been studied when combined with other vaccines, such as an adenovirus expressing P. yoelii CS protein and a Sindbis virus expressing circumsporozoite (CS) protein, with similar effects as the α-GalCer/γ-spz vaccine. Splenocytes from mice that received α-GalCer/γ-spz showed induction of IFN-γ-producing CS-specific $CD8^+$ T cells in

comparison to mice that only received γ-spz. Furthermore, CD1d-deficient mice and IFN-γ receptor-deficient mice confirmed that the adjuvant effect of α-GalCer depended on the activation of NKT cells. The study found that IFN-γ production by NKT cells mobilises NK cells to also secrete IFN-γ, thus enhancing protective anti-malaria immunity.

However, a further study examined C-glycoside in comparison with α-GalCer as a subsequent investigation into malaria vaccines. This study was performed using either α-GalCer or C-glycoside i.p. injection alone two days before they immunised with live P. yoelii sporozoites (221, 415). This study showed that C-glycoside resulted in a more profound Th1 bias than α-GalCer. In addition, IL-12 was shown to be a key factor in enhancing immunity, and that the use of C-glycoside resulted in higher levels of IL-12 compared to α-GalCer.

This model of using C-glycoside as a therapeutic agent proposed that DCs present C-glycoside to NKT cells. The activated NKT cells were found to produce IFN-γ and overexpress the IL-12 receptor. This interaction also activated DCs resulting in IL-12 expression. The events between these cells lead to suppressing the development of the malarial liver stage and enhancing the antimalarial response, as it is highly biased towards the Th1 responses. The study concluded that C-glycoside holds much promise to enhance anti-malaria immunity upon vaccination. Consistence with this, our data showed that activation of iNKT cells by C-glycoside lead to increase adjuvanticity compared to α-GalCer as a result of Th1 cytokine secretion induced by C-glycoside. This led to further increased the titre and longevity of serum IgG2C and IgA Ab compared to that seen with α-GalCer. This marked the requirement of the early production of IFN-γ via C-glycoside activated NKT cells as extremely important as IFN-γ is associated with the eradication of infection.

7.2.5. The use of α-GalCer or its analogues as vaccine adjuvants against viral pathogens

Activation of NKT cells have been reported to have potential effect on current HIV vaccines due to its ability to direct cytotoxic anti-viral activity. This has led many researchers to use α-GalCer as an adjuvant to potentially improve HIV vaccines.

DNA vaccines encoding the gag and env proteins of HIV-1 co-immunised with α-GalCer were found to enhance the response of IFN-γ-secreting, epitope-specific $CD8^+$ and $CD4^+$ in comparison to the DNA vaccine alone (343). This finding was supported by the enhancement of Gag-specific antibody responses in co-immunised mice. In addition, in a protocol that involved a prime-boost strategy there was a further enhanced production of Gag-specific-IgG responses in mice co-immunised with α-GalCer/DNA and boosted with DNA only. Taken together, the study concluded that co-immunisation with α-GalCer and DNA resulted in a balanced Th2/Th1 response as well as augmentation of the production of IgG against HIV-1 gag protein. This study supported a previous study finding that concluded that CD1d/NKT cells lead to the rapid production of cytokines that enhance the humoral immune response and immunogenicity for a wide range of vaccines.

α-GalCer has also been assessed as an adjuvant in a nasally-administered vaccine composed of formalin-inactivated PR8 influenza virus (346). BALB/c mice were co-immunised with inactivated PR8 with and without α-GalCer and a boost with live PR8 virus two weeks later. The study found that a single low dose of α-GalCer with the vaccine showed a higher reduction in viral titres compared to the vaccine alone. The study found that co-immunisation of inactivated PR8/α-GalCer enhanced the PR8-specific IgG and IgA Ab responses in comparison to the vaccine alone. In vitro re-stimulation of iNKT cells from the spleen and lymph nodes of such mice revealed a higher Th2 cytokine responses (IL-4 and IL-5 levels) in comparison with the high levels of IFN-γ production seen in vaccines alone.

These findings of higher survival rates alongside lower viral titres highlighted the importance of Th2 responses in influenza viruses, thus the addition of α-GalCer favoured a Th2-like cytokine environment which favoured IgA switching which has recently been shown to be critical for protection against influenza virus (436).

A subsequent study reported that the use of C-glycoside had similar effects when combined with a PR8 influenza vaccine (424). They reported that IFN-γ-dependent $CD8^+$ T cell responses have a significant role in boosting the immune response. Surprisingly, these findings are the opposite of the previous findings reported by Youn et al (346). However, these studies need to be improved by demonstrating a direct comparison between C-glycoside and α-GalCer as well as including $CD8^+$-specific responses together. Such research would help determine the precise role of cytokines in Th1/Th2 responses and the cytotoxicity effect of these adjuvants.

7.2.6. The use of α-GalCer or its analogues as vaccine adjuvants against tumours

The potent anti-tumour activity of NKT cells was first seen when injection of α-GalCer was shown to reduce the tumour burden in a B16 melanoma mouse model (216, 437). It has also been recognised that cancer patients display a large reduction in NKT cell numbers, no matter what cancer type or load they were diagnosed with (438). Observations such as these has led to attempts to utilise NKT cells as a therapeutic strategy against tumours either directly or indirectly by the use of agonist glycolipids as adjuvants in vaccines.

One effective strategy involved a DC-based vaccine to hepatocellular carcinoma where DC were pulsed with α-GalCer and HBV antigens before being injected into mice. (439). This led to tumour regression that was dependent on improved cytotoxic $CD8^+$ T cell responses along with IFN-γ and IL-12 secretion.

Autologous tumour-specific antigens were used as another strategy to target lymphoma tumours (440). These antigens show poor immunogenicity even though they are highly effective and

specific, however, the addition of α-GalCer loaded A20 lymphoma cells produced a robust, effective immune response to lymphoma in mice that was highly dependent on $CD4^+$ T cells.

7.3. Future Work

The work contained in this thesis has led to the further hypothesis that immunisation with C-glycoside+FliC would provide a durable and complete protection against STm infection. However, due to the time-constraints of the project these experiments were not performed. Therefore, the next set of experiments would aim to determine the protective capacity of glycolipid/FliC vaccination where mice will be immunised with C-glycoside/FliC or FliC only and receive a further dose of FliC 14 days later before being infected with an attenuated form of STm at day 28. The infection would be monitored via analysis of stool samples and dissemination determined by assessment of STm CFU in the spleen and liver at defined timepoints after infection.

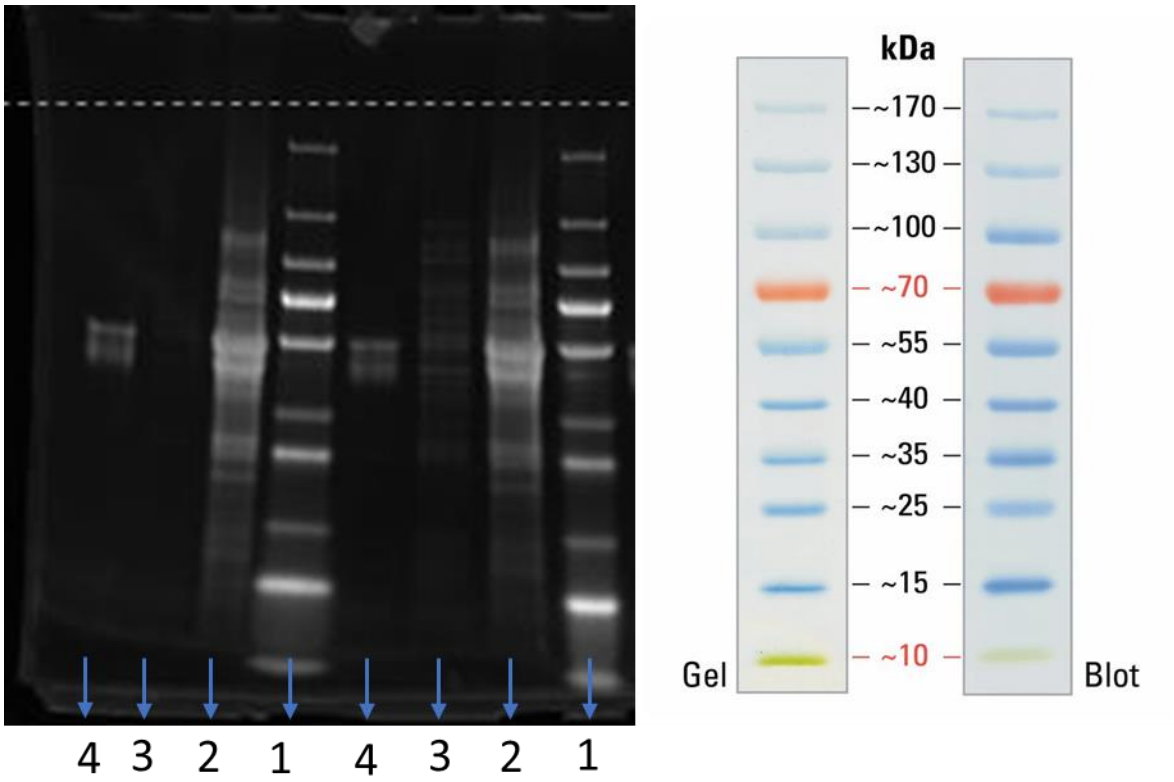
The other main strand of research would be to further improve iNKT cell agonist adjuvanticity for FliC immune responses by targeting both C-glycoside and FliC to the same cDC2 and perhaps limiting off-target effects by using the TLR5-binding capacity of FliC to target the SI. To this end we plan to engineer a conjugate flagellin-C-glycoside conjugate molecule. We postulate that the administration of this molecule will lead to a degree of gut tropism, due to the limited expressing of TLR5, as well as the co-presentation of both C-glycoside/CD1d and FliC peptide/MHC class II on cDC2. We hypothesise that this strategy will further enhance the adjuvanticity of iNKT cell agonism and improve vaccine responses to FliC.

7.4. Conclusion

We have shown that NKT cells are pivotal to successful vaccination using soluble FliC. NKT cells are required for the GC reaction and much of the Ab secretion seen upon vaccination with FliC moreover the use of an IFNγ-inducing glycolipid such as C-glycoside has been shown to be a viable candidate for improving vaccination with FliC to protect against subsequent STm infection.

APPENDICS

Appendix I SDS-PAGE



Assessment of sFliC via SDS-PAGE following purified via His-Tag purification and affinity chromatography.
1) Ladder. 2) Flow Through before affinity chromatography 3) Wash sFliC post affinity chromatography. 4) purified sFliC post affinity chromatography.

Appendix II Solutions

cRPMI media

1. L-glutamine 2mM (Sigma Aldrich)
2. Penicillin-streptomycin 100 U ml-1 (Sigma Aldrich)
3. FCS 10% (vol/vol)
4. 1X RPMI 1640 Sodium Bicarbonate without L-Glutamine (Sigma)
5. 2-mercaptoethanol 5.5 μM

Luria-Bertani (LB) broth

1. 1 L distilled H2O
2. 20g of powder LB broth (Sigma Aldrich)

FACS buffer

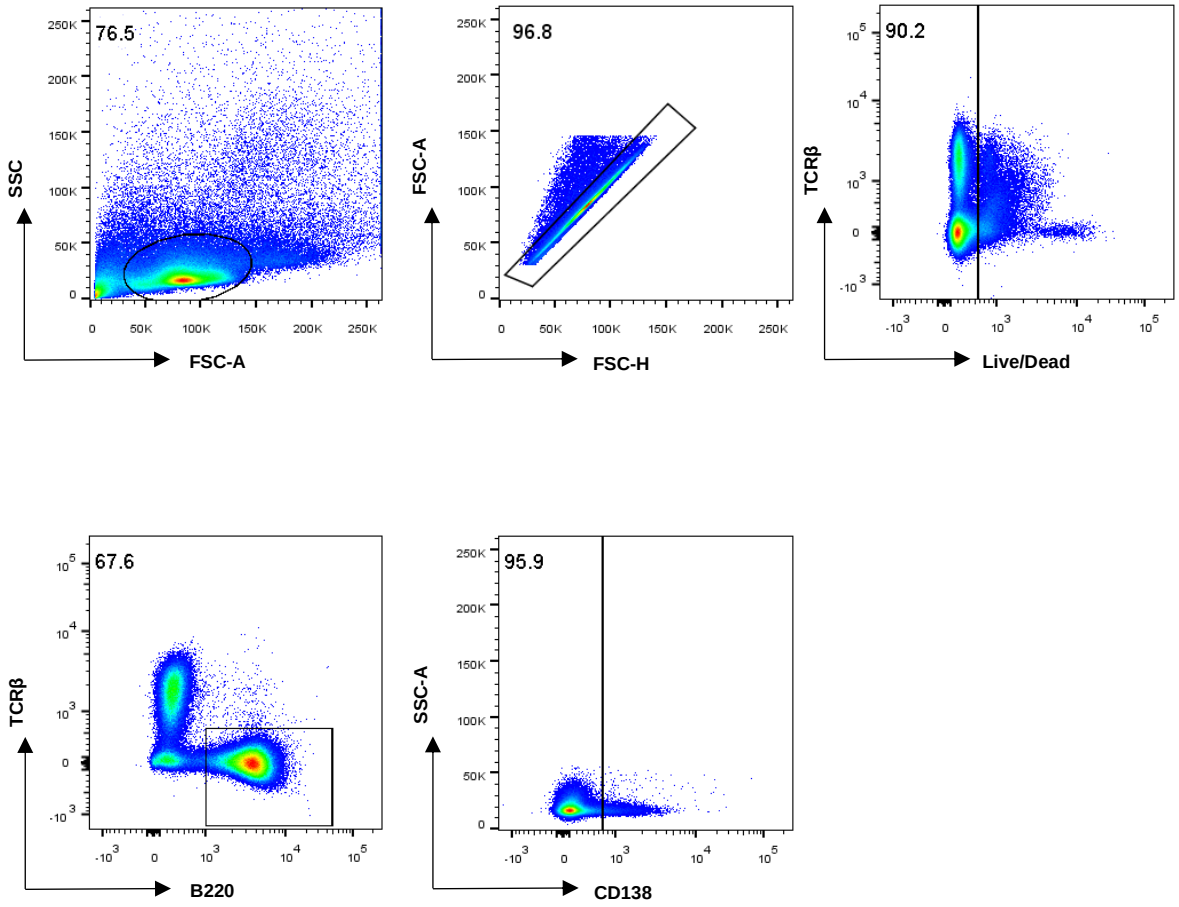
1. Azide 0.02% (w/v) (Sigma Aldrich)
2. 1X PBS

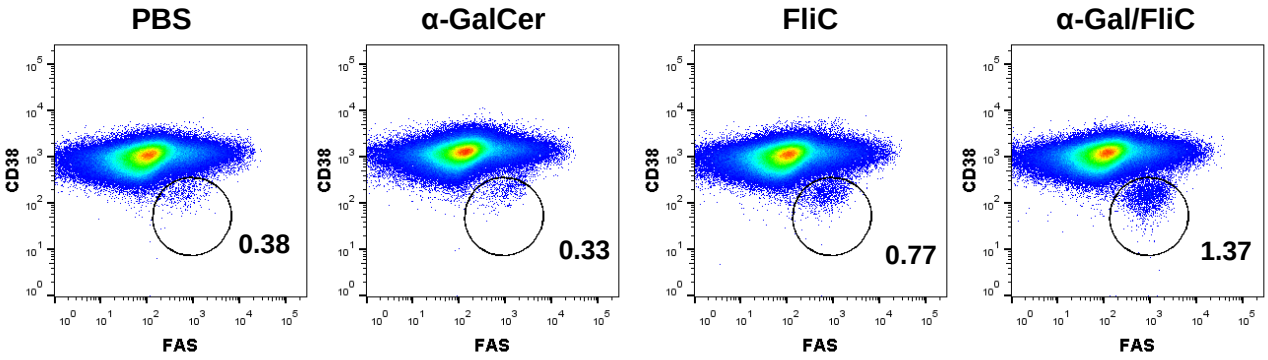
SDS Sample Buffer (4X)

1. 0.8g SDS
2. 1 M Tris HCl (pH 6.8)
3. 25% Glycerol
4. 0.5 M EDTA
5. 8 mg Bromophenol Blue
6. 14.7 M β-mercaptoethanol.

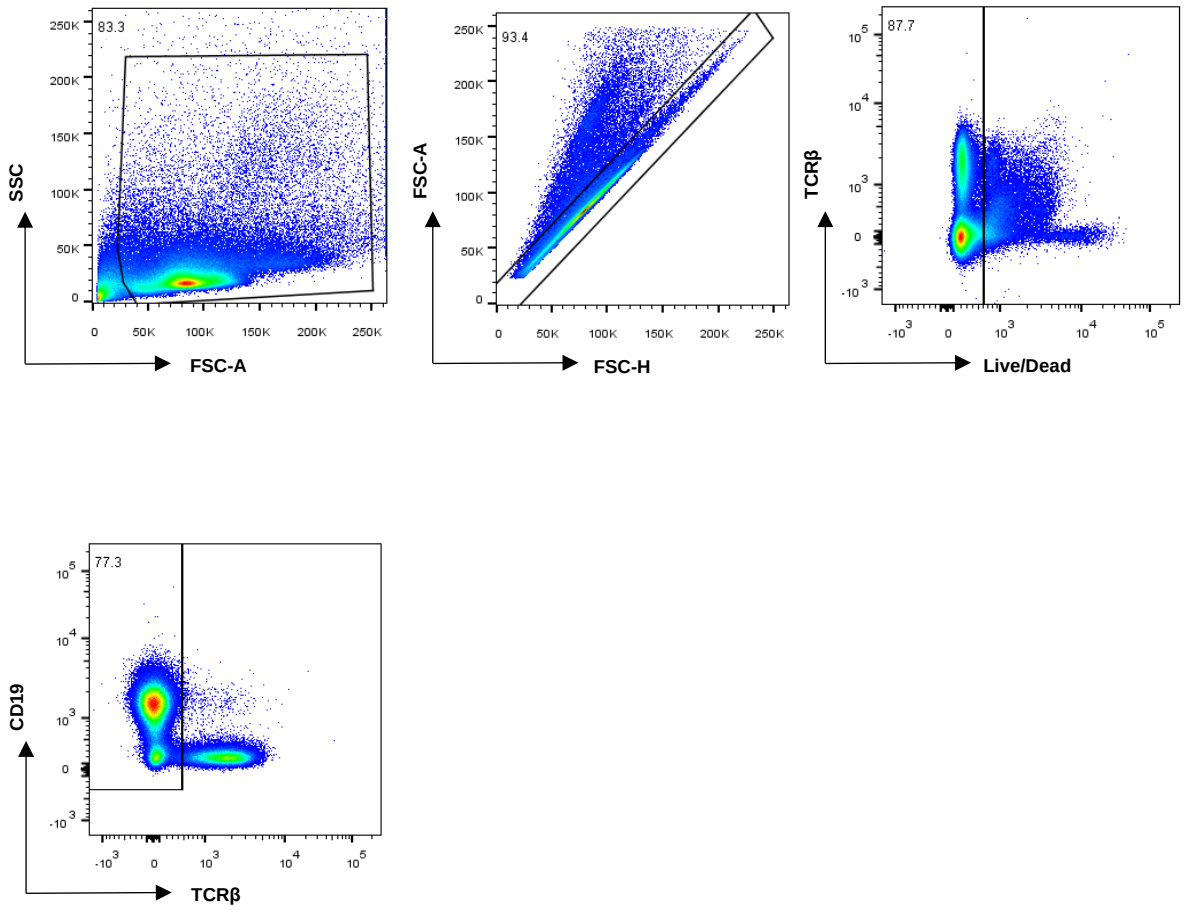
Appendix III Gating strategies

Germinal centre B cells gating strategy

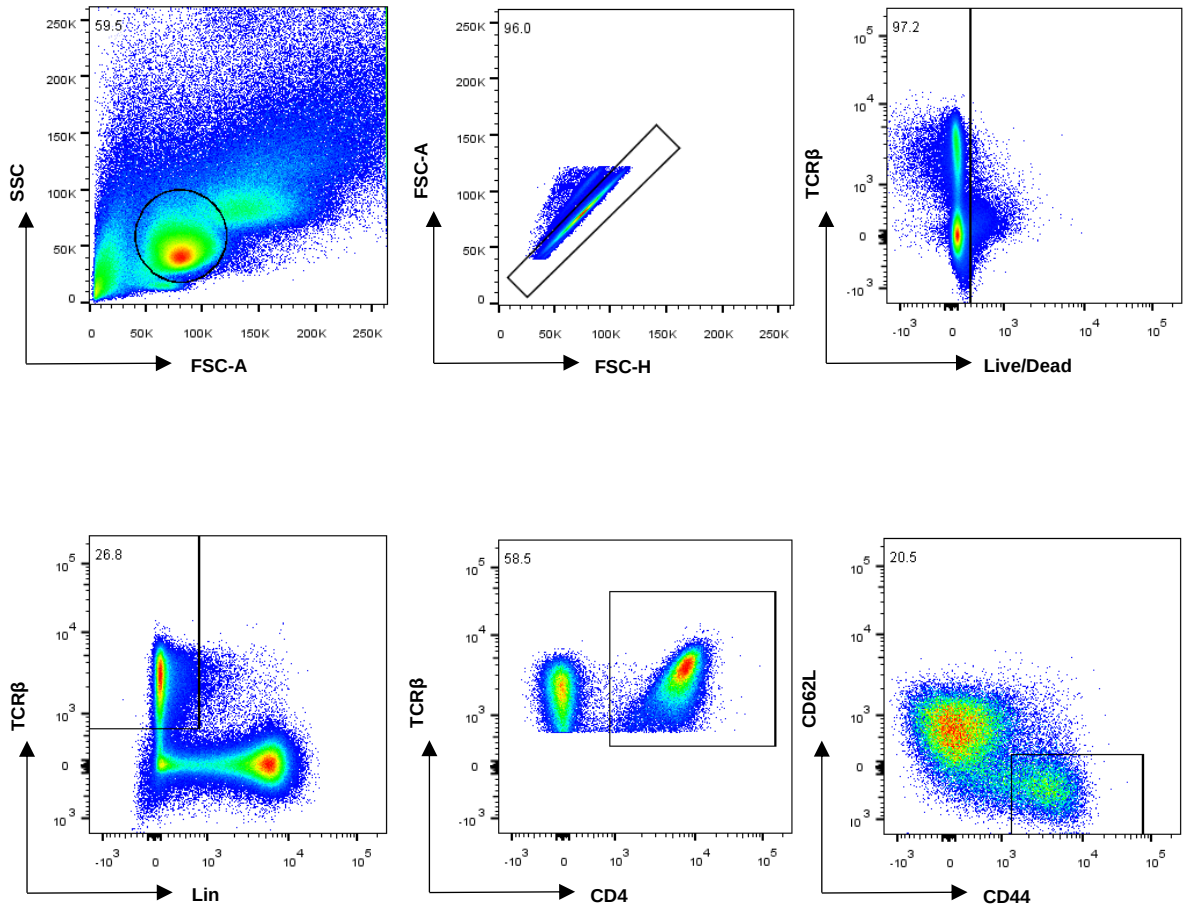


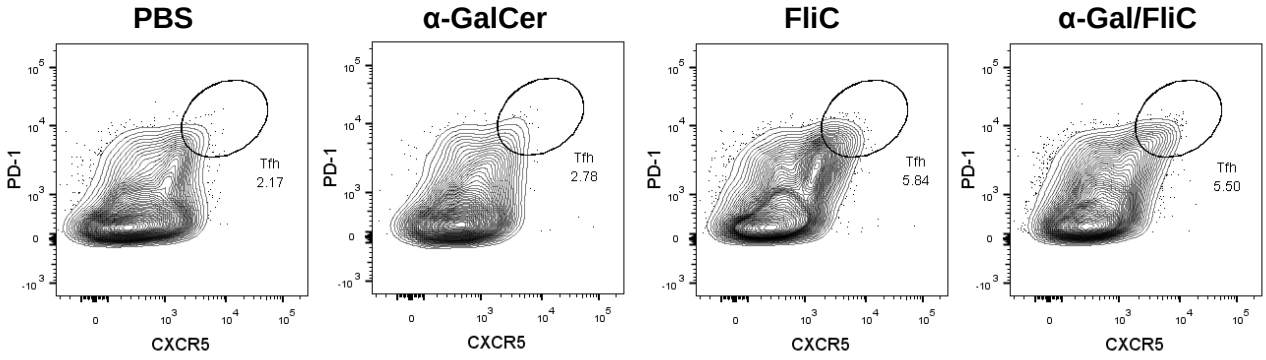


Plasma cells gating strategy

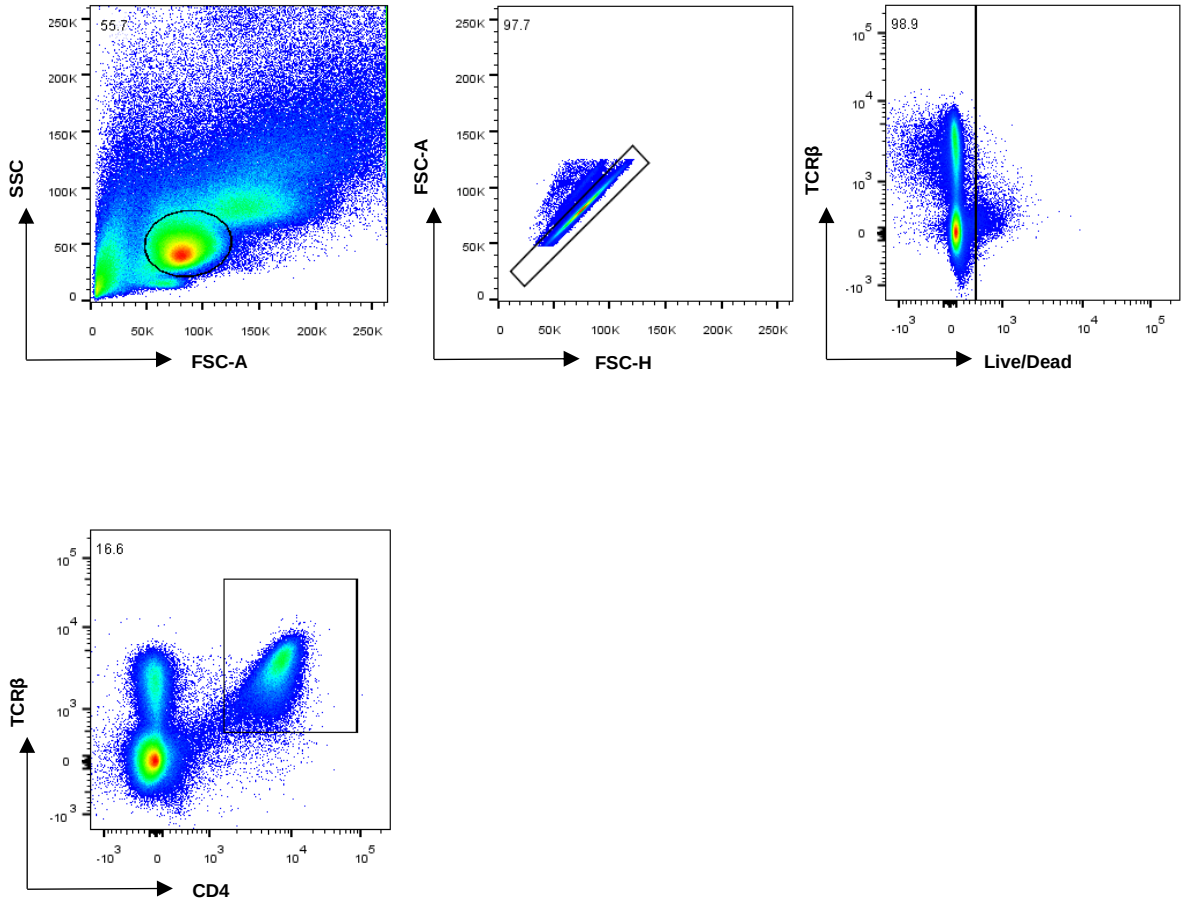


Memory B cells and Tfh cells gating strategy





CD4 T cells and Treg cells gating strategy



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