Revisiting Development and Homeostasis of Thymic Regulatory
T Cells in Type 1 Diabetes

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Master in Science by Research

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Biology

November 2017

Abstract

Regulatory T cells (Tregs) seem to play a protective role in the pathogenesis of type 1 diabetes (T1D), a serious autoimmune disease. Studies using T1D murine models such as the non-obese diabetic (NOD) mouse have suggested that T1D is initiated partly due to either paucity and/or defectiveness of Tregs in the periphery, although other reports contradict this. In contrast Treg development in the thymus is thought to be normal in T1D-prone mice. However, these latter studies neglect to investigate different thymic Treg subpopulations such as precursor Tregs, newly developed Tregs and thymic resident Tregs. It is therefore important to establish a pattern of Treg populations for a better understanding of the relationship between regulatory cells and T1D.

Using for the first time RAG-GFP reporter mice, this project re-investigated the Treg frequency and number in primary and secondary lymphoid organs in NOD mice in comparison to non-diabetes prone control mice. Time course flow cytometry studies revealed that there was a developmental fault in Treg generation in NOD mice; both precursor and mature Treg frequencies and numbers were increased with respect to control mice. However, further analysis of the mature Treg thymocytes demonstrated there was a paucity in newly developed Tregs compared to control mice, and concomitantly an increase in thymic resident Tregs in the NOD mice. These events were not related to intra-thymic IL-2 levels. Interestingly, B cells may have a role in Treg development and/or peripheral homeostasis in an age-dependent manner.

The data presented in this project provides new evidence on the inticacies of the relationship between Tregs and T1D, and emphasize the importance of succinct analyses of distinct thymic Treg subpopulations to validate this relationship.

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Abbreviations

Abs	Antibodies
AIRE	Autoimmune regulator gene
ALPs	All-lymphoid progenitors
BACs	bacterial artificial chromosomes
BB	Biobreeding rats
BcR	B cell receptor
beta2m	beta 2-microglobulin
B-KO	B cell knockout
BLPs	B-cell biased lymphoid progenitors
BrdU	bromodeoxyuridine
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CD45R	Cluster of differentiation 45R
CD4SP	CD4 single positive cells
c-Kit	Stem cell growth factor receptor
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CNS1	conserved non coding sequence 1
CRP	C-reactive protein
cTECs	Cortical epithelial cells
CTLA-4	cytotoxic T lymphocyte associated protein 4
CTLs	Cytotoxic T lymphocytes
CXCL12	CXC chemokine ligand 12
DCs	Dendritic cells
DN	Double negative cells
DP	Double positive cells
Ebf1	Early B cell factor 1
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETPs	Early thymic progenitors

Flt3	Fms-like tyrosine kinase 3
Foxo1	Factor forkhead box protein 1
Foxp3	Forkhead box P3
GAD	glutamic acid decarboxylase
GFP	Green fluorescent protein
GMPs	Myelomonocytic progenitors
HLH	Helix-loop-helix
HSA	Heat stable antigen CD24
HSCs	Haematopoietic stem cells
IA-2	insulinoma antigen-2
Idd3	insulin-dependent-diabetes 3
IFN- γ	interferon γ
Ig	Immunoglobulin
IGRP	islet-specific glucose-6-phosphatase catalytic subunit-related protein
Ikzf2	Helios
IL-2	Interleukin 2
IL-7R	Inerleukin 7 receptor
ILNs	Inguinal lymph nodes
iTregs	Peripherally induced regulatory T cells
JAK-STAT	Janus Kinase/Signal Transducer and Activator of Transcription
LCMV	lymphocytic choriomeningitis virus
Lin	Lineage
LMPPs	Lymphoid-primed multipotent progenitors
LT-HSCs	Long-term haematopoietic stem cells
mAbs	monoclonal antibodies
Mac1	Macrophage 1 antigen
MBL	Mannan-binding lectin
MEPs	Megakaryotic/erythroid progenitors
MHC	Major histocompatibility complex
MiDReG	Mining developmentally regulated genes
MPO	Myeloperoxidase
MPPs	Multipotent progenitors
mTECs	Medullary epithelial cells
mTregs	Mature regulatory T cells

ND	Newly developed
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NOD	Non-obese diabetic mouse
Notch1	Notch homolog 1
Nrp1	neuropilin 1
nTregs/tTregs	Naturally derived thymic regulatory T cells
PAMPs	Pathogen-associated molecular patterns
Pax5	Paired box gene 5
PBS	phosphate buffered saline
PD-1	programmed cell death-1
PLNs	Pancreatic lymph nodes
PRRs	Pattern recognition receptors
pTregs	Precursor regulatory T cells
RAG	Recombinase-activating gene
RIP	rat insulin promoter
RSS	Recombination signal sequences
RTEs	Recent thymic emigrants
SAP	Serum amyloid protein
Sca1	Stem cells antigen-1
SCF	Stem cell factor
scid	severe-combined immunodeficient
SP	Single positive
SR-A	Scavenger receptor type A
STAT5	signal transducer and activator of transcription 5
ST-HSCs	Short-term haematopoietic stem cells
STZ	streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCF-1	T cell factor 1
TcR	T cell receptor
TdT	Terminal deoxynucleotidyltransferase
Tfh	Follicular helper T cells
TGF-β	transforming growth factor β
Th	Helper T cell

Th-POK	T-helper-inducing POZ/Kruppel-like factor
TLRs	Toll-like receptors
TNF- α	tumour necrosis factor α
Tregs	Regulatory T cells
TSAs	Tissue specific self-antigens
TSPs	Thymic settling progenitors

Acknowledgements

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other University. All sources are acknowledged as references.

Introduction

1.1 Diabetes

Diabetes mellitus commonly referred to as diabetes, is a metabolic disorder characterized by chronic hyperglycemia (increased blood sugar levels). On the report of Diabetes UK, only in the UK there are 4.6 million people with diabetes including 1 million speculated undiagnosed patients due to their unawareness of their condition. In addition, they state that since 1996 the number of diabetic people has increased more than 2-fold at an alarming rate (www.diabetes.org.uk) Diabetes is a result of either lack of and/or resistance to the hormone insulin. There are a few types of diabetes the two main types being type 1 diabetes (T1D) and type 2 diabetes (T2D), and others such as gestational diabetes that develops during pregnancy and fewer rare ones (www.diabetes.org.uk). T1D is an autoimmune condition in which insulin-secreting β cells are destroyed by the body's own immune system resulting in diminished levels or lack of insulin (Devendra 2004). On the other hand, in T2D insulin resistance and insulin-producing cell dysfunction are the major pathophysiologic factors driving this condition (Taylor 2013). Nonetheless, both types result in hyperglycemia (www.diabetes.org.uk). Insulin is a hormone produced by the pancreatic β cells situated in the Islets of Langerhans and is vital for normal growth and development by maintaining normal homeostasis of glucose, fat and protein metabolism (Le Roith and Zick 2001). Insulin's main function is to inhibit excessive glucose being produced when blood glucose is high by impeding glycogenolysis (breakdown of glycogen stores), proteolysis (breakdown of proteins), lipolysis (breakdown of fats) and glucagon production, an 'anti-insulin' hormone (Sonksen and Sonksen 2000). Hyperglycemia is the cause of many complications seen in diabetic patients. As a result, monitoring and controlling blood glucose levels via appropriate nutrition and/or medication such as insulin administration, depending on the type and severity of diabetes in patients is crucial. Some common symptoms of diabetes are excessive urination, thirst and drinking alongside with short and long term effects. In the short term, excessive

or insufficient blood glucose can result in hyperglycemia and hypoglycemia respectively that leads to diabetic ketoacidoses in T1D or hyperosmolar hyperglycemic state in T2D patients if not treated (Daneman 2006, Longmore et al. 2014). In the long term, serious microvascular or macrovascular problems arise such as retinopathy, nephropathy, neuropathy, stroke, renovascular disease, limb ischaemia and enhanced risk of heart disease (Longmore et al. 2014). Therefore, the continuation of diabetes research aiming to find a cure is of great importance.

1.1.1 Type 1 Diabetes

This project is solely concerned with T1D that accounts for 5-10% of all diabetes cases. Nonetheless, T1D is a serious autoimmune condition where pancreatic insulin-producing β cells situated in the islets of Langerhans are destroyed by the host's immune system (Daneman 2006). Furthermore, T1D patients are required to monitor their blood glucose levels via regular finger stick blood testing followed by insulin injections due to insufficient or lack of insulin resulting from β cell destruction (Atkinson and Eisenbarth 2001, Oram et al. 2014). Ultimately, T1D impacts the quality of life of T1D patients and restricts their activities due to the short and long-term implications discussed above (Alvarado-Martel et al. 2015).

1.2 The immune system

1.2.1 Role

The immune system is a well-balanced, complex host defence mechanism against a variety of pathogenic microorganisms such as fungi, bacteria, protozoa and viruses that threaten normal host functions. Once these pathogens invade the host, they are recognised by the immune system and are subsequently eliminated via a variety of mechanisms before they cause damage to the host. The immune system is sub classified into two main systems known as the innate immune system and the adaptive immune system. The innate immune system is the initial, nonspecific host response whereas the adaptive immune system is the

following response expressed after the innate response of the host and is highly specific against unique pathogen antigens. Although each system has distinct pathways and cells involved, effective elimination of pathogens depends on the synergy between the innate and adaptive immune system via cell-cell interactions and crosstalk as well as cytokine and chemokine secretion (Parkin and Cohen 2001, Pancer and Cooper 2006, Chaplin 2010).

1.2.2 The innate and adaptive immune system

The innate immune system is identified as a rapid response, lacking specificity and is unable to form immunological memory of infection. There are several features composing the innate immune system's defense mechanisms. For instance, physical barriers such as the tight cell-cell contacts of the epithelial cell layers or the overlaid secreted mucus on epithelial respiratory, gastrointestinal and genitourinary tracts, prevent pathogen invasion. In addition, several soluble proteins that are either present in biological fluids or released by activated cells contribute in the capture and elimination of pathogens trying to invade the host (Chaplin 2010). However, in the case of pathogens invading successfully the host, innate immune cells such as natural killer (NK) cells, mast cells, eosinophils and phagocytes (macrophages, monocytes, dendritic cells (DCs) and neutrophils) take action (Parkin and Cohen 2001). In order for these cells to be activated they need to be able to recognise invading pathogens. Microbial pathogens express unique molecular structures intracellularly or extracellularly, that allow the innate immune system cells to distinguish between self and nonself. These unique molecular structures are known as pathogen-associated molecular patterns (PAMPs). The cells of the innate immune system have a variety of numerous cell membrane receptors such as C-type lectins , scavenger receptor type A (SR-A), Toll-like receptors (TLRs), serum amyloid protein (SAP), mannan-binding lectin (MBL), C-reactive protein (CRP) etc., expressed on their cell surface, intracellular compartments or secreted in the bloodstream, collectively known as pattern recognition receptors (PRRs) that are capable of recognising PAMPs (Janeway and Medzhitov 2002, Iwasaki and Medzhitov 2004). Once PAMPs are recognized by PRRs, inflammatory and immune responses are initiated due to a cascade of activated signalling pathways transmitted into the cells resulting in upregulation of costimulatory and MHC molecules, the induction of inflammatory cytokines, antimicrobial genes and the adaptive immune system as well as mediating

phagocytosis of pathogens. Furthermore, due to lack of the innate immune system specificity, sometimes host tissue and cells are damaged and the immune system is capable of removing damaged host cells in order to prevent inflammation and potential responses against self-molecular structures (Parkin and Cohen 2001). Damage-associated molecular patterns on damaged host cells act as endogenous danger signals in order for the innate immune system to recognise them through PRRs and remove them (Shin et al. 2015).

The adaptive immune system in contrast to the innate immune system, is a slower response with higher specificity against pathogens and is capable of forming immunological memory to infections (Chaplin 2010). Only higher animals have adaptive immunity as part of their immune system, in which immune responses are funnelled towards a specific pathogen (Parkin and Cohen 2001). The adaptive immune system is composed of T and B cell lymphocytes that have unique receptors against specific antigens on cells, yet each cell has its own specificity that might differ from another cell's, against a certain antigen. Collectively among the cells, there is an enormous receptor repertoire with different antigen specificity expressed by T and B cells that allows them to recognise a vast number of antigens. This is by virtue of rearranged variable, diversity and joining gene segments, also known as V, D and J, respectively (see below) (Xu and Selsing 1994, Pancer and Cooper 2006). In order for T and B cells to function they need to be activated first in order to drive targeted responses. Once T and B cells associate with their cognate antigen (an antigen specific to a certain receptor), leads to priming, activation and differentiation. The effector response occurs in which activated T cells migrate from the lymphoid tissue to the infected area and B cells differentiate into plasma cells which secrete antibodies (Abs) in the blood and tissue fluids homing to the infected area. However, despite the similar gene rearrangement process of T and B cell receptors, antigens are recognised differently. T cells receptors are able to bind to small linear peptides, whereas antibodies recognise the shape of epitopes (part of the antigen recognised by the immune system). Furthermore, there is a variety of functions of T and B cells depending on their subtype. In regards to T cells, T helper (Th) cells are responsible for orchestrating cells of the immune response as well as activating B cells whereas cytotoxic T cells are capable of killing directly cells that bear their cognate antigen. In regards to B cells, they

predominantly produce antibodies that are the secreted form of B cell receptor and are able to neutralise toxins produced by invading microorganisms, enhance phagocytosis by labelling bacteria (opsonization), prevent adhesion of organisms to mucosal surfaces and collectively acting to enhance features of the innate immune system (Parkin and Cohen 2001).

As mentioned previously, both T cell receptors (TcRs) and B cell receptors (BcRs) are antigen-specific resulting from a similar process of random rearrangement and splicing of several DNA segments encoding the antigen binding areas of the receptors. This results in the production of a huge receptor repertoire of over 10^8 TcRs and 10^{10}-10^{13} Ab BcRs, sufficient to respond to a range of pathogens encountered throughout life (Parkin and Cohen 2001, Pieper et al. 2013). In regards to their structure, T cells have two forms of receptors, each consisting heterodimeric chains. The most common one is the α and β chain receptor and the second one is the γ and δ chain receptor. Similarly, BcRs have two chains known as the heavy chain and light chain. These chains are composed of constant and variable regions mediating effector functions and form antigen recognition binding sites of the antibody, respectively (Pieper et al. 2013). Due to the random somatic gene rearrangement and joining known as V(D)J recombination, results in an enormous repertoire of receptors. The heavy chain encodes V, D and J segments whereas the light chain encodes only V and J segments. In essence, individual V,D and J segments are randomly selected and spliced to form VDJ or VJ gene sequences that will be transcribed to produce different receptors contributing to clonal diversity (Fugmann 2014). This process is catalyzed by recombinase-activating gene enzymes (RAG) (Chaplin 2010, Fugmann 2014). The VDJ segments are recognised by RAG enzymes via their recombination signal sequences (RSS) adjacent to the each segment. Once the RAG enzymes bind to RSS and juxtapose them bringing the chosen gene segments together to be recombined, the DNA is cleaved by RAG enzymes exactly at the junction of the gene segment and its RSS motif, creating a DNA hairpin in order to join the segments. Subsequently, nucleases cleave the DNA hairpins at the end of the segments allowing them to join via non homologous end joining DNA repair factors (Schatz and Ji 2011). In addition, an enzyme called terminal deoxynucleotidyltransferase (TdT) is recruited and adds random nucleotides to the end of the DNA strands contributing to the diversity of the

rearranged VDJ gene (Chaplin 2010). Collectively, these processes result in the formation of a vast number of possible receptors.

1.3 B cells

B cells, so-called because discovered in Bursa of birds (Cooper 2015), major function is the production of antibodies although they have another physiological role as APCs. As previously mentioned, antibodies are able to eliminate pathogens via a variety of mechanisms such as opsonization enhancing phagocytosis as well as neutralising toxins including other mechanisms that help protect the host, as part of the adaptive immune response, against different pathogens including bacteria and viruses. In addition, B cell development initially occurs in the bone marrow and subsequently complete their development in the spleen.

1.3.1 Development

Haematopoietic stem cells (hereafter called HSCs) are situated in the bone marrow and are the progenitors of all major blood cell types such as erythrocytes (red blood cells), myeloid cells (monocyte/macrophage and granulocytes), megakaryocytes/platelets, natural killer (NK) cells, dendritic cells (DCs), mast cells and both T and B lymphocytes (Seita and Weissman 2010, Morrison and Scadden 2014). There are two classes of HSCs; long-term and short-term HSCs (hereafter called LT-HSCs and ST-HSCs, respectively). The LT-HSC subset is highly self-renewed throughout the host's lifespan while the ST-HSC subset is able to self-renew roughly 8 weeks after differentiating from LT- HSCs (Morrison and Weissman 1994). The LT-HSCs are characterized by the expression of Thy-1.1^{lo}, lineage (Lin)$^-$, stem cells antigen-1 (Sca1)$^+$, stem cell growth factor receptor (c-Kit)$^+$, fetal liver kinase-2 (Flk-2)$^-$ where ST-HSCs express Thy-1^{lo}, macrophage 1 antigen (Mac1)^{lo}, Lin$^-$, Sca1$^+$, c-Kit$^+$ and Flk-2$^+$ cell markers, but are both devoid of fms-like tyrosine kinase 3 (Flt3) expression (Weissman 2000, Christensen 2001, Kumar et al. 2008). Collectively Lin$^-$Sca1$^+$c-Kit$^+$ expressing cells are also known as LSK cells (Kumar et al. 2008). Henceforth, each step of differentiation of these multipotent cells results in a functionally irreversible maturation. Once LT-HSCs differentiate to ST-HSCs, they can give rise to multipotent progenitors

(hereafter called MPPs) that lack the self-renewing capacity of HSCs, yet are able to differentiate into oligolineage progenitors giving rise to all types of blood cells due to MPPs' full-lineage differentiation potential (Seita and Weissman 2010). MPPS are also LSK cells and acquire the expression of Flt3. Throughout lymphoid commitment of HSCs, erythroid and megakaryocytic potential gradually decreases (Kumar et al. 2008). Following MPP development, the classical and currently prevailing haematopoietic commitment and blood lineage development, supports distinct myelopoiesis and lymphopoiesis originating from common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), respectively (Adolfsson et al. 2005). CMPs have the capacity to give rise to either myelomonocytic progenitors (GMPs) or megakaryotic/erythroid progenitors (MEPs) whereas CLPs can give rise to T lymphocytes, B lymphocytes or natural killer (NK) cells. In addition, CLPs are distinguished based on their phenotype of interleukin-7 receptor (IL-7 R)$^+$, Lin$^-$, Thy1.1$^-$, Sca-1^{lo}, c-Kit^{lo} and Flt3$^+$ (Weissman 2000). Through the use of Flt3 knock-out mice resulting in B and T cell progenitors as well as CLPs reduction in mice, the significance of Flt3 expression on CLPs was revealed, as Flt3 synergizes with IL-7 and stem cell factor (SCF) during the early stages of lymphopoiesis in order to promote lymphocyte proliferation (Borge et al. 1999, Adolfsson et al. 2005, Holmes et al. 2006). Therefore in essence, the initial lineage of multipotent cells is LT-HSCs → ST-HSCs → MPPs → CMPs/CLPs. Adding to the complexity of lineage commitment, further studies have revealed heterogeneity of the CLP population. For instance, MA. Inlay et al. using a new computational method of mining developmentally regulated genes (MiDReG), identified Ly6D expression of CLPs as a potential surface marker for B cell progenitors (Inlay et al. 2009). In their investigation they illustrated how CLPs are subdivided into two populations, the Ly6D$^-$ and Ly6D$^+$ subset. Examining the potential of each subset to produce T, B and NK cells, revealed the ability of Ly6D$^-$ CLPs to keep their full lymphoid potential and give rise to all three lymphocyte types hence called all-lymphoid progenitors (ALPs). Whereas Ly6D$^+$ CLPs produced mostly B cells, hence called B cell-biased lymphoid progenitors (BLPs) (Inlay et al. 2009). Subsequently, R. Mansson et al. using λ5 (a gene of B cells) reported transgenic mice crossed with RAG-1-GFP knock in mice in order to track λ5 and RAG-1 co-expression, identified three phenotypically distinct CLP subsets; λ5$^-$Rag1^{low}, λ5$^-$Rag1^{high}, and λ5$^+$Rag1^{high} cells (Mansson et al. 2010). Clonal in vitro differentiation showed that

$\lambda5^-$Rag1^{low} cells were capable of producing T, B and NK cells alike, $\lambda5^-$Rag1^{high} cells could give rise to T and B cells but not NK cells whereas $\lambda5^+$Rag1^{high} cells were restricted to B cell lineage generation (Mansson et al. 2010).On the other hand, more recently Q. Zhang et al. sought to observe three well established differentiation milestones in B cell commitment, Ftl3, RAG-1 and Ly6D in order to chart the main differentiation pathways (Zhang et al. 2013). Based on their findings in agreement with previous studies (Inlay et al. 2009, Tsapogas et al. 2011), restricted B cell lineage potential was highly correlated with RAG-1 and Ly6D expression on CLPs. In addition, these CLP subpopulations expressed high levels of B cell gene transcripts such as early B cell factor 1 (Ebf1), RAG-1 and paired box gene 5 (Pax5), suggesting CLP commitment towards the B lymphocyte lineage (Zhang et al. 2013).

Collectively, it seems that within the CLP stage the hierarchy is not as clear-cut as once thought to be and their fate depends on which lineage their subpopulations leans to. Therefore, combining these evidence together it is plausible that B cell restricted progenitor could be distinguished based on Sca-1^{lo}, c-Kit^{lo}, Flt3$^+$, IL-7R$^+$, Ly6D$^+$ and RAG-1 expression together with high levels of B cell gene transcripts.

Immediately following the CLP stage, cluster of differentiation 45R (CD45R) isoform B220 expression distinguishes cells restricted to B cell lineage, prior to CD19, a molecule expressed on all later B cell lineage stages (Hardy and Hayakawa 2001). In addition, evidence of a CLP subpopulation characterised as Lin$^-$, B220$^-$, CD19$^-$, CD127$^+$, Flt3$^+$, Sca-1^{lo} and c-Kit^{lo} was able to express B cell lineage-associated genes, suggesting that B cell lineage commitment occurs prior to B220 and CD19 expression that were initially thought to be the hallmark of B cell lineage commitment (Mansson et al. 2008). Nonetheless, followed by the B cell lineage commitment and the expression of both B220 and CD19, development of B cells within the bone marrow progresses through a series of major stages known as pro B cell, pre B cell and immature B cell (Li et al. 1996). Each stage has its own distinct phenotypic characterisation based on B220, CD19, leukosialin (CD43), heat stable antigen CD24 (HSA) and immunoglobulin M (IgM) expression amongst others. For instance, together with B220 and CD19 expression throughout these stages, pro B cells are characterised as

CD43$^+$HSA$^+$IgM$^-$, pre B cells as CD43$^-$HSA$^+$IgM$^-$ and immature B cells as CD43$^-$HSA$^+$IgM$^+$ (Hardy et al. 1991, Li et al. 1996, Loder et al. 1999, Carsetti 2000, Hardy and Hayakawa 2001). Once immature B cells are formed, they migrate to the spleen via the bone marrow sinusoids (a type of small blood vessel) in order to complete their maturation process. Recent bone marrow B cell immigrants in the spleen are known as T1 B cells and B cells found exclusively in the primary follicles of the spleen are known as T2B cells (Loder et al. 1999).

Collectively, studies have shown that B cell development processes seem to be governed by the expression of certain genes and transcription factors in addition to the presence of cytokines contributing to their developmental process (Li et al. 1996, Hardy and Hayakawa 2001, Tokoyoda et al. 2004). Throughout the bone marrow, a small amount of stromal cells express CXC chemokine ligand 12 (CXCL12) that is vital during B cell development as it maintains them spatially within certain niches in the bone marrow during their development progression (Tokoyoda et al. 2004). In regards to gene expression, inactivation of the E2A gene that is vital in Ig gene rearrangement during the pro and pre B cell stage, was demonstrated to halt the early B cell developmental stages where B220 expression was being upregulated (Zhuang et al. 1996, Bain et al. 1997). On the other hand, although mice lacking EBF had more B lineage cells at the B220 stage, they failed to progress to subsequent stages possible due to the failure of Ig rearrangement (Lin and Grosschedl 1995). Another important transcription factor for the generation of both myeloid and lymphoid lineages is PU.1 that regulates the differentiation and the cytokine-dependent proliferation of precursor cells (DeKoter et al. 1998). In addition, differing concentrations of PU.1, low or high, determine whether the cells will develop into either B cell or macrophage, respectively (DeKoter and Singh 2000). Low levels of PU.1 allow lymphoid commitment due to upregulation of IL-7Rα, EBF and Pax5 transcription factor expression, which are vital in the process of B cell lineage commitment and ultimately their development (DeKoter et al. 2002, Hagman and Lukin 2006). In contrast to PU.1 which acts prior to CLPs on progenitors enhancing CLP differentiation, E2A, EBF1 and Pax5 factors act downstream the CLP stage in order to promote B cell lineage progression. E2A transcription factor was implicated in early B cell lineage development due to the activity of its binding function once B lymphopoiesis is initiated. In addition, E2A consists of two basic-

helix-loop-helix (HLH) proteins known as E12 and E46 that have been shown to be equally important in B cell development as demonstrated using mice lacking either E12 or E47 (Bain et al. 1997, Hagman and Lukin 2006). At the beginning of B lymphopoiesis, alongside with E2A activity is EBF1, and both of them coordinately activate the expression of B cell genes crucial for Ig heavy chain gene rearrangements (Busslinger 2004). In addition, factor forkhead box protein 1 (Foxo1) seems to be part of the transcription factors promoting commitment to B cell lineage, by activating the expression of RAG-1 and RAG-2 (Amin and Schlissel 2008, Lin et al. 2010). Furthermore, EBF1 initiates the expression of Pax5 transcription factor that is also important in B cell lineage restriction while being regulated by PU.1 (Lin et al. 2010). In essence, Pax5 represses the expression of non-B cell lineage genes such as Notch1, myeloperoxidase (MPO) and M-CSFR, while activating B cell lineage specific genes such as BLNK, Igα and CD19 (Busslinger 2004). In addition, using a retroviral vector in order to force Pax5 expression in HSCs resulted in blockade of T cell lineage development but not NK development, in favor to B cell lineage fate (Cotta 2003). Later on, mice lacking Pax5 in mature B cells due to conditional deletion resulted in the formation of functional T cells with Ig heavy and light chain rearrangements suggesting dedifferentiation of mature peripheral B cells into multipotent progenitors (Cobaleda et al. 2007). Therefore Pax5 seems to be a crucial transcription factor more for the progression rather than commitment that was believed previously, of B cell lineage (Welinder et al. 2011). Nonetheless, transcription factors and activation of B cell lineage specific genes, allow the progression of B cell progenitors to subsequent developmental stages.

1.3.2 B cell receptor

BcR rearrangement of the IgM heavy chain is an essential step initiated at the pro B cell stage in order to progress to the pre B cell stage (Raff et al. 1976, Grawunder et al. 1995). Followed by IgM heavy chain rearrangement, a surrogate light chain (composed of the λ5 and VpreB proteins) that is structurally similar to a light chain, is coupled with the heavy chain to form a pre-BCR. Prior to the light chain rearrangement of pre-B cells, surrogate light chain genes are silenced which terminates the expression of pre-BcR (Burrows et al. 2002). As mentioned previously, pathogen recognition by B cells is possible due to their highly specific receptor and numerous complex mechanisms also result in highly

diverse BcR repertoire capable of recognizing a vast amount of pathogens (Xu and Selsing 1994, Pancer and Cooper 2006). The BcR is a transmembrane protein and similarly to an antibody's structure, it is composed of two parts, two heavy chains and two light chains. Both the heavy and light chains consist of constant and variable regions in which the variable regions of the chains are positioned next to each other. The variable regions of the chains combined form the antigen binding site. Once an antigen binds on the BcR, a cascade of signalling pathways results in the differentiation of B cells into plasma cells. Subsequently, antibodies are secreted by plasma cells into the bloodstream in order to eliminate pathogens (Parkin and Cohen 2001, Pieper et al. 2013). There is a variety of antibody isotypes such as IgA, IgD, IgE, IgG and IgM due to different types of heavy chains: α, δ, ε, γ, μ, respectively (Janeway 2005). The function and niche of each antibody isotype differs among them. For instance IgA is vital for mucosal immunity (Underdown and Schiff 1986) and IgE is involved in allergic responses (Pier et al. 2004). Nonetheless, V (D) J recombination in order to form a vastly diverse receptor repertoire results in the formation of autoreactive B cells as well, targeting self-antigens (Goodnow 1992). Therefore, self-tolerance mechanisms evolved in order to bypass the actions of these self-reactive cells and ultimately prevent autoimmune diseases. These censoring mechanisms include clonal deletion, receptor editing, receptor dilution and clonal anergy (Hartley et al. 1993, Cornall et al. 1995, Goodnow et al. 2009, Manjarrez-Orduño et al. 2009). Regarding B cell clonal deletion, when the IgM of immature B cells situated in the bone marrow binds and responds to self-antigens, results in downregulation of IgM expression and maturation arrest leading to premature autoreactive B cell elimination (Hartley et al. 1993, Cornall et al. 1995). However, autoreactive B cells get another chance to escape elimination by rearranging their autoreactive light chain to a non-autoreactive one (Cornall et al. 1995). Clonal anergy of autoreactive B cells in essence implies the inability of B cells to respond to their antigen due to chronic stimulation making them functionally inactive (Cornall et al. 1995, Hardy and Hayakawa 2001, Manjarrez-Orduño et al. 2009). In addition, higher affinity interactions to self-antigens by IgM seem to result in deletion and lower-affinity interactions result in either receptor editing or clonal anergy (Hardy and Hayakawa 2001). Receptor editing of autoreactive B cells can only occur within the bone marrow whereas the rest of self-tolerance mechanisms can occur in the periphery as well (Cambier et al. 2007).

1.4 T cells

1.4.1 Development

The majority of hematopoietic lineages develop in the bone marrow however, T cells complete their development in the thymus, hence called T cells. There is a constant input of T cell progenitors migrating from the bone marrow via the bloodstream to the thymus. This is necessary as thymocytes are not self-renewing in contrast to the bone marrow hematopoietic stem cells (Heinzel et al. 2007). However, the exact identity of the cell type that migrates from the bone marrow, settles in the thymus and acts as a progenitor of T cells is still undisclosed (Zlotoff and Bhandoola 2011).

As mentioned in Section 1.3, all blood cells including lymphocytes originate from the LSK (Lin^- Sca^+ c-Kit^+) HSCs situated in the bone marrow (Kumar et al. 2008). Subsequently, LSK HSCs differentiate into the non self-renewing MPPs that can give rise to all types of blood cells (Seita and Weissman 2010). Thereafter MPP differentiation, a more recent alternative and more complex model of HSC lineage commitment, supports the formation of lymphoid-primed multipotent progenitors (LMPPs) prior to CLP generation (Adolfsson et al. 2005, Drissen et al. 2016). LMPPs are phenotypically characterised as Lin^- Sca^+ c-Kit^+ $Flt3^+$ whereas CLPs are characterised as Lin^- $Thy1.1^-$ Sca-1^{lo} c-Kit^{lo} $Flt3^+$ and IL-$7R\alpha^+$ (Weissman 2000). LMPPs can give rise to lymphocytes, NK cells, macrophages and granulocytes whereas CLPs are capable of only producing T cells, B cells and NK cells (Weissman 2000, Adolfsson et al. 2005, Drissen et al. 2016).

In an effort to search for thymic settling progenitors (TSPs), Flt3 was found to be expressed in all TSPs in addition to chemokine receptor (CCR) 7 and chemokine receptor 9 that seem to be important for efficient thymic settling (Adolfsson et al. 2005, Zlotoff et al. 2010). Both chemokine receptors are required for an effective thymic settling as using CCR7 and CCR9 double knockout mice resulted in severely reduced levels of thymic settling (Zlotoff et al. 2010). Therefore, CCR7 and CCR9 expressing LMPPs or MPPs, could be potential TSPs. After thymic entry by TSPs, development of early thymic progenitors (ETPs) is followed by a well-defined intrathymic T cell development hierarchy known as the double

negative (DN) phase that is subdivided in four stages (DN1-4) where neither CD4 nor CD8 molecule is expressed. Henceforth, the spatial location of T cells through distinct thymic microenvironments is crucial for the perception of differentiating signals and ultimately their successful development progression. TcR rearrangement initiates during the DN phase and in particular in DN3, by the mechanisms described previously (see section 1.2) (Starr et al. 2003). Ensuing TcR rearrangement and other T cell developmental checkpoints, leads to the co-expression of CD4 and CD8 molecules, a phase known as double positive (DP). During the DP phase, T cells are subjected to another developmental checkpoint known as positive selection in which thymic cortical epithelial cells (cTECs) expressing major histocompatibility complex (MHC) class I and II, play a major role in T cell fate. At this point, TcRs that are able to recognise either MHC-class I or MHC-class II at a moderate affinity, will receive survival and maturation signals allowing them to progress with their development. Depending on the strength of interaction between MHC-class and TcR was able to associate with, will also direct the type of T cells they become such as CD4$^+$ or CD8$^+$. For instance during positive selection, TcR that associates MHC-class I or MHC-class II will develop into CD8 (CD8$^+$) or CD4 (CD4$^+$) single positive (SP) T cells, respectively. However, TcRs that are unable to recognise either MHC-class or bind too avidly, fail to receive survival signals or receive a death signal, respectively and die by apoptosis (Starr et al. 2003, Zlotoff et al. 2010). Collectively, positive selection allows immature DP T cells that express TcRs with intermediate affinity towards MHC complexes to differentiate into mature SP thymocytes (Starr et al. 2003, Klein et al. 2014). Succeeding positive selection, T cells migrate from the thymic cortex to the thymic medulla where negative selection of SP T cells is initiated by dendritic cells and thymic medullary epithelial cells (mTECs) (Brocker 1997). Autoimmune regulator (AIRE) gene is critical in negative selection as it is capable of expressing tissue specific self-antigens (TSAs) that are displayed by mTECs (Anderson and Su 2011). mTECs can then present TSAs alongside MHC molecules to T cells in order to check if they would react against the host's antigens and depending on the affinity between the TcR and the TSA will determine their fate in development (Nossal 1994, Anderson and Su 2011). Developing T cells that react to TSA-MHC complex with high affinity are then eliminated by receiving death signals in order to prevent their maturation and migration in the periphery. In the case of these self-reactive T cells escaping to

the periphery, could result in damage of the host's self-tissue as they cannot distinguish between self and nonself antigens. This aberrant response is known as autoimmunity (Nossal 1994, Starr et al. 2003). Collectively, although negative selection is crucial for the release of non autoreactive T cells in the periphery, it also contributes as a first line of defence preventing autoimmunity via T cell tolerance (see section 1.3) (Walker and Abbas 2002).

Similarly to B cell development (section 1.3), expression of certain genes and transcription factors, is critical for T cell development as they navigate from progenitors into lineage commitment and progression in subsequent development. As previously mentioned, followed by TSPs entering the thymus, comes the development of ETPs. However, in order for TSPs to progress into the ETP stage, a number of vital interconnected transcription factors are required such as T cell factor (TCF-1), GATA-binding protein 3 (Gata3) and Notch homolog 1 (Notch1) including many others. Deficiencies in any of these transcription factors have no effect on ETP progenitors such as bone marrow and blood LSK progenitors however, results in a decrease of the ETP population (Busslinger 2004, Sambandam et al. 2005, Hosoya et al. 2009, Zlotoff et al. 2010, Weber et al. 2011). For instance, Notch signalling is crucial for imposing T cell identity and maturation initiating from the bone marrow continuing to the thymus, as events involved in thymic precursors progression, are driven by this pathway and prevents apoptosis of developing T cells (Deftos et al. 1998, Busslinger 2004, Yui and Rothenberg 2014). In addition, it seems that some of the T cell progenitors at early stages of the DN phase, may still exhibit B cell lineage capacity (Porritt et al. 2004). However, T cell development is promoted due to the expression of Notch1 in the bone marrow as it was observed to suppress B cell lineage commitment by suppressing transcription factors that are essential in B cell development, such as Pax5. Furthermore, constitutive expression of Notch1 results in a halt of B cell lymphopoiesis and ectopic T cell development of immature $CD4^+CD8^+$ T cells in the bone marrow whereas inactivation of Notch1 results in deficient T cell fate and development as well as ectopic development of B cells in the thymus (Radtke et al. 1999, Pui et al. 1999, Han et al. 2002). Collectively, these observations illustrated the critical role of Notch1 expression for T cell development in addition to the importance of a balanced Notch1 expression for normal lymphocyte development. Additionally,

Notch signals upregulate the expression of TCF-1 that is also vital in T cell commitment by assisting in the expression of T-lineage genes, including GATA3 and Bcl11b transcription factors. GATA3 is required for the generation of ETPs and subsequently control directly or indirectly the development of T cells (Ting et al. 1996, Hosoya et al. 2009). In addition, Bcl11b deletion was shown to result in either diversion to NK cell lineage development or halt of T cell development, illustrating Bcl11b's crucial role in maintaining T cell lineage commitment (Weber et al. 2011). Furthermore, as previously discussed, E12/E46-composed E2A protein is not only important in B cell lymphopoiesis (see section 1.3), but also in T cell lymphopoiesis and development as well (Dias et al. 2008). E2A protein was illustrated to be needed for proper LMPP development as well as assisting the transition from the DN phase to the DP phase (Bain et al. 1997, Dias et al. 2008). Another essential transcription factor for T cell development and differentiation, is the runt-related transcription factor known as RUNX. RUNX was illustrated to contribute in the initial progenitors response towards T cell lineage commitment as well as transition from DN2 to DN3 phase (Rothenberg et al. 2008). Moreover, the synergy between RUNX, GATA3 and Th-POK (T-helper-inducing POZ/Kruppel-like factor) is vital for T cell development as they assist in determining the CD4/CD8 lineage commitment (Collins et al. 2009). Altogether, T cell development depends on a vast number of transcription factors working together in order to allow complex T cell specific development processes to take place.

1.4.2 T cell receptor development

The production of self-tolerant yet versatile TcRs relies on accurate coordination of the antigen-receptor recombination process, differentiation as well as selection processes taking place during development in the thymus. As previously mentioned, TcR development initiates at the early stages of T cell development during the DN phase (Ebert et al. 2010). The TcR is composed of two disulphide-linked heterodimeric membrane proteins, TCRα and TCRβ chains or TCRγ and TCRδ chains (Nemazee 2006). T cells expressing TCRα and TCRβ are referred to as αβ T cells and T cells expressing TCRγ and TCRδ are referred to as γδ T cells with αβ T cells being the most common ones found in circulation (Pieper et al. 2013). Furthermore, TcR construction is crucial for T cell development as it allows T cells to progress through development checkpoints in the thymus. For

instance, following TCRβ rearrangement, during the DN phase thymocytes with a functional TCRβ chain can then express a pre-TCR that is a complex composed of CD3 molecules, a pre-TCRα chain that is invariant and an incipient TCRβ chain that is critical for early T cell development (Yamasaki and Saito 2007). Subsequently to the generation of pre-TCR, DN cells are then allowed to transition from the DN phase to the DP phase and this process is known as β-selection. Using pre-TCRα or TCRβ KO mice, suggested a key role of pre-TcR as a molecular sensor for subsequent T cell development by triggering survival, proliferation and expression of either CD4 or CD8 as well as TCRα rearrangement (Mombaerts et al. 1992, Yamasaki and Saito 2007). After β-selection, during the DP phase TCRα rearrangement occurs that results in the generation of a mature αβTcR (Jones and Zhuang 2007). DP cells will then undergo positive selection as mentioned previously that will filter out DP cells with non functional TcRs and enable DP cells with functional TcRs to become either CD4$^+$ or CD8$^+$ SP cells. Finally, T cells expressing a functional TcR will undergo negative selection in order to eliminate autoreactive cells prior to migration in the periphery (Starr et al. 2003, Jones and Zhuang 2007).

1.4.3 Subsets of T cells

Following T cell commitment into either CD4$^+$ or CD8$^+$ T cells, depending on the expression of either CD4 or CD8 glycoprotein surface molecule, respectively, comes the sub-classification of T cells into different types such as Th cells, cytotoxic T cells, regulatory T cells (Tregs) and memory T cells, each exhibiting different functions (Golubovskaya and Wu 2016). For instance, Th cells can differentiate further into different subsets such as Th1, Th2, Th3, Th9, Th17, Th22, follicular helper T cells (Tfh) as well as Tregs. Each subset can be distinguished based on their cytokine profile and have a different effect on the adaptive immune response. Th cells are vital to the immune system especially adaptive immunity as they assist in B cell activation and ultimately antibody secretion, as well as in elimination of targeted infected cells by assisting in the activation of cytotoxic T cells. Therefore, in essence Th cells assist other immune cells to do their function via cytokine secretion. However, prior to this, Th cells need to be activated by APCs displaying peptide antigens by their MHC-class II surface molecules for Th cells to respond to via their TcR and CD4 surface molecule acting as a coreceptor to stabilize this interaction by binding to MHC-

class II (Alberts et al. 2002, Golubovskaya and Wu 2016). In regards to cytotoxic T cells (CTLs), characterised by the expression of CD8, monitor all the body's cells and can either directly or indirectly destroy infected cells i.e. by a virus, by recognising antigens presented by MHC-class I molecules, in order to prevent viral spread to other cells and protect the host's integrity. There are three distinct pathways CTLs can eliminate targeted cells, one involving cytokine secretion such as interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α), and the other two involve direct cell-cell contact between CTLs and target cells which ultimately result in apoptosis of targeted cells. However, sometimes CTLs can inflict cellular damage towards a host's organ due to the recognition of a self-antigen unique to that organ as non-self that initiates an immune response against it. This can ultimately result in an autoimmune disease, such as T1D (see section 1.7) (Andersen et al. 2006). Another subtype of T cells are Tregs. Tregs are vital in balancing immune responses of the immune system so that there is minimal self-tissue damage. In addition, Tregs are required for maintaining peripheral tolerance and ultimately preventing autoimmune diseases such as T1D and limit chronic inflammatory diseases (see section 1.7) (Vignali et al. 2008). Suppression of harmful immune responses are speculated to occur either via the production of inhibitory cytokines such as IL-10 and transforming growth factor β (TGF-β) (Walker and Abbas 2002) or cytotoxic T lymphocyte associated protein 4 (CTLA-4) (McCoy and Le Gros 1999). Tregs are thought to also suppress effector T cells (Th and CTLs alike) by inducing cell death either via cytokine deprivation by 'consuming' local IL-2 that is vital for other T cells survival or cytolysis (Vignali et al. 2008, Shalev et al. 2011). Additionally, Tregs are thought to regulate either the maturation and/or function of DCs that are required for effector T cells activation (Vignali et al. 2008). However, there are many biological questions regarding Treg function and even their development that will be discussed in more detail later on.

1.4.4 Activation of T cells

Antigen presentation is a crucial process by which cells of the innate immune system communicate with the adaptive immune system. This way the adaptive immune system is alerted and able to mount an appropriate response against invading pathogens. Professional APCs such as DCs and additional cell lines with antigen-presenting capabilities such as B cells and macrophages, search for

invading pathogens that once encountered and recognised via APCs' PRRs (see section 1.2), are then internalised (Kapsenberg 2003, Mogensen 2009). Subsequently, pathogens are broken down internally by APCs and the pathogen-associated antigens are displayed by either MHC-class I or II that will activate CD8^{+} or CD4^{+} T cells, respectively (Underhill et al. 1999, Kapsenberg 2003). In addition to T cells recognising their cognate antigen displayed by the peptide/MHC complex situated on APCs, there are two more signals required in order for T cells to become activated. Therefore, three distinct signals are needed in total for the activation of naive T cells (Corthay 2006). Signal one involves the antigen-specific recognition of peptides by TcRs, that are presented by MHCs on APCs. Signal two, is provided by the binding of co-stimulatory molecules CD80 and CD86 found on APCs, and CD28 molecule found on T cells. In addition, costimulatory molecules of APCs are only expressed when activated during an inflammatory response which ensures T cell activation only when necessary. Lastly, signal three relies on released cytokines by APCs that bind on their cognate receptor on T cells and direct T cell differentiation in different subtypes. For instance, T cell differentiation into various effector phenotypes such as Th1 or Th2 including others, is determined by the signals provided by APCs and the most appropriate immune response required that is dictated by the innate immune response via APCs (Scott 1993, Corthay 2006). However, in contrast to CD4^{+} T cells that recognise and respond to MHC-class II, CD8^{+} T cells will respond to MHC-class I in a process known as cross-presentation in which for instance, exogenous antigen taken up by an APC is then cross-presented on MHC-class I to activate CD8^{+} T cells (Rock and Shen 2005). Generally, endocytosed or phagocytosed antigens originating from outside the cell also known as exogenous antigens, are processed and subsequently displayed by MHC-class II, whereas processed antigens originating from within virus-infected or abnormal/cancerous cells also known as endogenous antigens, are expressed on MHC-class I (Wieczorek et al. 2017). Additionally, MHC-class I complexes are presented on all nucleated cells which allows them to alert activated CD8^{+} cytotoxic T cells in the case of viral infection or transformed in an offending state such as cancer (Rock and Shen 2005). Therefore, following recognition of MHC-class I by CD8^{+} T cells in combination with binding to costimulatory molecules mentioned previously present on APCs, naive CD8^{+} T cells are activated and differentiate into effector CTLs (Kaech and Ahmed 2001). CTLs are then capable

of eliminating target cells expressing their cognate antigen displayed on MHC-class I complex via highly specific mechanisms such as TNF-α and IFN-γ cytokine secretion that ultimately result in target cell apoptosis, cytolytic activity leading to cell death either by caspase cascade or CTL release of cytotoxic perforins and granzymes (Andersen et al. 2006).

1.4.5 T cell tolerance

As in BcR diversity of B lymphocytes (see section 1.3), similarly T cells possess highly diverse TcRs due to somatic gene rearrangement that can also potentially result in the formation of autoreactive TcR clones against host self-antigens. Therefore, in order to avert the generation, spread and action of these unwanted autoreactive T cells, a variety of self-tolerance mechanisms evolved (Abbas et al. 2004). As mentioned previously, negative selection is a crucial educational step for thymocytes in order to prevent autoreactive T cells escaping to the periphery. However, this process is not 100% effective due to different factors such as the inability to express all self-antigens in the thymus, therefore lacking T cell tolerance towards those missing self-antigens. In addition, negative selection is not as stringent in deleting all developing thymocytes with autoreactive receptors, therefore some thymocytes with a degree of autoreactivity escape to the periphery and are left to be dealt with by other backup self-tolerance mechanisms. If negative selection was stricter with a lower binding affinity threshold between TcR and MHC/peptide complexes, would result in a narrower repertoire and ultimately reduce their ability to fight against pathogens. Therefore, peripheral tolerance mechanisms evolved in order to maintain self-reactive cells that have escaped central deletion (Walker and Abbas 2002). Such mechanisms include deletion by apoptosis, induction of functional anergy leading to unresponsiveness towards antigens or suppressive action by Tregs (Abbas et al. 2004). Failure of self-tolerance mechanisms would potentially result in the accumulation of uncontrolled autoreactive lymphocytes, therefore is thought to be correlated with autoimmune diseases.

1.5 Regulatory T cells

The existence of a T cell subpopulation (now known as Tregs) with immunosuppressive abilities, was uncovered by the seminal observation in 1969 by Y. Nishizuka and T. Sakakura. A severe organ-specific autoimmune disease was documented followed by neonatal thymectomy (removal of the thymus), yet was prevented by CD4$^+$ T cell transfer from wild type healthy mice (Nishizuka and Sakakura 1969). This documentation elucidated the thymic origin of Tregs capable of preventing autoimmunity by suppressing immune responses.

1.5.1 Development

Up until the TcR gene rearrangement phase, T cells and Tregs seem to exhibit the same developmental pathways (See section 1.4). During TcR gene rearrangement of developing T cells results in the generation of TcRs capable of recognising self-antigens at different intensities and timescales. T cells that recognise self-antigens with either really high affinity or really low affinity are eliminated via apoptosis or death by neglect, respectively. However, the most recognised model in regards to Treg development suggests that Tregs originate from developing T cells stimulated via their TcR at relatively high affinity against self-antigens, yet below the threshold needed to promote apoptosis (Ohkura et al. 2013). This stimulation seems to activate the expression of the forkhead box P3 (Foxp3) transcription factor by CD4$^+$ SP T cells, that is essential in Treg development and function (Hori 2003, Fontenot et al. 2003, Ohkura et al. 2013). On the other hand, others have suggested an alternative model in which Treg development is significantly determined by unknown trans-acting factors acting on DN cells prior to the expression of TcR (Pennington et al. 2006). Nonetheless, the mechanisms how TcRs are then biased towards Treg lineage is unclear therefore the previous model is more accepted due to more supporting evidence. In an effort to elucidate Treg development, investigations have identified CD4$^+$ CD25^{hi} Foxp3$^-$ T cells as Treg precursors as this subset was observed to be highly enriched with cells that would subsequently express Foxp3 and following TcR selection would be termed as mature Foxp3$^+$ Tregs (Fontenot et al. 2005, Maynard et al. 2007, Lio and Hsieh 2008).

Although engagement with the TcR is essential for thymic Treg development, it is not sufficient and therefore additional signalling pathways are needed. An example is the epigenome that seems to play a key role in Treg development. For instance, TcR stimulation at a certain period of time assembles a Treg-cell-type DNA hypomethylation pattern resulting in accessibility and enhancing transcription of certain genes associated with Treg-specific molecules or Treg function such as Foxp3, Eos and CTLA-4 (Ohkura et al. 2012). Therefore, collectively with the epigenomic events and Foxp3 expression, developing T cells are driven towards a stable Treg lineage. Interestingly, Foxp3 expressing T cells without the Treg-specific epigenome results in the generation of unstable Tregs whereas T cells with Treg-specific epigenome yet lacking initial Foxp3 expression, can still express Foxp3 and differentiate into functional Tregs (Ohkura et al. 2013). Furthermore, cytokines such as IL-2, IL-7 and IL-15 seem to play a key role in thymic Treg development since mice with either deficient IL-2, IL-7 or IL-15 receptors, demonstrated a significant decrease in thymic Treg numbers (Bayer et al. 2007, Vang et al. 2008, Apert et al. 2017). IL-2 is the most vital cytokine for Treg development as Tregs classically express CD25 which is the high affinity subunit of the IL-2 receptor for IL-2 and is required for Foxp3 expression allowing generation of functional Tregs (Stephens and Mason 2000, Burchill et al. 2007). Additionally, there are signalling pathways downstream of the TcR and cytokine receptors involved in Treg development such as IL-2, that have also been suggested to have a role in Treg development such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Janus Kinase/Signal Transducer and Activator of Transcription (JAK-STAT) pathways, respectively (Hayden and Ghosh 2011, Gilmore et al. 2016; Mahmud et al. 2013). For instance, the NF-κB family of transcription factors are a key regulator of genes expressed that are essential for the development and function of the immune system (Gilmore et al. 2016). TcR signalling can activate various pathways and one of them is the NF-κB signalling pathway that has been suggested to be involved in Treg development (Feuerer et al. 2009). NF-κB seems to be the pioneer transcription factor of Foxp3 expression as it enhances the accessibility of the Foxp3 locus to the general transcription machinery (Long et al. 2009). As a result differentiation of thymocytes into Foxp3 expressing Tregs depends on NF-κB signalling pathway. In regards to signalling pathways downstream of cytokine receptors involved in Treg development, the JAK-STAT

pathway seems to be important. The JAK-STAT pathway is one of the many pleiotropic signalling cascades essential for development and homeostasis in animals (Eyles and Hilton 2003). Regarding Treg development, IL-2 signalling activates JAKs which then phosphorylate STATs in particular STAT5a/b. Using STAT5a/b knockout mice and tissue-specific Stat5 deletion, STAT5a/b was illustrated to be important for both the development and maintenance of Tregs as well as Foxp3 expression (Yao et al. 2007). In addition, STAT3 was found to regulate Foxp3 by attenuating its expression via the mediation of IL-6 signals (Kasprzycka et al. 2006). Alongside these signalling pathways, CD28-CD80/CD86 and CD40-CD154 pathways seemed to play a role in Treg development (Salomon et al. 2000, Tai et al. 2005, Spence and Green 2008). Deletion of genes encoding CD28 or its ligands CD80 resulted in significant reductions of thymic and peripheral Tregs elucidating their importance in Treg development (Salomon et al. 2000, Tang et al. 2003). Furthermore, CD28 signals were linked with earlier stages of Treg development at the $CD25^+Foxp3^-$ precursor stage prior to TcR signalling and is also suggested to protect developing Tregs from negative selection (Lio et al. 2010, Klein and Jovanovic 2011). On the other hand, although deficiency in CD40 or CD154 resulted in roughly 50% reduction of thymic and peripheral Tregs (Guiducci et al. 2005), CD40-CD154 signalling pathway did not seem to be required for precursor Treg development, but instead having an effect on later stages in Treg development (Cuss and Green 2012). Nonetheless, the documented peripheral Treg reduction is not clear whether it is due to decrease in thymic output or decrease in peripheral Treg homeostasis (Guiducci et al. 2005, Spence and Green 2008). Ultimately, once Tregs are mature and functional they can either migrate from the thymus to the periphery in order to maintain immune responses or may be retained in the thymus (Cuss and Green 2012).

As mentioned previously briefly, additionally to the development of naturally derived thymic Tregs (nTregs or tTregs) described above, Tregs can also be induced in the periphery from naive conventional T cells and are known as iTregs or adaptive Tregs (Bluestone and Abbas 2003, Klein and Jovanovic 2011) (Ohkura et al. 2013). TGF-β was found to be involved in many aspects of T cells such as their development, homeostasis and function (Gorelik and Flavell 2000, Li and Flavell 2008). In addition, several studies have illustrated the importance

of TGF-β molecule in causing Foxp3 upregulation in peripheral naive T cells resulting in the generation of functional iTregs (Chen and Konkel 2015). T cell-specific deletion of TGF-β suggested that TGF-β was critical for the homeostasis and maintenance of Foxp3 of peripheral Tregs as there were no detrimental effects upon thymic Treg development (Li et al. 2006, Marie et al. 2006). In regards to the mechanisms underlying the activation of Foxp3 by TGF-β, seems to involve Smad3 and NFAT transcription factors downstream TGF-β and the TcR, respectively, are thought to co-operatively bind directly to conserved non coding sequence 1 (CNS1) that is an enhancer element in the Foxp3 gene, and activate it (Tone et al. 2008). However, TGF-β is also involved in the differentiation of naive T cells into Th17 cells (Mangan et al. 2006), suggesting the involvement of other signals such as retinoic acid that inhibits Th17 development (Xiao et al. 2008). Collectively, peripheral Tregs are comprised of two distinct developmental origins, mature nTregs that developed in the thymus and subsequently migrated in the periphery, or iTregs generated from peripheral naive CD4$^+$ T cells. Nonetheless, identifying markers to distinguish among nTregs and iTregs was challenging as they are both phenotypically very similar (Lin et al. 2013). Although both nTreg and iTregs express CD25, Foxp3, GITR and CTLA-4, nTregs seem to have higher expression of neuropilin 1 (Nrp1), Helios (Ikzf2), programmed cell death-1(PD-1) and CD73 in comparison to iTregs with only Helios and Nrp1 being able to individually provide distinction between nTregs and iTregs (Yadav et al. 2012, Lin et al. 2013).

1.5.2 Role and function

As previously mentioned, CD4$^+$ Tregs are mostly involved in the control of autoreactive T cells, thus maintaining immunologic self-tolerance (Sakaguchi 2004). Other suggested functions of Tregs include modulating the magnitude of immune responses, oral tolerance, feto-maternal tolerance as well as suppression of allergy, asthma and pathogen-induced immunopathology (Corthay 2009). Tregs can affect proliferation, differentiation and effector function of multiple immune cell types (DiPaolo et al. 2005, Sojka et al. 2008). Several mechanisms have been proposed for Treg function yet there is still a debate whether Tregs inhibitory activity depends on direct cell-cell contact. One of the suggested Treg mechanisms suppressing effector T cells is depriving them from IL-2 that is vital for their survival and proliferation and ultimately results in

apoptosis (Vignali 2008). Additionally, other studies have suggested that Tregs have cytolytic activity and can directly eliminate target cells via granzyme/perforin dependent way (Gondek et al. 2005, Cao et al. 2007). Furthermore, generation of pericellular adenosine nucleosides by coexpressed CD39 and CD73 on Tregs was shown to suppress activated effector T cell function via type 1 purinergic adenosine A2A receptor expressed on effector T cells (Deaglio et al. 2007). On the other hand, others have suggested that an indirect way of immunological suppression by inhibiting APC (such as DCs) maturation and/or function that could potentially attenuate effector T cell activation by DCs (Tang and Bluestone 2006, Tang et al. 2006). Moreover, Tregs secrete IL-10 and TGF-β inhibitory cytokines suppressing effector T cells while generating Tr1 and Th3 Treg subpopulations, respectively (Chen and Wahl 2002, Vignali 2008). Additionally, IL-35 was also described as an inhibitory cytokine secreted by Tregs contributing in their suppressive function (Collison et al. 2007). In summary, Tregs can potentially exert their suppressive effect on effector T cells either directly or indirectly via a variety of mechanisms however, whether all these mechanisms are critical for Treg function is still unclear. Concluding, Tregs are capable of modulating immune responses that are deleterious to the host as well as playing an indispensable role in the regulation of autoreactive T cells and prevent autoimmune diseases (DiPaolo et al. 2005, Sakaguchi et al. 2008). Impairment in either the development or function of Tregs seems to be the primary cause of autoimmune and inflammatory diseases in both humans and animals (Sakaguchi et al. 2008).

1.6 The Non-obese diabetic mouse

1.6.1 Model of Type 1 Diabetes

In 1974 at the Shionogi Research labs in Japan, a mouse strain now known as non-obese diabetic (NOD) mouse, was developed as a model for T1D in which autoreactive cells such as CD8^{+} CTLs target the insulin producing β cells (Hanafusa et al. 1994, Makino et al. 1980, Lieberman et al. 2003). Since then, NOD mice have become a well described mouse model used for understanding human T1D as they share various genetic and immunologic traits with the human form ofT1D (Hanafusa et al. 1994, Thayer et al. 2010). NOD mice are genetically

predisposed to spontaneously develop T1D in a series of well described chronological stages referred in this document as sensitisation, regulation and aggression stages. In brief, during the sensitisation stage around 3-4 weeks of age the initial priming of autoreactive naive anti-β cell T cells occurs, which takes place in the pancreatic lymph nodes (PLNs) (Gagnerault et al. 2002). Subsequently, from 3-4 weeks of age, non deleterious infiltration of lymphocytes in the pancreatic islets of Langerhans known as insulitis occurs (Faveeuw et al. 1994) and during the regulation stage between 5-12 weeks of age insulitis of autoreactive cells carries on however, their destructive functions are suppressed by transient regulatory mechanisms that prevents attack to the host's β cells (Gagnerault et al. 2002). Once the exerted self-tolerance by the regulatory mechanisms breaks down around 12 weeks of age, activated CD8^{+} T cells differentiate into CTLs that target β cells and results in their destruction and the onset of diabetes, hence referred to as aggression stage (Bach 1995, Delovitch and Singh 1997, Cnop et al. 2005).

Among other rodent models used for research of T1D such as biobreeding (BB) rats that also spontaneously develop T1D (Kolb et al. 1986) and LEW.1AR1/-iddm rats as well as other chemically induced models of T1D, NOD mice are the most commonly used autoimmune model for T1D where females predominantly develop diabetes in comparison to male mice. Once NOD mice are overtly diabetic, they lose weight fast and insulin injections are vital for their survival (King 2012). Furthermore, MHC-class II gene variants which are major loci in both human and NOD mice in the disease process, are conserved (Todd and Wicker 2001). In summary, although NOD mice have a significantly different islet histopathology compared to humans with T1D, they still share several similar genetic and immunological characteristics. Therefore, NOD mice have been useful in studying the etiopathogenesis of T1D by dissecting pathways and mechanisms, providing insights to the human disease (In't Veld 2014).

1.6.2 Initiation of Type 1 Diabetes and insulitis

The exact cause of T1D is unknown. However, it is speculated that susceptibility genes in combination with environmental factors (such as viruses) trigger the onset of T1D pathogenesis (Filippi and von Herrath 2008, Van Belle et al. 2011). In addition, both the innate and adaptive immune system seem to play a role in

the initiation of T1D via PRR binding that leads to upregulation and secretion of proinflammatory cytokines and chemokines and ultimately activates T and B cells in an effort to eliminate the virus which leads to pancreatic inflammation and subsequently the onset of T1D (Green and Flavell 1999, Zipris 2011). Furthermore, self-antigens such as insulin, glutamic acid decarboxylase (GAD), tyrosine phosphatase-like proteins insulinoma antigen-2 and 2β (IA-2 and IA-2β) as well as imogen-38 and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), seem to be involved in the initial lymphocyte responses against β islets in T1D (Green and Flavell 1999, Roep and Peakman 2012). Under normal conditions, these autoantigens would not be exposed to immune cells, yet in T1D are uncovered as a result autoreactive cells can become activated in response to autoantigens and initiate an autoimmune response. One of the hypothesis explaining the exposure of self-antigens is, followed by viral infections, apoptotic events of the islets take place which releases islet self-antigens and potentially enhances self-antigen presentation by APCs (Filippi and von Herrath 2008, Morse and Horwitz 2017). However, recently another study suggested that exposure to sequestered self-antigens is not adequate to mount T1D (Ono et al. 2017). Therefore, both genetic predisposition as well as environmental factors, seem to play an important role in T1D induction.

Until around 3-4 weeks of age there seems to be no abnormal lymphocytic infiltration in the islets (Gagnerault et al. 2002). However, subsequently to the sensitisation stage, non-destructive or benign insulitis occurs, where initially APCs such as DCs and macrophages start invading the edges of islets known as peri-insulitis, which are then followed by CD4^{+} and CD8^{+} T cell and B cell invasion (Thomas and Kay 2000, Brodie et al. 2008). Gradually immune cells infiltrate within the pancreatic islets during the pre-diabetic stage. At this point during insulitis it is speculated that tolerance is lost towards β cell antigens leading to β cell destruction by activated CD8^{+} CTLs (André et al. 1996, Brodie et al. 2008). This pathogenesis of infiltrated islets by APCs, CD8^{+} T cells capable of differentiating into CTLs and other lymphocytes observed in NOD mice, was also observed in pancreatic samples of diabetic patients which allows to gain some insight of the human disease using NOD mice instead (Hanafusa and Imagawa 2008).

1.6.3 Beta cell destruction

Following non-destructive insulitis, β cells begin to be destroyed by aggressive autoreactive immune cells. In both NOD and human pancreatic islets, β cell destruction is thought to be mediated by CD8^{+} CTLs (Thomas and Kay 2000, Hanafusa and Imagawa 2008, Brodie et al. 2008). By the end of this process, hardly any β cells survive (In't Veld 2014)

1.7 T cell and B cell involvement in Type 1 Diabetes

1.7.1 T cell involvement

T cells pathogenic role has been well established in T1D where ultimately autoreactive T cells infiltrate the pancreatic islets of Langerhans and destroy insulin-producing β cells (Roep 2003). In addition, both pathogenic CD4^{+} and CD8^{+} T cells play distinct roles in mediating diabetogenesis. In regards to CD4^{+} T cells, as mentioned previously, once activated CD4^{+} T cells can differentiate into different subtypes characterised based on their cytokine production and immunological function (Alberts et al. 2002, Golubovskaya and Wu 2016). For instance, Th1 cells can induce antibodies such as IgG2a/c in mice as well as produce IL-2, IFN-γ, TNF-β amongst others, which activate macrophages that aid innate immunity to clear intracellular pathogens (Romagnani et al. 1999, Hollo et al. 2000, Stout et al. 2009, Walker and von Herrath 2016), whereas Th2 cells produce IL-4 which leads to macrophage activation, IL-5, IL-6, IL-10, IL-13 amongst others, that are associated with stimulation of the humoral immune system which involves the production and secretion of antibodies by B cells against extracellular pathogens (Lafaille et al. 1998, Janeway et al 2005, Smith et al. 2000). In addition, the secreted products of these Th subtypes can reciprocally inhibit the development of the other (Mosmann 1989). Several studies documented altered Th1/Th2 balance which has been speculated to be involved in the pathogenesis of T1D (Walker and von Herrath 2016). However, there is a debate whether T1D is mediated by Th1 or Th2 or even both (Anderson et al. 1993, Shimada et al. 1996, Azar et al. 1999). Originally, it was proposed that autoreactive Th1 cells would promote T1D, whereas Th2 cells would protect mice from the disease by dampening the activity of Th1 effectors (Liblau et al. 1995). Using T cell cultures expressing diabetogenic TcRs, J. Katz *et al.* demonstrated

that neonatal NOD mice would elicit diabetes when T cells differentiated towards Th1 but not Th2 phenotype as Th1 cells actively promoted diabetes (Katz et al. 1995). Consistent with this concept, IFN-γ that is produced by Th1 cells seemed to be particularly involved in the disease process. For instance, progression of T1D in NOD mice was directly correlated with increase in IFN-γ levels (Rabinovitch 1994). Additionally, in virus-induced T1D models such as rat insulin promoter (RIP) lymphocytic choriomeningitis virus (LCMV) transgenic mice, IFN-γ seemed to be required for the development of insulitis and T1D (von Herrath and Oldstone 1997). Blockade of IFN-γ expression seemed to prevent diabetes (Debray-Sachs et al. 1991, Campbell et al. 1991), whereas ectopic expression of IFN-γ seemed to be adequate to cause development of diabetes in mice (Sarvetnick et al. 1988). IFN-γ was also implicated in homing of diabetogenic T cells in the pancreatic islets of NOD mice as well as β cell destruction (Savinov et al. 2001, Chong et al. 2001, Barral et al. 2006). Nonetheless, although various studies support the notion of Th1 bias in T1D, there are studies that disagree. For example, some studies claim that β cell destruction was mediated by Th2 cells rather than Th1 cells or that both subtypes are involved (Anderson et al. 1993) (Azar et al. 1999, Poulin and Haskins 2000). Long term cultured Th2 cell clones bearing a diabetogenic TcR obtained from diabetogenic Th1 cell clones, were demonstrated to rapidly cause diabetes in neonatal NOD mice (Poulin and Haskins 2000). Moreover, deficiency in IFN-γ or the β chain of its receptor did not seem to prevent insulitis or diabetes development in NOD mice (Hultgren et al. 1996, Serreze et al. 2000). Additionally, IL-4 which promotes Th2 generation, was demonstrated to prevent evolution of insulitis (Rapoport et al. 1993, Mueller 1996). However, deficiency of IL-4 using IL-4 KO NOD mice as well as recombinant IFN-γ injection, did not seem to accelerate or intensify insulitis (Satoh et al. 1989, Wang et al. 1998). Furthermore, paradoxically to the previous notion of a deleterious effect of IFN-γ in T1D, DO. Sobel *et al.* suggested that on the contrary IFN-γ could inhibit the diabetic process in NOD mice by decreasing the activity of effector cells against islets (Sobel et al. 2002). Collectively, these data suggest that the involvement of Th cells in T1D is much more complex than what was previously believed.

Following the discovery of Th17 cells and their secretion of IL-17 (Park et al. 2005, Harrington et al. 2005), the Th1 paradigm was challenged as Th17 cells

were proposed to be involved in T1D pathogenesis. However, the role of Th17 in T1D is unclear. Initially IL-17 was speculated to be associated in the pathogenic process of T1D (Jain et al. 2008, Emamaullee et al. 2009), yet a subsequent study by J. Joseph *et al*. in which IL-17 was silenced by RNA interference, suggested that IL-17 was dispensable in the pathogenesis of T1D and did not protect NOD mice from the disease (Joseph et al. 2011). Moreover, interestingly further investigations by MA. Kriegel *et al*., II. Ivanov *et al*. and K. Lau *et al*., suggested that IL-17 could protect T1D murine models from T1D or even delay T1D development than promoting T1D (Ivanov et al. 2009, Kriegel et al. 2011, Lau et al. 2011). Interestingly, in the gut of patients with Crohn's disease, cells capable of expressing both IL-17 and IFN-γ were identified and subsequently observed in the colon of an adoptive cell transfer mouse model (Annunziato et al. 2007, Ahern et al. 2011). These cells sometimes referred to as Th1/17 cells, were suggested to be present in the T1D setting according to L. Reinert-Hartwall *et al*. who observed in T1D children IL-17^{+} cells to have a higher predisposition in making IFN-γ in comparison to healthy controls (Reinert-Hartwall et al. 2015). Similarly, separate studies using T1D patients, also implicated Th1/17 cells in T1D (Honkanen et al. 2010, Ferraro et al. 2011, Arif et al. 2011). More recent evidence suggest that Th17 cells have distinct plasticity depending on the inflammatory setting (Hirota et al. 2011). Nonetheless, further investigations are required to clarify the role of Th1, Th2 and Th17 in the pathogenesis of T1D.

Furthermore, more recently another T cell subtype that is speculated to be involved in T1D development is the follicular helper T (Tfh) cells that provide assistance in B cell activation and ultimately antibody production (Vinuesa et al. 2005). Using a transgenic mouse model in order to investigate the gene expression profile of islet-specific T cells via microarray analysis, identified a signature of Tfh cell differentiation. In addition, purified memory T cells from T1D patients seemed to have higher mRNA levels of Tfh cell markers such as CXCR5, ICOS, PDCD1, IL-21 as well as BCL6 that is a master transcription factor for Tfh cell differentiation, in comparison to healthy controls (Kenefeck et al. 2015). Data from mouse models provided evidence which suggest that Tfh cells are partly responsible for T1D pathology. For instance, Roquin is a gene that when expressed Tfh cells are generated and when mutated formation of Tfh cells is exaggerated resulting in systemic autoimmunity (Vinuesa et al. 2005).

Using a TcR transgenic diabetes mouse model, mutation of Roquin gene not only caused immoderate formation and activity of Tfh cells but also fast progression to T1D (Silva et al. 2011). Overall, it has been illustrated that an expansion of cells with Tfh phenotype seem to be a T1D characteristic in both mouse models and humans (Walker and von Herrath 2016).

In T1D autoreactive CD8^{+} T effector cells have been established to play a fundamental role in targeting and subsequently eliminating pancreatic β cells. Various investigations using the NOD mouse have contributed in the understanding of the mechanisms involved in the activation, homing and β cell destruction attributes of CD8^{+} T effector cells (Tsai et al. 2008). For instance, pancreatic islets of recently diagnosed T1D patients were significantly infiltrated with CD8^{+} T cells which suggested that CD8^{+} T cell were involved in the onset of T1D (Hänninen et al. 1992, Itoh et al. 1993). Studies using NOD mice in order to elucidate the contribution of CD8^{+} T cells in T1D pathogenesis provided evidence which suggested that both CD4^{+} and CD8^{+} T cells were required in diabetogenesis (Bendelac 1987, Yagi et al. 1992, Christianson et al. 1993, DiLorenzo et al. 1998). This conclusion was assessed via adoptive transfer studies in which the ability to transfer diabetes of each NOD T cell subset into neonatal NOD and severe-combined immunodeficient NOD (NOD.scid) was compared. For example, diabetes was induced less efficiently when only splenic CD4^{+} T cells were transferred from female prediabetic NOD mice into NOD.scid mice in comparison to co-transfer of both CD4^{+} and CD8^{+} splenic T cells that induced diabetes more efficiently (Christianson et al. 1993). Similarly to this notion, NOD islet-derived β cell-specific CTL clones seemed to be able to transfer diabetes especially when co-injected with unstimulated polyclonal CD4^{+} T cells (Nagata et al. 1994, Utsugi et al. 1996). In addition, beta 2-microglobulin (beta2m)-deficient NOD mice lacking MHC-class I molecule therefore no generation of CD8^{+} T cells, did not develop T1D (Sumida et al. 1994, Serreze et al. 1994). Moreover, interactions between CD8-APCs or CD8-β cells seem to contribute in T1D development as NOD mice lacking MHC-class I on either mature APCs or β cells, never became diabetic (Hamilton-Williams et al. 2003, de Jersey et al. 2007). Collectively CD8^{+} T cells are crucial contributors of T1D

progression as they have been implicated in both early and late stages of the disease.

1.7.2 B cell involvement

B cells seem to play a vital role in T1D onset although it is considered to be a T cell-mediated autoimmune disorder (Hinman and Cambier 2014). For example, in 1996 D.V. Serreze *et al.* generated B cell deficient NOD mice that seemed to be resistant to T1D. These mice had a functionally inactivated Ig μ heavy chain and therefore were unable to generate B cells. This strain of mice was known as NOD.Ig μ null, they retained all known susceptibility genes of T1D and although they lacked B cells they had normal numbers of T cells (Serreze 1996). Later on, H. Noorchashm *et al.* induced in vivo depletion of B cells using anti-Ig μ antibody in female NOD mice and demonstrated abrogation of insulitis and sialitis development in contrast to control mice. In addition, they found that discontinuation of anti-Ig μ antibody treatment resulted repopulation of B cells and re-appearance of insulitis and sialitis (Noorchashm et al. 1997). Subsequent studies with various methods depleting B cells in NOD mice, for instance using anti-CD20 (Hu et al. 2007), anti-CD22 (Fiorina et al. 2008), blocking BAFF (a vital B cell survival factor) using BCMA-Fc (Mariño et al. 2009) or anti-BAFF monoclonal antibodies (mAbs) (Zekavat et al. 2008), results in similar protective effects against T1D. Similarly in newly diagnosed T1D patients, the use of rituximab results in depletion of B cells as well as transient preservation of β cell function (Pescovitz et al. 2009). Further evidence also suggested that the BcR may change the development of T1D in mice depending on the specificity against islet antigens of the BcR repertoire produced (Hulbert et al. 2001). Based on several studies it was speculated that B cells antibody production, cytokine secretion and auto-antigen presentation properties are implicated in T1D pathogenesis (Hinman et al. 2014). For instance, in regards to antibody production, although autoantibodies against islet antigens were detected in early stages of T1D and correlated in T1D pathogenesis, autoantibodies do not seem to play a primary diabetogenic role of B cells as transfer of autoantibodies from diabetic NOD mice to NOD.Ig μ null mice did not alter their disease protection (Serreze et al. 1998, Côrte-Real et al. 2009). In regards to B cell antigen presenting function, in order to investigate the contribution of antigen presentation versus autoantibody production, F. S. Wong *et al.* used transgenic

NOD mice that expressed IgM heavy chain yet were unable to to secrete antibodies. In comparison to B cell deficient NOD mice, the transgenic NOD mice exhibited an increased incidence of T1D in comparison to B cell deficient NOD mice, indicating that specific antigen presenting function of B cells was implication in diabetogenesis (Wong et al. 2004). Further to this, it was speculated that B cells were essential for the initial T cell priming to pancreatic β cell GAD self-antigen, as transfer of NOD B cells into B cell deficient NOD mice restored T cell responses against GAD that were absent in B cell deficient NOD mice (Serreze et al. 1998). Interestingly, another study suggested that B cells are not a prerequisite for T1D in NOD mice. This was speculated as subsequently to T cell transfer from NOD mice into NOD.scid mice that lacked functionally mature T and B cells and usually do not develop T1D, still developed insulitis and ultimately T1D although they lacked B cells (Charlton et al. 2001). Nonetheless, this could be explained by a seeming ability of other subpopulations of APCs such as macrophages and DCs, to elicit a T cell response against GAD following initial priming by processing and presenting GAD, yet less efficiently (Serreze et al. 1998). Furthermore, both MHC-class I and II have been suggested to be involved in diabetogenic T cell activation as deficiency in either MHC-class I or II of B cells in NOD mice have been observed to result in a decrease of activated CD8$^+$ T cells or resistance to T1D, respectively (Noorchashm et al. 1999, Stratmann et al. 2000, Marino et al. 2013). Collectively, up to date, evidence support the notion of antigen presenting function of B cells being their primary pathogenic action in T1D.

1.8 Regulatory T cell overview in Type 1 Diabetes

Since the discovery of Tregs (see section 1.5), various studies have speculated their vital involvement in T1D among other autoimmune diseases (Paschou 2011). In regards to T1D, despite the ongoing infiltration of pancreatic β cells by several immune cells prior to the onset of diabetes, overt diabetes does not occur until months later. This suggested that peripheral immunoregulatory mechanisms keep autoreactive T cells in check yet due to progressive breakdown of those mechanisms results in part to T1D progression. Initial evidence from co-transfer studies indicated the involvement of Tregs in the delay of β cell destruction and

disease development. For instance, C. Boitard *et al.* and colleagues transferred splenocytes from diabetic NOD mice into pre-irradiated adult NOD mice as a model of accelerated T1D. They then transferred splenocytes from non-diabetic donors and observed a delay in disease development. Subsequent depletion experiments showed a CD4^{+} cell-dependent transient protection originating from the thymus of non-diabetic donors (Boitard 1989). Similarly, Hutchings and Cooke *et al.* showed that transfer of splenocytes from young non-diabetic NOD mice into irradiated recipients followed by a transfer of splenocytes from diabetic NOD mice, did not exhibit hyperglycaemia and demonstrated a role of CD4^{+} cells in this protective effect (Hutchings and Cooke 1990). Additionally, later on the removal of CD62L-expressing CD4^{+} T cells (a marker expressed by Tregs, naive and memory T cells) (Fu et al. 2004, Surh 2008, Matsushita 2008) from splenocytes of pre-diabetic mice enhanced the generation of diabetogenic lymphocytes whereas expression of CD62L exhibited enriched protective or suppressive capacity (Lepault et al. 1995, Lepault and Gagnerault 2000). Similar effects were observed in NOD mice in which depletion of CD25-expressing T cells (the α-chain of IL-2 receptor exressed by Tregs and other activated T cells) (Sakaguchi et al. 1995, Asano et al. 1996) or disruption of a crucial pathway involved in Treg development, B7/CD28 pathway in NOD mice would result in acceleration of T1D onset (Suri-Payer et al. 1998, Salomon et al. 2000, Tang et al. 2003). Moreover, Z.Chen *et al.* using Foxp3-deficient NOD mice documented an accelerated onset of destructive insulitis and therefore T1D progression that again illustrated a role of Tregs in T1D pathogenesis (Chen et al. 2005). Further to this, adoptive transfer of ectopic Foxp3-transduced T cells with islet antigen specificity reversed the disease of recently developed diabetic mice (Jaeckel et al. 2005). However, despite the known suppressive Treg mechanisms discussed in section 1.3, the exact mechanism(s) involved in this protection against the development of the disease, is unclear.

Following the established Treg involvement in T1D, subsequent mounting evidence supported the notion of either quantitative and/or qualitative defects in Tregs during T1D development. Therefore, the disease progression is speculated to be in part due to an imbalance in either the number and/or function of Tregs towards autoreactive effector T cells (Cabrera et al. 2012). For instance, in regards to Treg function, S. Gregori *et al.* suggested an age-dependent decline in

Treg suppressive activity as co-transfer of Tregs and splenocytes depleted of Tregs from 8 week old NOD mice into NOD.scid mice delayed the onset of diabetes, whereas co-transfer of the same populations from 16 week old NOD mice did not (Gregori et al. 2003). Additionally, S. You *et al.* suggested that decline of Treg suppressive activity does not solely explain the onset of T1D. Instead, he also suggested a possible progressive resistance of autoreactive effector T cells against the inhibitory activity of TGF-β that is secreted by Tregs and in combination with Treg activity decline could result in T1D progression (You et al. 2005). Further to this, M. Tritt *et al.* also supports the notion of a qualitative rather than a quantitative change in Tregs of NOD mice as they demonstrated an age-dependent decline of Treg functional potency (Tritt et al. 2007). Qualitative changes of Tregs have also been observed in T1D patients (Kukreja et al. 2002, Brusko et al. 2005, Lindley et al. 2005). Interestingly, R.J. Mellanby *et al.* suggested that there is no qualitative change in the in vitro Treg suppressive activity of diabetic NOD mice towards effector T cells in comparison to Tregs from non-diabetic NOD mice. In addition, following cotransfer of Tregs and effector T cells into NOD.scid mice, although they documented a decline in Treg suppressive activity they speculate that this does not indicate a decline in Treg function as contamination from non-Foxp3^{+} populations could skew the results (Mellanby et al. 2007). On the other hand, some studies support the notion of a quantitative change in Tregs as well, that contributes in T1D development. For instance, S.M. Pop *et al.* demonstrated a temporal percentage decline of islet infiltrating $CD4^+$ $CD25^+$ $CD62L^{hi}$ T cells as well as FoxP3 and TGF-β co-expressing T cells inside pancreatic lymph nodes in combination with an age-dependent decline of these Treg populations TGF-β suppressive activity (Pop et al. 2005). Further to this, B.K. Nti et al. suggested that migration of Tregs in PLNs might play a potential role in diabetogenesis as they documented a decrease in PLN Treg numbers of NOD mice possibly due to the downregulation of relative chemokines such as SDF-1 (Nti et al. 2012). In addition, R.K. Gregg *et al.* documented an abrupt decline of membrane-bound TGF-β Tregs alongside with decline in their suppressive function during the transition from benign insulitis to deleterious insulitis and diabetes onset (Gregg et al. 2004). In contrast, other studies support no alterations of primary and secondary lymphoid tissue Treg frequencies in an age-dependent manner (Tritt et al. 2007) threrefore, we cannot

arrive at a consensus as to whether diabetes is associated with changes in Treg numbers.

In regards to the thymus, thymic development of Tregs in NOD mice seems to be normal (Sgouroudis and Piccirillo 2009, Dardenne et al. 1989) if not enhanced (Feuerer et al. 2007). Assessing the frequency and function of thymic Foxp3-expressing nTregs in NOD mice and non-diabetes prone control mice, M. Tritt *et al.* suggested that there are no alterations in either the frequency or function of nTregs in the thymus of pre-diabetic NOD mice (Tritt et al. 2007). However, A.J. Wu *et al.* demonstrated a decrease in thymic Tregs of NOD mice characterised as $CD4^+$ $CD25^+$ in comparison to non-diabetes prone mice that may however be explained by the reduced thymus size of 3 week old NOD mice that potentially resulted in reduction of all thymocyte populations including Tregs (Wu et al. 2002). In comparison to more recent evidence provided by J. Tellier *et al.*, they documented an increase in frequency of thymic Foxp3-expressing Tregs (Tellier et al. 2013). Additionally, T.M. McCaughtry *et al.* used RAG2p-GFP reporter mice in order to distinguish between thymic Foxp3-expressing Tregs that have been newly developed (ND) or Tregs that are present in the thymus subsequent to their development (referred to as resident Tregs in this document) which adds another layer of complexity in Treg analysis (McCaughtry et al. 2007). In brief, GFP is coexpressed when RAG genes are active during TcR rearrangement and although GFP transcription terminates with RAG activity, GFP protein levels gradually decline as its half-life is estimated to be ~56 hours in conventional CD4SP thymocytes (Mombaerts et al. 1992, Monroe et al. 1999, McCaughtry et al. 2007). Therefore, this system would allow examination of thymic ND and resident Tregs, which have been neglected by previous studies investigating Tregs in T1D.

1.9 Project aims

Numerous investigations undertaken to understand the involvement of Tregs in T1D pathogenesis, lead to the mounting contradictory evidence in regards to thymic and peripheral Treg function and/or number using different T1D mouse models at various stages of the disease. We hypothesise a loss of Tregs in pre-

diabetic NOD mice that may be correlated with T1D progression. Therefore, due to the contradictory findings and the importance of Tregs in T1D development, this project aimed to reinvestigate the development and proliferative homeostasis of thymic Tregs as well as the proliferative homeostasis of Tregs in secondary lymphoid tissues, using for the first time RAG-GFP expressing NOD mice in order to dissect any alterations in ND and resident Tregs that have not been taken into account by previous studies.

These are the main questions that this project aimed to investigate:

1) Are there any alterations in the Treg proportion/number between NOD and non-diabetes prone mice, if so, is it age-dependent?

2) Do B cells affect Treg development or homeostasis in NOD mice?

Materials and Methods

2.1 Animals

C57BL/6 (B6), NOD, NOD-RAG2p-GFP, NOD.µMT$^{-/-}$-RAG2p-GFP, Friend Virus B (FVB)-RAG2p-GFP mice were used in this project.

Diabetes incidence in our female NOD mouse colony is 95%, approximately 3% of mice develop T1D at 12-14 weeks of age, 85% at 18-20 weeks of age with the remaining 7% of females progressing to T1D by 23 weeks of age. Animals not diabetic by 23 weeks of age rarely develop T1D. The data is based on a cohort of 200 animals. These statistics highlight that 12-14 weeks of age in our colony represents late insulitic-preultimate diabetic stage, a critical time when immunoregulation of the autoreactive response starts to breakdown. Thus, in our initial studies we focused on two major time points; the pre-early insulitic phase (4-6 weeks) and the post-insulitic/pre-diabetic phase (12-14 weeks) to assess Treg proportions in primary and secondary lymphoid tissues.

C57BL/6 mice were provided by the Biomedical Services Facility, University of York. FVB-RAG2p-GFP mice (hereafter referred to as FVB-RAG-GFP) were purchased from the Jackson Laboratory and thereafter supplied by the Biomedical Services Facility, University of York. FVB-RAG2p-GFP mice have been described previously by Yu *et al.* 1999 and used by McCaughtry *et al.* (Yu et al. 1999, McCaughtry et al. 2007). NOD-RAG2p-GFP reporter mice (hereafter referred to as NOD-RAG-GFP) were a product of FVB-RAG2p-GFP mice backcrossed 12 generations (N$_{12}$) to the NOD mouse. NOD.µMT$^{-/-}$-RAG2p-GFP reporter mice (hereafter referred to as B-KO) were a product of N$_{12}$ NOD-RAG2p-GFP mice crossed with NOD.µMT$^{-/-}$ resulting in heterogeneous mice which were backcrossed with NOD.µMT$^{-/-}$ in order to get a homogeneous colony. Mouse genotypes were determined by flow cytometric analysis of blood samples to assess B-cells and/or GFP presence depending on the strain of mouse. Unless otherwise stated in the results, slightly younger adult female mice used for

experimental purposes were between 8 and 14 instead of 12 and 14 weeks of age due to time limitations, yet still representing late insulitic-preultimate diabetic stage, and were closely age matched within experiments.

All animals were bred and maintained under specific pathogen-free barrier conditions, under a 12 hour light/dark cycle and fed normal chow at the Biological Services Facility, University of York. All experimental procedures were conducted in accordance with UK Home Office and ARRIVE regulations (https://www.nc3rs.org.uk/arrive-guidelines).

2.2 Cell preparation

Single cells were prepared from the thymus, spleen and lymph nodes. For the thymus, the tissues were removed and either homogenised or digested with digestive enzymes. For homogenisation thymi were placed in an 100µm EASYstrainer^{TM} (Greiner bio-one) on top of a 50 ml falcon tubes and the tissue was pressed through the strainer using a plunger from a 5 ml syringe. The cells were resuspenced in 15 ml of 1X phosphate buffered saline (PBS)/1% FCS. For digestion, isolated thymi were transferred into a 24 well plate containing 500 µl of digestion media (RPMI supplemented with 0.325 Wunsch U/ml Liberase TM and 50 Kunitz/ml DNAseI) and then cut into small pieces (+/- mm^3). The tissue pieces were transferred with the digestion media to a 50ml falcon tubes, placed in a shaker for 50 minutes at 1800 rpm at 37°, then 10 µl of 50 nM stock concentration of ethylenediaminetetraacetic acid (EDTA) with a final concentration of 1.96nM, was added for 5-10 minutes on ice to dissociate the suspension into single cells.

For the spleen, the single cells were prepared by homogenising as above. For both the thymus and spleen red blood cells were removed. The single cell suspensions were centrifuged at 300g for 10mins, 4^{0}C and cell pellets resuspended in 1 ml of ACK lysing buffer (Gibco) for 1 minute for the thymi and 5 minutes for the spleens. 30 ml of 1X PBS/1% FCS was added to each tube and the cells were then centrifuged as before, supernatant discarded and the cells were resuspended in 1 ml 1X PBS/1% FCS as appropriate for subsequent procedure.

For the lymph nodes, single cells were prepared by grinding the lymph nodes between two frosted glass slides (Thermo scientific). Single cells were then resuspended in 500 µl of 1X PBS/1% FCS.

Following the preparation of single cell suspensions, a small volume of the samples was stained with 1:20 dilution of Triton blue (cell suspension:Triton blue). Thymic and splenic cell numbers were measured automatically using a live cell imaging chamber ViCell and the cell number of lymph nodes was counted manually using a haemocytometer. All cell sample data were acquired using Becton Dickinson Fortessa X-20 with 4 lasers and 16 detectors and subsequently cell sample analysis was done using FlowJo software.

2.3 Flow cytometry

Single cells prepared as in Section 2.2, were resuspended in x1 PBS/1% FCS. Then 100 µl of cell suspension (equivalent to 10^6 to 10^7 cells) was transferred to an appropriate vessel and CD16/CD32 (Fc block) antibodies added for 20 minutes at 4°C in order to block the Fc receptors. The cells were then centrifuged for 7 minutes at 300g, the supernatant discarded and the cell pellets were resuspended in 100 µl 1X PBS/1% FCS. Subsequently, surface molecules were incubated with appropriate fluorescently labelled antibodies and incubated for 30 minutes at 4°C in the dark. All antibodies used in this study were assessed and optimised for use against isotype control. A list of antibodies used is shown in Table 2.1. Following incubation, the cells were then washed 2X as before and resuspended in 500µl 1X PBS/1% FCS ready for analysis.

For detection of intracellular Foxp3 detection, surface molecules were stained as above, the cells were centrifuged as before and resuspended in fixation/permeabilisation buffer (eBioscience) overnight at 4^0C. The next day, the cells were washed 2X in 1X permeabilisation buffer (eBioscience) then incubated with fluorochrome labelled Foxp3 antibodies for 1 hour at room temperature. The cells were washed 2X in permeabilisation buffer then resuspended in 100 µl x1 PBS/1% FCS ready for analysis.

For dual detection of GFP and Ki67, the surface molecules were stained as above and the cells centrifuged as before. The cells were fixed, permeabilised and incubated with Ki67 as for the Foxp3 staining protocol above. Following this, the cells were centrifuged and cell pellets resuspended in 200 µl of 1x fixation buffer (Biolegend) for 10 mins at room temperature. A dual fixation was required in order to detect GFP as a single fixation protocol with the eBioscience fixation/permeabilization buffer instead of the Biolegend fixation buffer, resulted in no detection of GFP. The cells were then centrifuged as before and washed 2X in 1X permeabilisation buffer (Biolegend). The cell pellet was resuspended in 200 µl of 1x Perm buffer (eBioscience) containing fluorochrome-labelled anti-GFP antibodies 1 hour incubation at 4°C in the dark. Anti-GFP was required in order to detect effectively GFP-expressing cells. Following incubation, the cells were washed 2 times with 200 µl of Perm buffer (Biolegend) and then washed twice in 200 µl 1x PBS/0.5% BSA before resuspending them in 100 µl 1x PBS/0.5% BSA to be analysed.

Antibody clones and suppliers are shown in Table 2.1.

Antibody target	Fluorochrome	Clone	Supplier
CD4	Brilliant Violet 650™(BV650)	RM4-5	Biolegend
CD8	eFluor 450™/Allophycocyanin (APC)	53-6.7	eBioscience
CD16/32	-------	93	eBioscience
CD25	PerCP-Cyanine5.5	PC61.5	eBioscience
Foxp3	Phycoerythrin (PE)/eFluor 450™	FJK-16s	eBioscience
GITR	Phycoerythrin-Cyanine dye 7(PE-Cy7)	DTA-1	eBioscience
Anti-GFP	Alexa Fluor 488	FM264G	Biolegend
Ki67	Phycoerythrin (PE)	B56	BD Pharmingen
LiveDead	eFluor 780™	-------	Invitrogen

Table 2.1: Antibody conjugated fluorochromes, clones and suppliers.

2.4 Preparation of cell extracts

Single cells were prepared from the thymus by homogenisation as before. Cell extraction protocol adopted from the splenic extraction protocol by T. Yi *et al.* (Yi et al. 2012). Each thymus mashed in separate 15 ml tubes was then centrifuged for 10 minutes at 300g in order to collect all the cells at the bottom of the falcon tube, supernatant was then discarded, and the pellet was resuspended in 1 ml 1x

PBS/0.5% BSA and then centrifuged for an additional 15 minutes at 3000g at 4°C in order to lyse the cells and separate the lysates from the cell extracts. Therefore, the extracted protein came from the same number of thymocytes for each sample. The supernatant was then removed to a new eppendorf tube and stored at -20 °C if not used immediately.

2.5 ELISA-IL-2

For the ELISA 100 μl of thymic cell extract was prepared as described above. IL-2 was detected using an IL-2 ELISA kit with 2pg/mL sensitivity, following manufacturer's guidelines (Mouse IL-2 ELISA Ready-SET-Go!® kit from affymetrix eBioscience). Briefly, the plate was coated with capture antibodies, washed three times with a PBS/1% Tween 20 buffer, the wells were blocked by incubation with 1X ELISA/ELISASPOT diluent for an hour at room temperature. The IL-2 standard was added and serial 2 fold dilutions performed generate a standard curve. Subsequently, 100 μl of the cell extracts were added to appropriate labelled wells and incubated at room temperature for 2 hours. Following incubation, the plate was washed 2X as before. A biotinylated anti-IL-2 antibody was added to the wells and incubated for 1 hour at room temperature. Following 2X washes as before, an avidin-HRP substrated was added to the plate for 30 minutes at room temperature. The plate was washed twice again, and bound cytokine detected with TMB solution. Finally, stop solution was added to each well prior to reading the plate at 450nm.

2.6 Statistical analyses

All graphs were drawn using GaphPad Prism and statistical analyses (Mann-Whitney and One way ANOVA Tukey's tests) were performed using built-in functions within the software.

Results

3.1 Type 1 Diabetes and the Thymus

3.1.1 Alterations of Treg numbers in the NOD mouse

Regulatory T cells (Tregs) have been described previously to play a major role in the processes averting autoimmune diseases such as Type 1 Diabetes (T1D) (Sakaguchi et al. 1995). As suggested by *You, S. et al.*, T1D progression in NOD mice is partly explained by a decline of peripheral Tregs' activity where others claimed that this might be a result of a decrease in peripheral Treg numbers instead (Sakaguchi et al. 1995, You et al. 2005). In regards to the thymus, others claimed no alteration in thymic Tregs (D'Alise et al. 2008). However, using for the first time RAG-GFP reporter mice that could distinguish between newly developed and resident Tregs in the thymus, we decided to re-examine the thymic Treg proportions and establish a pattern of Treg homeostasis in NOD mice in order to enhance our knowledge on T1D pathogenesis and ultimately find a cure. Therefore, flow cytometric analysis was used to quantify the thymic total Treg population of 5 male C57BL/6 (B6) 8 week old auto-immune free strain used as a control, and 4 female NOD 10 week old mice. Over the years Tregs have been phenotypically defined to express a variety of cell markers such as CD25, CD45RB^{lo}, CTLA-4, CD62L, CD103 and GITR (Powrie et al 1193, Fehérvari and Sakaguchi 2004) on their cell surface of CD4 single positive (CD4SP) cells. However, some of these molecular markers are not unique to this T cell lineage and can be also found on activated, effector or memory T cells as well (Hori 2003, Zhan et al. 2007). Therefore, initially we decided to look at CD25 that is constitutively expressed on the majority of Tregs, and GITR coexpression on CD4SP cells in order to be more restrictive in Treg identification than other studies that only used CD4 and CD25 coexpression. The final gate of total Treg population was defined by gating on the thymic lymphocytes, excluding doublets and dead cells, focusing on CD4SP expression and sequential GITR and CD25

expression (Fig. 1.1A). Identical gating strategy was applied for both strains, B6 and NOD (Fig. 1.1B). There was a trend towards increase and a significant increase in the frequency and the absolute number, respectively of thymic total Tregs of NOD in comparison to B6 (Fig. 1.2A and B). In order to validate that this increase in NOD Tregs was not due to an increase in NOD thymic cellularity, we counted the absolute number of the total thymic cells as one would expect a universal direct increase in lymphocytes with cellularity (Fig. 1.2C). However, there was no significant difference in the total thymocyte number between B6 and NOD mice (Fig. 1.2C). This indicated that the changes in frequency and absolute number of NOD total Treg was due to alterations within the CD4SP population rather than changes in total thymic cellularity.

Foxp3, an intracellular transcription factor, was established to be specifically expressed in Tregs which allows the sub-classification and detection of precursor (pTregs) (GITR^{hi} CD25$^+$ Foxp3$^-$ CD4SP) and mature (mTregs) (GITR^{hi} CD25$^+$ Foxp3$^+$ CD4SP) Tregs (Williams and Rudensky 2007, Thiault et al. 2015). The addition of Foxp3 in our markers panel not only resolved the Treg population analyzed from other lymphocytes mentioned above (activated, effector or memory T cells), but also allowed analysis of pTregs and mTregs that have been neglected in other murine T1D studies. Hence, flow cytometry was used to measure the thymic pTregs and mTregs of 5 female B6 and 5 female NOD mice of 13 weeks of age, in order to observe whether pTregs or mTregs accounted for the increase in NOD Tregs we documented initially. Gating strategy was identical to the previous experiment (refer to Fig.1.1A), with the only difference of the final gate focusing on the Foxp3 expression of CD4SP CD25$^+$ GITR^{hi} cell population instead (Fig.1.3). We were able to replicate the pattern observed in Figure 1.2A and B, but in this experiment the increase of total Tregs in NOD mice compared to B6 mice was statistically significant in both their frequency and absolute number (Fig.1.4A and B). No significant difference was observed in either the frequency or the absolute number of pTregs between B6 and NOD mice (Fig.1.4A and B). However, there was a significant increase in both the frequency and the absolute number of mTregs of NOD in comparison to B6 mice (Fig.1.4C and D). Once again, there was no significant difference in the total thymocyte number between B6 and NOD mice (Fig.1.4E). This verifies that changes of NOD mTregs are not affected due to total thymocyte number difference, but alterations

within the CD4SP population. In addition, the total Treg increase in NOD mice observed previously (Fig.1.2A and B) is most likely due to NOD mTregs increase and not pTreg alterations (Fig.1.4).

3.1.2 Treg development compromised in the NOD-RAG-GFP mice

RAG2p-GFP reporter mice have been used to identify novel Treg populations. The RAG2p promoter drives GFP expression in RAG2p-GFP mice (Monroe et al. 1999). RAG genes are active during TcR rearrangement (Mombaerts et al. 1992), therefore GFP begins to be coexpressed with RAG expression at the late CD4$^-$ CD8$^-$ double-negative stage and throughout the CD4$^+$CD8$^+$ double-positive (DP) stage of intrathymic T cell development (Boursalian et al. 2004, Matthews and Oettinger 2009). Although GFP transcription terminates immediately after the DP stage with RAG activity, the GFP protein has a half-life of ~56 hours in conventional CD4SP thymocytes (McCaughtry et al. 2007). Thus, GFP expression of RAG2p-GFP mice gradually declines and acts as a "molecular timer" of thymocyte maturation (McCaughtry et al. 2007). This allowed the identification of newly developed (GFP$^+$) and resident (GFP$^-$) Tregs. However, observing these Treg subpopulations in NOD mice has not been examined before. To assess whether alterations occurred in these subpopulations, flow cytometry was conducted.

Female mice 13-14 weeks of age, 5 FVB and 3 NOD expressing RAG2p-GFP (hereafter called FVB-RAG-GFP and NOD-RAG-GFP, respectively), were used to observe the GFP expression of total Tregs. Flow cytometric analysis showed that the GFP percentage frequency of CD4SP thymocytes was lower than the double positive (DP) population yet greater than the double negative (DN) population, as predicted as DP thymocytes are actively transcribing GFP (Fig.1.5A). For this experiment only the GFP expression of total Tregs (i.e. CD4SP CD25$^+$ GITR$^+$) could be quantified, as Foxp3 was not detected using the GFP staining protocol. The final gate of GFP$^-$ and GFP$^+$ total Treg populations was applied for both strains, in which there was a subtle difference in the intensity of GFP fluorescence between FVB-RAG-GFP and NOD-RAG-GFP mice

observing higher intensity of GFP fluorescence in FVB-RAG-GFP mice than NOD-RAG-GFP mice (Fig.1.5B). Nonetheless, in contrast to the significant difference observed in total Tregs of B6 and NOD mice (Fig. 1.2), there was no significant difference in either the frequency or absolute number of total Tregs between FVB-RAG-GFP and NOD-RAG-GFP mice (Fig.1.6 A and B) that could be explained either by the use of a different strain as a control or by the slight age difference between the mice used depicted in Figure 1.2 and 1.6. C57BL/6 strain would have been more appropriate to be used as a control, if there was a C57BL/6-RAG-GFP available, due to the fact that FVB-RAG-GFP strain has a slightly wider spread as seen in some of our results than C57BL/6 mice. An additional reason explaining this non-significance is the fact that a total of only 3 NOD-RAG-GFP mice were used for this experiment which was due to the limited number of mice available at the time, that is insufficient to detect differences between the groups. However, there was significant decrease in both the frequency and absolute number of newly developed total Tregs of NOD-RAG-GFP mice compared to FVB-RAG-GFP mice (Fig. 1.6C and D). As mentioned earlier, the use of GFP allowed us to distinguish newly developed from resident Tregs and based on this we observed a significant increase in the frequency and a trend towards increase in the absolute number of resident total Tregs (Fig. 1.6E and F) of NOD-RAG-GFP mice. In order to rule out the possibility that these alterations were due to changes of the total cell number of the thymocytes, we counted the total thymocyte number of both strains. However, the total thymocyte number of FVB-RAG-GFP and NOD-RAG-GFP mice did not significantly differ (Fig. 1.6G). Therefore, the decrease of newly developed total Tregs observed in NOD-RAG-GFP mice is a genuine alteration within the Treg subpopulation that is not accounted by a change in thymocyte cellularity.

3.2 Type 1 Diabetes and the Periphery

3.2.1 Tissue-specific Treg alterations

As mentioned previously in Section 1.1, T1D is a chronic autoimmune condition in which the destruction of the beta cells situated in the pancreatic islets of Langerhans, results in high blood sugar levels due to a paucity of insulin

(Daneman 2006, Van Belle et al. 2011). Using Tet-TNFa/CD80 mice, it was suggested that accumulation of Tregs in pancreatic lymph nodes (PLNs) during the infiltration and insulitis (regulation) stage of T1D, might delay diabetes progression in NOD mice (Green et al. 2002). However, other studies using NOD mice have observed either a decrease or no alterations in peripheral Tregs residing in either the PLNs or the spleen (Wu et al. 2002, Alard et al. 2006) (D'Alise et al. 2008, Pop et al. 2005, Nti et al. 2012, Mellanby et al. 2007). The RAG-GFP expression in NOD mice provides a means to re-assess these findings while looking at Treg GFP expression in secondary lymphoid organs such as spleen, ILN and PLN, in addition to the thymus which has not been observed before. In order to determine if there was a Treg deficiency among the secondary lymphoid organs, flow cytometry was conducted initially using FVB-RAG-GFP and NOD-RAG-GFP mice.

To overcome the previous staining issue encountered with Foxp3 and GFP, originally for this experiment both precursor/mature Tregs as well as GFP expression of those populations, were to be detected using separately α-Foxp3 and α-GFP antibodies in two distinct plates undergoing different optimal protocols depending on the stain (Foxp3 plate and GFP plate).The idea was that in theory, the gating strategies applied on the cells stained in each plate, that came from the same single cell suspensions but aliquoted in two separate plates, would have similar cell frequencies if the same gating strategy was applied on the cells from each plate. Therefore, gates identifying Foxp3 expression for instance (hence Foxp3 population) from the Foxp3 plate would determine, theoretically, the Foxp3 expressing cell population in the GFP plate, that was not stained with Foxp3, when similar gating strategy was applied. However, analysis of the data obtained using this approach were shown to be unreliable as the results would vary among the experiments as a significant amount of Foxp3 expressing cells was unintentionally excluded from the final gate. This emphasized the need of an optimisation protocol in which both Foxp3 and GFP would be detected simultaneously. Therefore, in this experiment only the GFP expression of Total Tregs is analysed from the GFP plate (Figure 1.8).

All female mice 9-11 weeks of age 5 FVB-RAG-GFP and 4 NOD-RAG-GFP, were used to observe the GFP expression of total Tregs. Data were pooled from two

experiments with identical protocol. Figure 1.7 shows the final gating strategy (refer to Fig.1.1) of total Treg GFP expression in secondary lymphoid organs. There was a significant increase in total Treg frequency of both NOD-RAG-GFP spleens and ILNs compared to FVB-RAG-GFP mice, in contrast to their absolute numbers where there was no significant difference (Fig.1.8A and B). This suggests that alterations within other lymphocyte populations may be occurring and not Tregs per se. In agreement to previous findings (Fig.1.6) there was no significant difference in either the frequency or absolute number of thymic total Tregs (Fig.1.8A and B). There was also no significant difference in either the frequency or absolute number of PLN total Tregs between these two strains (Fig.1.8A and B). In addition, the mean of the absolute number of PLN total Tregs of NOD-RAG-GFP mice is elevated compared to the FVB-RAG-GFP mice however, this is not statistically significant (Fig.1.8B). The small number of mice used for this experiment could explain the lack of significant difference henceforth, the trend of data will be discussed in the upcoming experiments as well despite non-significance. There was no significant difference in either the frequency or absolute number of total Treg GFP$^+$ of spleens and ILNs (Fig.1.8C and D). In contrast to previous findings (Fig.1.6C and D), there was no significant decrease in either the frequency or absolute number of thymic ND total Tregs (Fig.1.8C and D). However, the mean absolute number of thymic ND Tregs seems to be similar to Fig.1.6 D findings. Interestingly, both frequency and absolute number of PLN total Treg GFP$^+$ have decreased, yet with significant difference only in the frequency (Fig.1.8C and D). The spleen and ILN total Treg GFP$^-$ frequencies' trend displayed a non-significant increase yet there was no significant difference in their absolute numbers (Fig.1.8E and F). In contrast to previous findings (Fig.1.6E and F), there was no significant difference in either the frequency or absolute number of thymic resident total Tregs (Fig.1.8E and F). Surprisingly, there was no significant difference in either the frequency or absolute number of PLN total Treg GFP$^-$ (Fig.1.8E and F). In addition, we sought to observe whether the genetic background of NOD mice influenced the cellularity of the whole organs by quantifying the total cell numbers that could potentially account for the alterations documented in Tregs (Fig. 1.8G). Overall, these preliminary findings of subtle tissue-specific changes in total Treg populations, especially in the PLNs, make one wonder whether is due to proliferation, survival and/or B cell interactions.

3.2.2 B cells may be involved in Treg alterations in the periphery

B cells seem to play a critical role in T1D. It has been noted that B cell-deficient NOD mice are resistant to T1D (Serreze 1996). A variety of ways depleting B cells such as: use of anti-CD20 (Hu et al. 2007, Xiu et al. 2008) or anti-CD22 (Fiorina et al. 2008) in addition to blockage of BAFF, a critical B cell survival factor, using BCMA-Fc (Mariño et al. 2009) or anti-BAFF mAb (Zekavat et al. 2008) has analogous protective effects against diabetes progression in the NOD mouse model. Furthermore, B cells also seem to promote pancreatic beta cell destruction via insulin-antigen presentation to both CD4$^+$ and CD8$^+$ T cells (Mariño et al. 2012). More recently, it was suggested that thymic B cells increase Treg numbers (Lu et al. 2015) and our group has also documented that thymic B cells accumulate in NOD mice in an age-dependent manner and may contribute in the impairment of negative selection of autoreactive T cells (Pinto *et al.* submitted). Therefore, due to insufficient number of investigations whether the presence of B cells affects Treg numbers in diabetically-prone mice, we used NODuMTKO-RAG-GFP mice for the first time where flow cytometry was conducted in order to quantify Treg populations within secondary lymphoid organs and the thymus.

A total of 6 female 9-11 weeks of age NODuMTKO-RAG-GFP mice (B-KO), were used to observe the GFP expression of total Tregs (Fig.1.8). Data for this experiment were combined with previously obtained data from FVB-RAG-GFP and NOD-RAG-GFP mice for visual comparison and statistical analysis. All protocols were identical. Interestingly, there is no significant difference in the frequency of splenic total Tregs between the FVB-RAG-GFP and B-KO mice in contrast to the NOD-RAG-GFP (Fig.1.8A). Based on the decrease of total splenocytes shown in Fig.1.8 G, a significant decrease of the absolute number in total Treg of B-KO mice was also observed which may signify an involvement of B cells in the maintenance of splenic Treg numbers (Fig.1.8 B). There was no significant difference in either the frequency or absolute number of thymic total Tregs among the three strains (Fig.1.8A and B). Similarly to the ILN total Tregs of NOD mice, there was also a significant increase in the frequency of ILN total Tregs of B-KO mice compared to FVB-RAG-GFP (Fig.1.8A). However, there was

no significant difference in absolute number of ILN total Tregs among the three strains (Fig.1.8B). There was also no significant difference in both the frequency and absolute number of PLN total Tregs among the three strains (Fig.1.8A and B). It is difficult to detect a trend in the absolute number of PLN total Tregs due to the spread of FVB-RAG-GFP mice. Therefore, repetition of this experiment with more mice would clarify whether the absolute number of PLN total Tregs in B-KO mice is more similar to the FVB-RAG-GFP mice compared to the NOD-RAG-GFP. No significant difference was noted in the frequency of total Tregs GFP$^+$ in either the spleen, thymus or ILN among the three strains (Fig.1.8C). In contrast, the frequency of PLN total Treg GFP$^+$ population was significantly less in both B-KO and NOD-RAG-GFP mice compared to the FVB-RAG-GFP mice (Fig.1.8C). Although there was no significant difference in the absolute number of total Treg GFP$^+$ populations of the lymphoid organs depicted in Fig.1.8D, there is a trend of decreased total Treg GFP$^+$ cells in both B-KO and NOD-RAG-GFP mice. This again could be clarified with the use of more mice. There was also no significant difference among the strains in the frequency of total Tregs GFP$^-$ in any lymphoid organ shown in Fig.1.8E. However, interestingly there seems to be a slight difference between the splenic total Treg GFP$^-$ B-KO and NOD-RAG-GFP mice (Fig.1.8E). No significant difference was observed in the absolute number of total Treg GFP$^-$ among the strains in the thymus, ILN and PLN (Fig.1.8F). There was again an expected significant decrease in the absolute number of splenic total Treg GFP$^-$ cells in the B-KO mice compared to the other strains (Fig.1.8F). In conclusion, these data on B-KO mice have not explained the thymic Treg alterations observed previously (Fig.1.3, 1.4 and 1.6) however, they provided valuable insights concerning Treg homeostasis in secondary lymphoid organs.

3.3 Thymic Treg alterations not due to interleukin 2 levels

Interleukin 2 (IL-2) is a pleiotropic hormone-like growth factor, which stimulates proliferation of activated T cells (Smith and Ruscetti 1981) as well as inducing naïve CD4$^+$ T cell differentiation into Tregs (Malek and Castro 2010). Studies have demonstrated IL-2 playing a critical role in the maintenance of natural immunologic self-tolerance, as the homeostatic maintenance of thymic and peripheral CD25$^+$ Foxp3$^+$ CD4$^+$ Treg cells is affected negatively in IL-2 depleted

mice (Setoguchi et al. 2005). In addition, low dose administration of IL-2 seemed to boost Treg survival in the spleen, PLNs and pancreatic islets and protected NOD mice from T1D progression (Tang et al. 2008). Furthermore, our group recently showed that ND and resident Treg proportions are affected differently by IL-2 availability and that decreased levels of IL-2 only affected resident but not ND Treg proportions (Cuss and Green 2012). Regarding B cells, it was suggested by *Ortega, G et al.* that activated B cells express IL-2 receptor (Ortega et al. 1984). Therefore, ELISA was used in order to quantify the IL-2 levels in RAG-GFP mice; FVB, B-KO and NOD, in order to explain the thymic Treg alterations observed previously (Fig.1.3, 1.4 and 1.6).

All female 8-11 weeks of age, 4 FVB-RAG-GFP, 4 NOD-RAG-GFP and 4 B-KO mice, were used to quantify the total thymic IL-2 levels. Data obtained using an ELISA plate reader depicted in Fig.1.9, showed no significant difference among the strains. Hence, changes in IL-2 levels do not appear to explain the thymic Treg alterations observed during T1D regulation stage.

3.4 Treg proliferation as a potential rationale for Treg alterations in the thymus and the periphery

Ki67 is a protein found in the cell nucleus during the interphase stage and sequentially relocates to the surface of condensed chromosomes during mitosis yet is absent in quiescent cells (Isola et al. 1990). Based on this it was defined as a cell proliferation marker (Starborg et al. 1996). Thymic and/or peripheral Treg alterations could be explained based on their proliferation status, therefore flow cytometry was conducted in order to quantify Ki67 expression of Treg subpopulations in the thymus and secondary lymphoid organs; spleen and PLN. All female 11 weeks of age 4 FVB-RAG-GFP, 3 NOD-RAG-GFP and 4 B-KO mice, were used for this experiment.

3.4.1 Abnormal proliferation of thymic mTregs but not pTregs does not explain Treg alterations in NOD mice

Following an optimised protocol, we were able to detect the expression of Foxp3, GFP and Ki67 simultaneously on Tregs. This is illustrated on Figure 1.10 in which Ki67 is detected clearly in all three groups of mice (FVB-RAG-GFP, NOD-RAG-GFP and B-KO) (Fig.1.10A) as well as GFP expression of Tregs (Fig.1.10B). Although there was no significant difference in either the frequency or absolute number of Ki67$^+$ mTregs, that may well be explained by the small number of mice used, there is a trend towards an increase in the frequency of proliferating cells of B-KO and NOD-RAG-GFP mice (Fig.1.11A and B). In addition, pTregs showed no abnormalities in regards to their proliferation status (Fig.1.12 G and H). Initially, the total thymocyte number was measured and surprisingly in contrast to our previous findings where the total thymus number of B6 and NOD mice were similar (Fig. 1.2C and 1.4E), there was a significant decrease in both B-KO and NOD-RAG-GFP mice in comparison to FVB-RAG-GFP (Fig.1.11I). Due to the fact that data were pooled from different experiments with identical protocol, experiment-to-experiment variation may be a major source of variation. Although there was no significant difference in either the frequency or absolute number of mTregs, there seems to be a slight increase in frequency of B-KO and NOD-RAG-GFP mice, yet the spread of data and small number of mice mean this is not statistically significant (Fig.1.11C and D). There was also no significant difference in both the frequency and absolute number of ND mTregs among the strains (Fig.1.11E and F). In addition, no significant difference was observed in either the frequency or absolute number of resident mTregs among the strains (Fig.1.11G and H). The initial smaller thymocyte number of both B-KO and NOD-RAG-GFP mice may account for the constant decrease in mTreg (Fig.1.11) and pTreg (Fig.1.12) absolute numbers in comparison to FVB-RAG-GFP mice.

3.4.2 B cells may be involved in splenic Treg proportion regulation via direct or indirect effect on their proliferation

GFP was not detected in the peripheral Tregs (data not shown) presumably due to a change in protocol that required extra staining and washing steps in order to include Ki67 and track the proliferation status of peripheral Tregs. As a result,

due to the absence of GFP, monitoring of recent thymic emigrants (RTEs) was not possible and could only observe total Tregs instead in secondary lymphoid organs. However future investigations observing RTEs are imperative as we previously observed alterations in RTEs of PLNs (Fig. 1.8C and D). Initially, the absolute number of splenocytes was observed to see if there are any defects at this level and a significant decrease was noted in B-KO mice compared to FVB-RAG-GFP and NOD-RAG-GFP mice (Fig.1.13A). There was also a significant increase in frequency of both B-KO and NOD-RAG-GFP splenic Tregs in comparison to FVB-RAG-GFP with B-KO splenic Tregs significantly more than NOD-RAG-GFP (Fig.1.13B). However, a significant decrease in absolute number of B-KO splenic Tregs was observed compared to the FVB-RAG-GFP mice and there was no significant difference with the NOD-RAG-GFP mice (Fig.1.13C). Ki67 expression showed an increase in the frequency of splenic Ki67$^+$ Tregs of B-KO mice in comparison to FVB-RAG-GFP (Fig.1.13D). In contrast, Figure 1.13E showed no significant difference in absolute number of splenic Ki67$^+$ Tregs among the strains which contrasts with the decrease in total splenocyte number (Fig.1.13A).

3.4.3 No detectable differences in Tregs residing in pancreatic lymph nodes of old NOD mice in comparison to FVB mice total PLN cell numbers

Although there was no significant difference in total PLN cell number among the strains, there seems to be a trend of decreased B-KO and NOD-RAG-GFP total PLN number that is not significant due to the spread in PLN cell numbers in FVB mice (Fig.1.14A). A significant decrease in frequency of B-KO PLN Tregs compared to both FVB-RAG-GFP and NOD-RAG-GFP mice was noted (Fig.1.14B). Similarly, there was a significant decrease in the absolute number of B-KO PLN Tregs compared to FVB-RAG-GFP mice with no significant difference with NOD-RAG-GFP mice (Fig.1.14C). In addition, there was also no significant difference in frequency of PLN Ki67$^+$ Tregs among the strains (Fig.1.14D). The absolute numbers of PLN Ki67$^+$ Tregs among the strains had a similar trend to the total PLN cell numbers (Fig.1.14A), with a significant decrease in the Ki67$^+$ Tregs in B-KO mice compared with the FVB-RAG-GFP mice (Fig.1.14E).

3.4.4 Treg proliferation status may be disease-stage dependent in B-KO mice

As mentioned previously in Section 1.6, T1D in NOD mice develops over a defined time course divided in three distinct periods; sensitisation, regulation and aggression. Thus far, homeostasis of Treg populations was observed during the regulation period, 8-14 weeks of age, a critical point of regulation in the PLNs and islets before aggression. The next question was whether Treg alterations observed during the regulation period applied in other periods as well. Therefore, it was decided to observe the homeostatic proliferation of thymic and peripheral Treg populations using RAG-GFP mice at a younger age, 4-6 weeks old, during the sensitisation period. A total of 18 female (7 FVB-RAG-GFP, 8 NOD-RAG-GFP and 3 B-KO) 4-6 weeks of age mice were used for this experiment. Gating strategy applied for this experiment was identical to the one using the older mice (Fig.1.10A).

3.4.5 B cells may play a role in thymic pTreg proliferation of young NOD mice

The total thymocyte number among the strains was similar with no significant difference (Fig.1.15A). There was also no significant difference in either the frequency or absolute number of mTregs among the strains with an unexpected level of variance of the NOD-RAG-GFP mice absolute number that could be explained by the fact that the data were pulled together from two experiments performed on two different days with an identical protocol (Fig.1.15B and C). ND and resident mTregs showed no significant difference in both frequency and absolute number among the strains (Fig.1.15D, E, F and G). There was a significant increase in frequency of B-KO Ki67$^+$ mTregs compared to FVB-RAG-GFP and NOD-RAG-GFP mice (Fig.1.15H). However, there was no significant difference in the absolute numbers of Ki67$^+$ mTregs among the strains, although this could be accounted for by the small number of B-KO mice used (Fig.1.15I). Data in figure 1.15 were pooled for different experiments with identical protocol. Surprisingly, there was a significant decrease in frequency of NOD-RAG-GFP pTregs in comparison to FVB-RAG-GFP and B-KO mice (Fig.1.16A). Although there was no significant difference in the absolute numbers of pTregs among the

strains, looking at the trend of NOD-RAG-GFP mice there seems to be a slight decrease compared to FVB-RAG-GFP mice (Fig.1.16B). A significant decrease in frequency of ND pTregs was also observed in NOD-RAG-GFP mice compared to the other strains (Fig.1.16C) however, there was no significant difference in the absolute number of ND pTregs among the strains (Fig.1.16D). Looking at the trend of ND pTregs, there seems to be a subtle decrease in the absolute number of NOD-RAG-GFP ND pTregs in comparison to the FVB-RAG-GFP mice (Fig.1.16D). Although there was a significant increase in frequency of resident pTregs of B-KO mice compared to the other strains, more mice should be used in the future in order to validate this observation as the spread was too wide and only three B-KO mice were available and used at the time (Fig.1.16E). No significant difference in the absolute number of resident pTregs was observed among the strains (Fig.1.16F). Surprisingly, the frequency of Ki67$^+$ pTregs of B-KO mice was significantly increased compared to the other strains (Fig.1.16G). There was also a significant difference in the absolute number of Ki67$^+$ pTregs between FVB-RAG-GFP and B-KO mice (Fig.1.16H).

3.4.6 Increase of NOD splenic Tregs is not due to proliferation abnormalities

The total splenocyte number showed an expected significant decrease in B-KO mice compared to the other strains (Fig.1.17A). Looking at the frequency of splenic Tregs, there was a significant increase in B-KO mice (Fig.1.17B). In contrast to their frequency, the absolute number of B-KO splenic Tregs is significantly decreased in comparison to FVB-RAG-GFP and NOD-RAG-GFP (Fig.1.17C). No significant difference was observed in splenic Ki67$^+$ Tregs (Fig.1.17D). However, there was a significant decrease in the absolute number of splenic Ki67$^+$ Tregs of B-KO and NOD-RAG-GFP mice compared to FVB-RAG-GFP. In addition, the absolute number of splenic Ki67$^+$ Tregs of NOD-RAG-GFP mice was significantly increased in comparison to B-KO mice (Fig.1.17E).

3.4.7 Treg levels in pancreatic lymph nodes of young NOD mice seem to alter after pre-insulitis stage

Similarly to the total PLN number of older mice (Fig.1.14A), there was also no significant difference among the strains in younger mice (Fig.1.18A). In addition, there was no significant difference in either the frequency or absolute number of PLN Tregs between the three strains (Fig.1.18B and C). These finding are in contrast to the ones observed in older B-KO mice that seem to have roughly a 25% proportional deduction in frequency between the older B-KO PLN Tregs and the other strains, that is not seen in younger mice (Fig.1.14B). Lastly, Ki67 expression showed no significant difference in both the frequency and the absolute number of Ki67$^+$ PLN Tregs among the strains (Fig.1.18D and E).

Figures

A
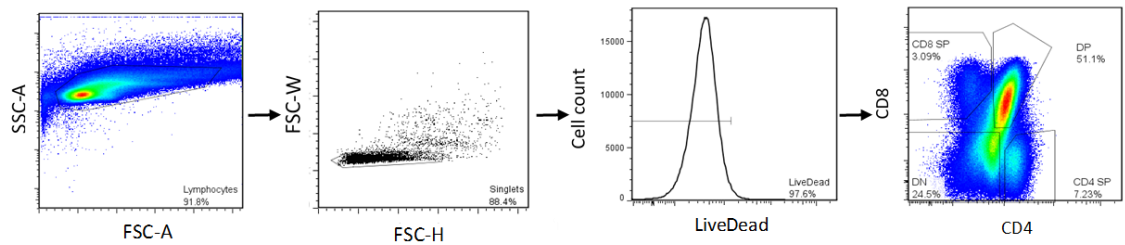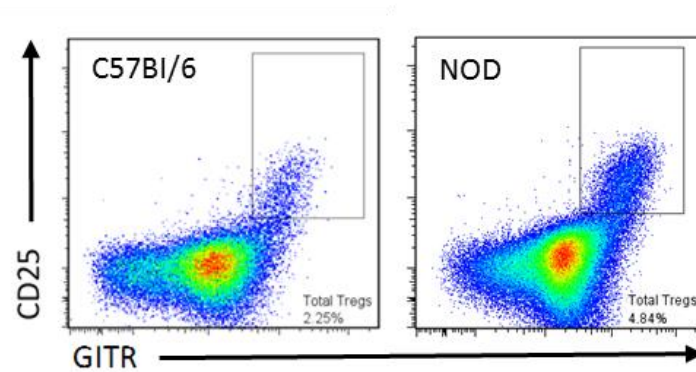

B

Fig.1.1 Gating strategies for measuring the frequency and absolute numbers of thymocyte subsets. (A) Representative flow plot of B6 thymocytes showing gating strategy; lymphocytes excluding doublets and dead cells, gating on CD4SP population (B) Representative flow plots showing the CD25 and GITR expression of CD4$^+$CD8$^-$ thymocytes, gate shows Total Tregs in B6 and NOD mice.
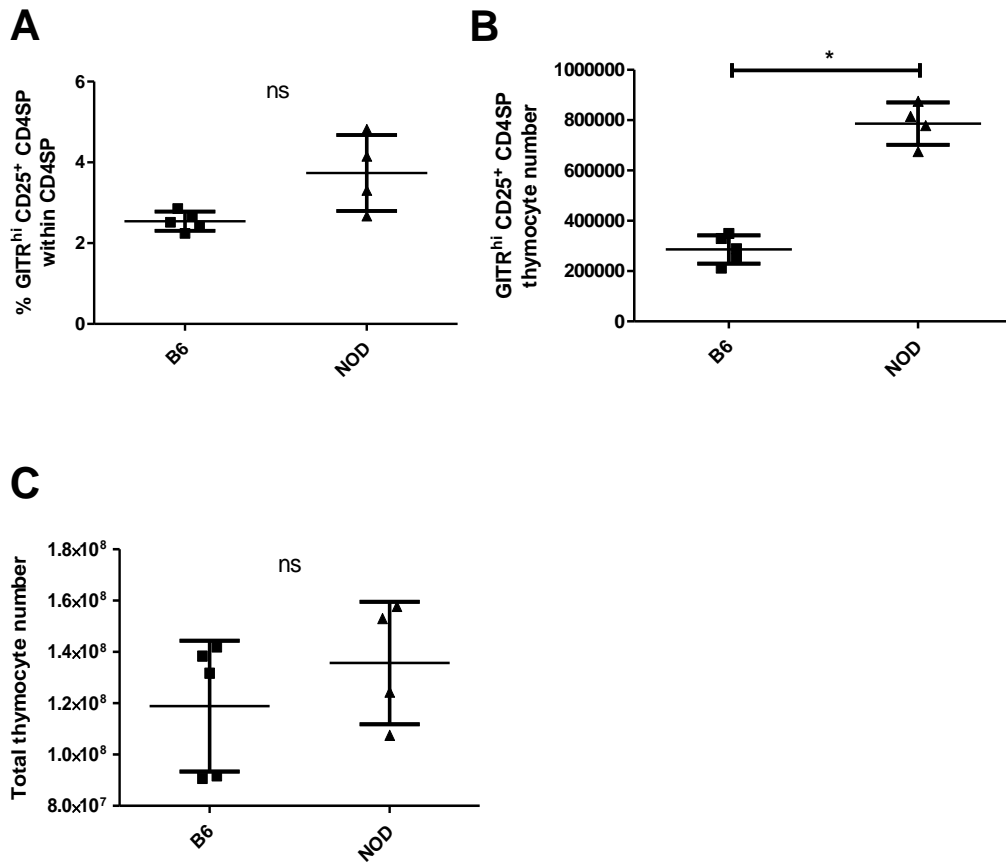
A

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Fig.1.2 Total Tregs increase in NOD compared to B6 mice. (A) Frequency of
GITR^{hi} CD25^{+} CD4SP cells, from B6 and NOD mice. Frequency shown is
percentage within CD4SP population. (B) Absolute number of GITR^{hi} CD25^{+}
CD4SP thymocytes, from B6 and NOD mice. (C) Absolute number of total
thymocytes, from B6 and NOD mice (mice age; 8-10 wks old, 5 B6 mice were
males and 4 NOD mice were females, from a single experiment). Data presented
as mean and scatter plot; p values were determined using non-parametric Mann-
Whitney U test, *p<0.05, ns= not significant, error bars = standard deviation.
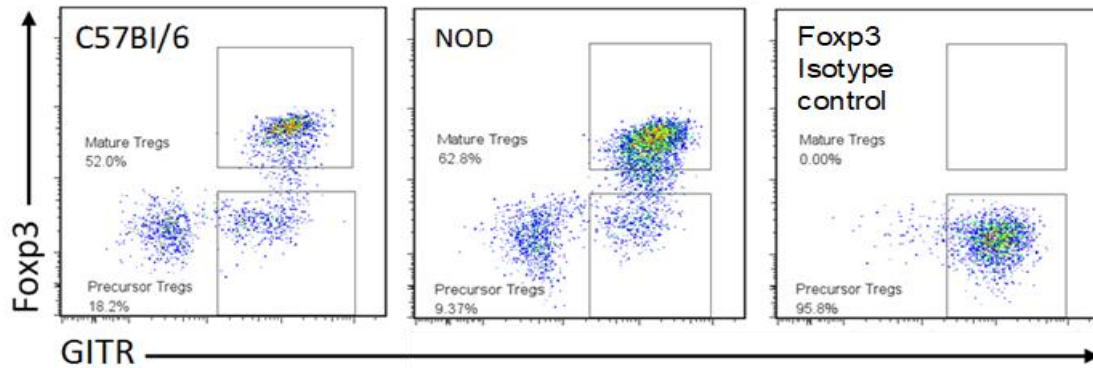
Fig.1.3 Gating strategy for measuring the frequency and absolute numbers of precursor and mature Tregs. Representative flow plots showing the Foxp3 and GITR expression gated on CD4SP CD25^{+} thymocytes showing precursor and mature Tregs within the subset.
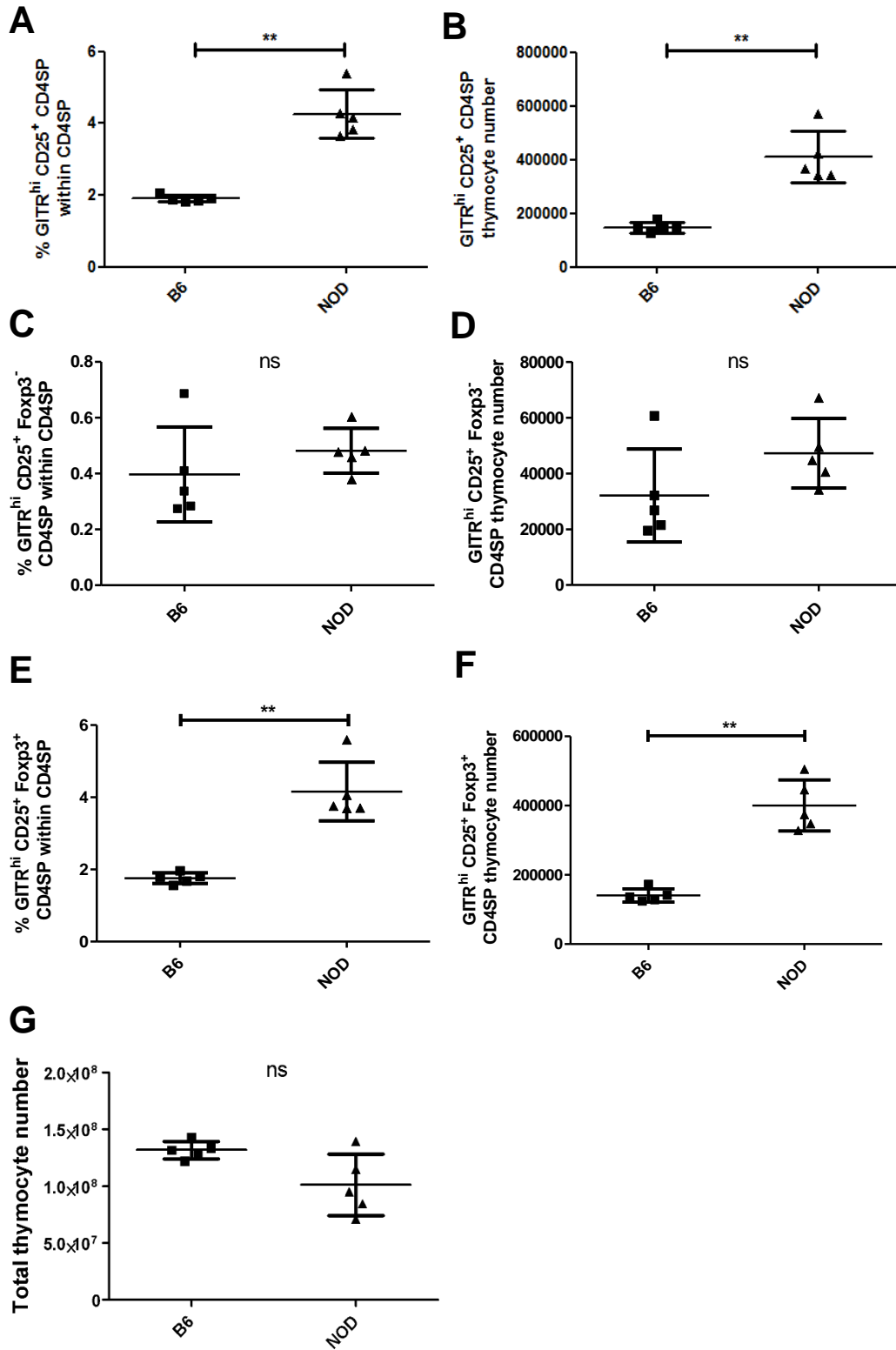
Fig.1.4 Thymic mature Tregs increase in NOD compared to B6 mice. (A) Frequency of CD4SP GITR^{hi}CD25$^+$ cells, from B6 and NOD mice. (B) Absolute number of CD4SP GITR^{hi}CD25$^+$ cells, from B6 and NOD mice. (C) Frequency of CD4SP GITR^{hi}CD25$^+$Foxp3$^-$ cells, from B6 and NOD mice. (D) Absolute number of CD4SP GITR^{hi}CD25$^+$Foxp3$^-$ thymocytes, from B6 and NOD mice. (E) Frequency of CD4SP GITR^{hi}CD25$^+$Foxp3$^+$ cells, from B6 and NOD mice. (F) Absolute number of GITR^{hi}CD25$^+$Foxp3$^+$CD4SP thymocytes, from B6 and NOD mice. (G) Absolute number of total thymocytes, from B6 and NOD mice. (mice age; 13 wks old, all female, n= 5 per group from a single experiment) Frequencies shown is percentage within CD4SP population. Data presented as mean and scatter plot; p values were determined using non-parametric Mann-Whitney U test, *p<0.05, ns= not significant, error bars = standard deviation.

A

B

Fig.1.5 Gating strategy for measuring the frequency and absolute numbers of newly developed and resident total thymocyte Tregs. (A) Representative histogram showing the GFP intensity of thymocyte populations; double-negative (DN), CD8 and CD4 single positive (CD8 SP, CD4 SP) and double-positive (DP), from FVB-RAG2p-GFP mice. (B) Representative flow plots showing the GFP expression gated on CD4^{+}CD8^{-}GITR^{hi}CD25^{+} thymocytes, gate shows newly developed (GFP^{+}) and resident (GFP^{-}) total Tregs.
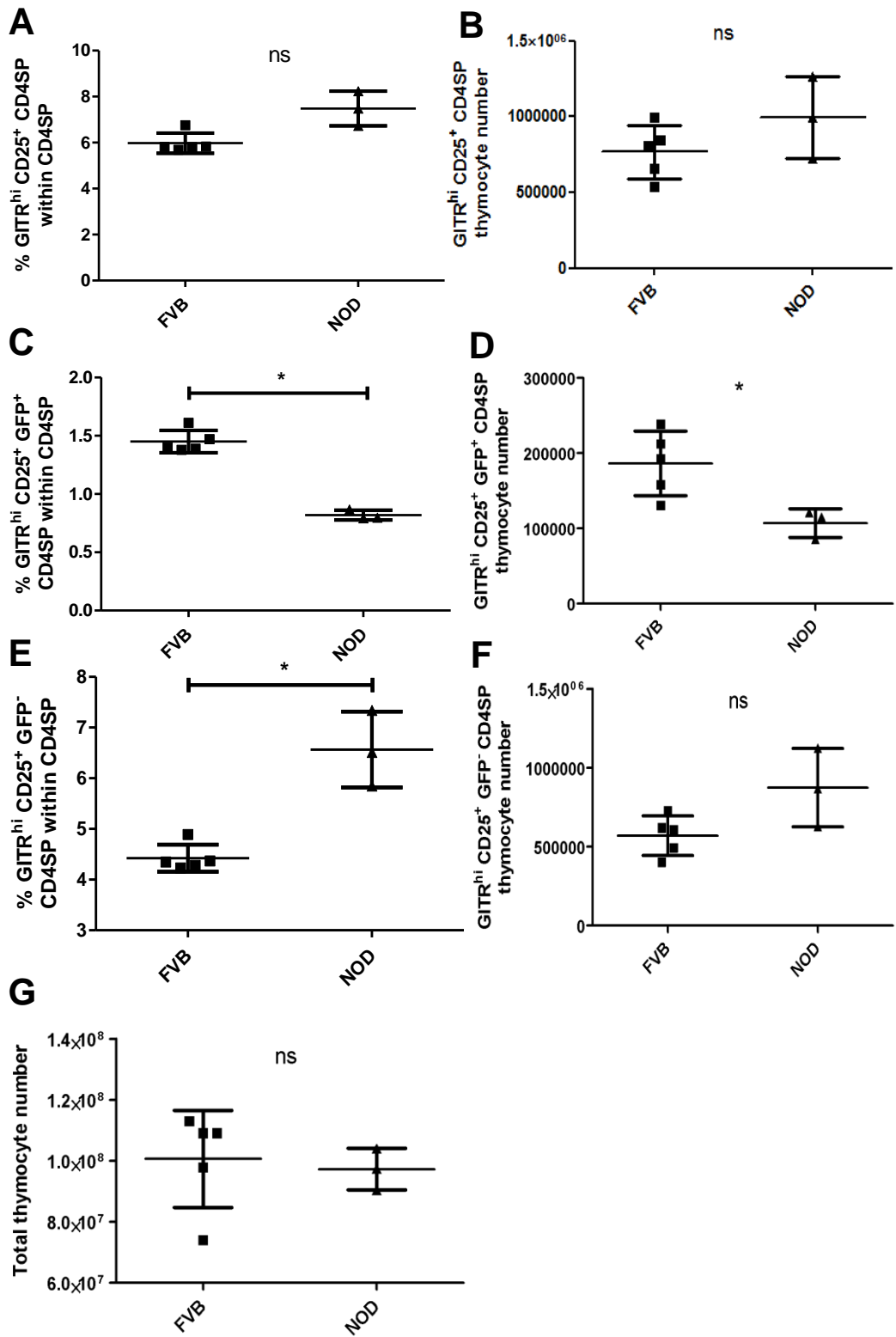
Fig.1.6 Abnormalities in Treg development of NOD mice. (A) Frequency of GITR^{hi}CD25^{+}CD4SP cells within CD4SP thymocytes population, from FVB-RAG-GFP and NOD-RAG-GFP mice. (B) Absolute number of GITR^{hi}CD25^{+}CD4SP thymocytes, from FVB-RAG-GFP and NOD-RAG-GFP mice. Frequency of GITR^{hi}CD25^{+}GFP^{+}CD4SP (C) and GITR^{hi}CD25^{+}GFP^{-}CD4SP (E) cells within CD4SP thymocytes population, from FVB-RAG-GFP and NOD-RAG-GFP mice. Absolute number of GITR^{hi}CD25^{+} GFP^{+}CD4SP (D) and GITR^{hi}CD25^{+}GFP^{-}CD4SP (F) thymocytes, from FVB-RAG-GFP and NOD-RAG-GFP mice. (G) Absolute number of total thymocytes, from FVB-RAG-GFP and NOD-RAG-GFP mice. (mice age; 13-14 wks old, all female, n= 3-6 per group from a single experiment) Data presented as mean and scatter plot; p values were determined using non-parametric Mann-Whitney U test, *p<0.05, ns= not significant, error bars = standard deviation.
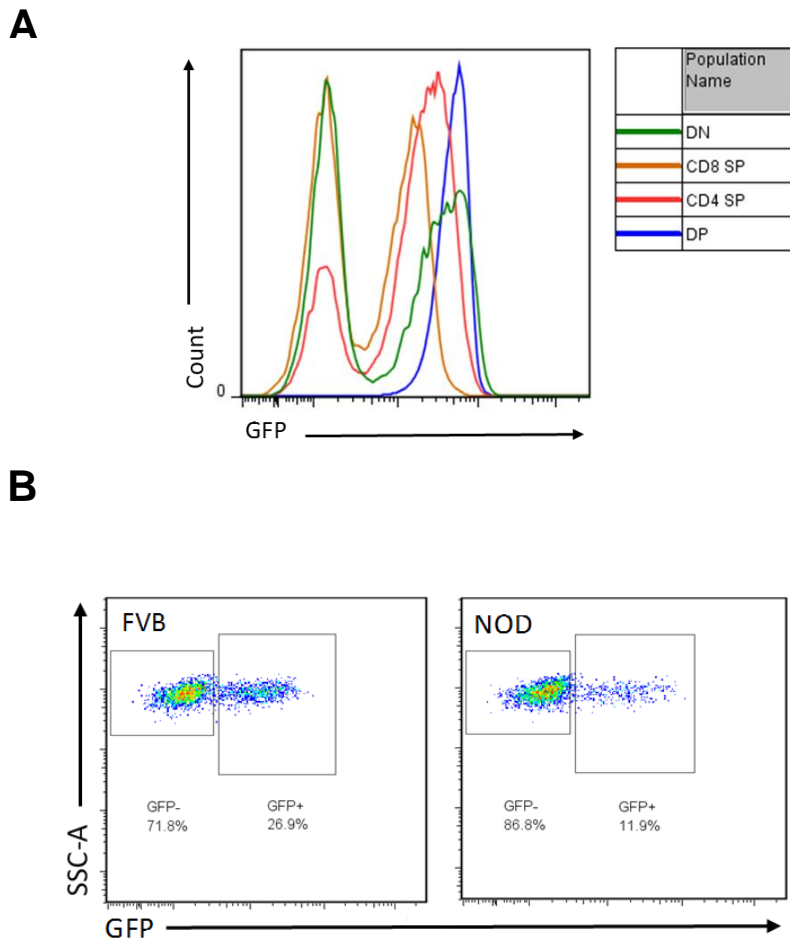
Fig.1.7 Gating strategy for measuring the frequency and absolute number of GFP expression in secondary lymphoid organs.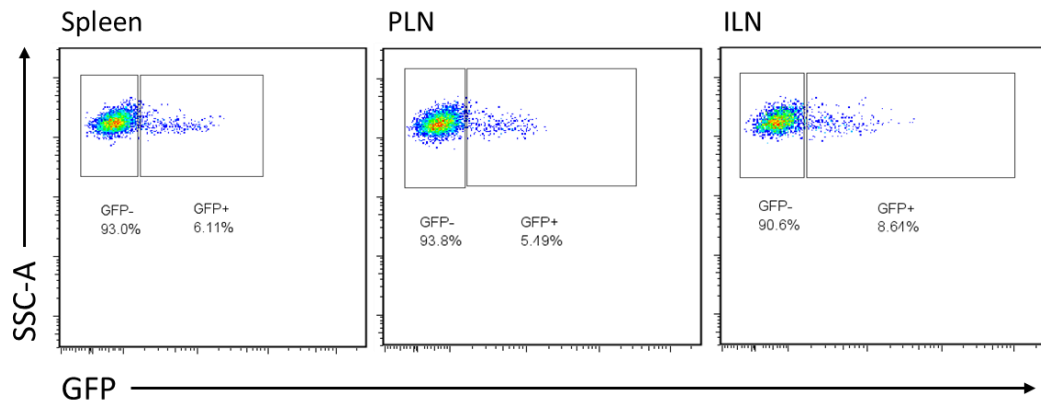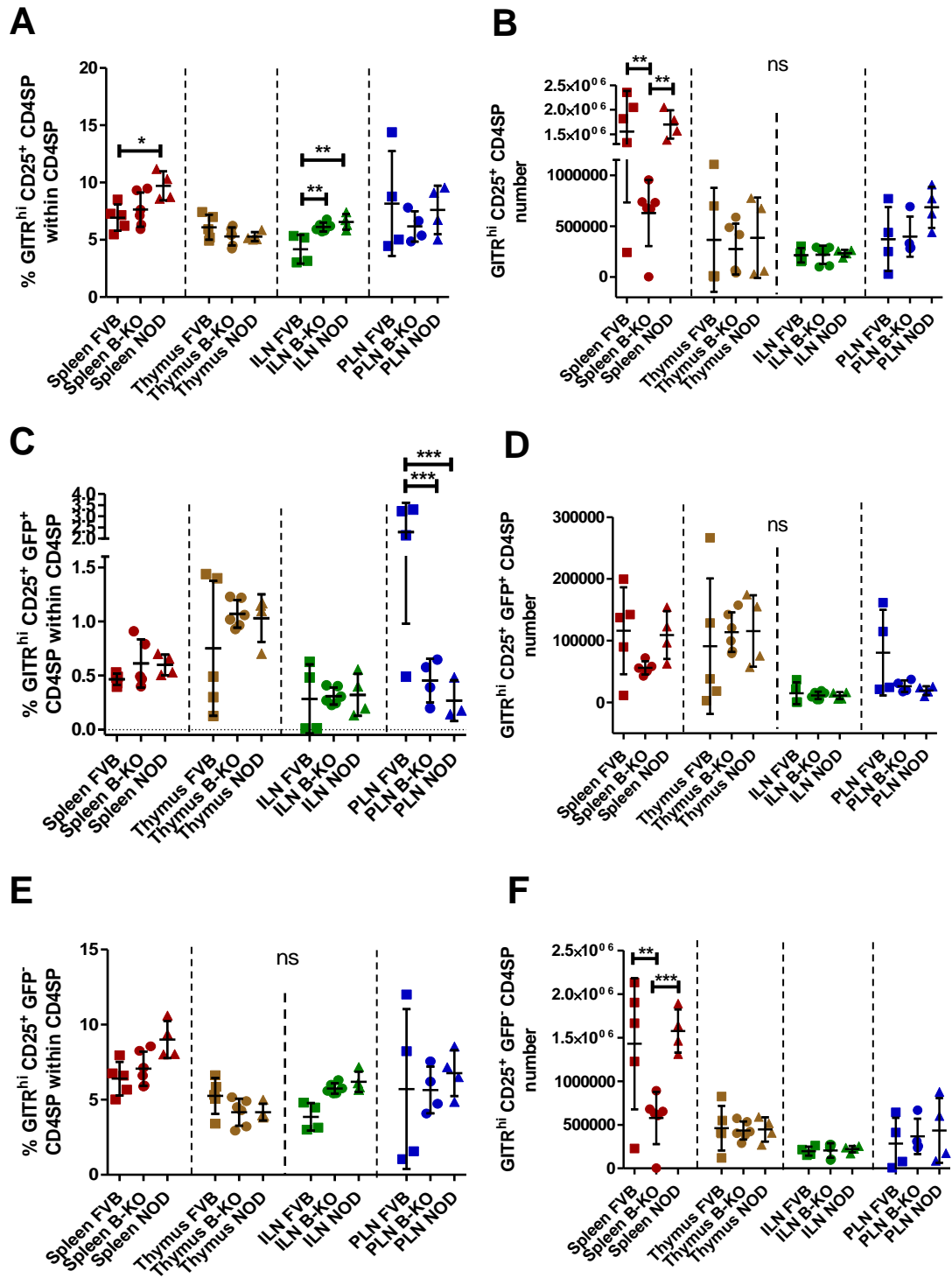 Representative flow plots of FVB-RAG-GFP mice gated on $CD4^+CD8^-GITR^{hi}CD25^+$ population showing the GFP expression, gate shows recent thymic emigrants (RTEs) (GFP^+) and resident (GFP^-) total Tregs in the spleen, pancreatic lymph nodes (PLN) and inguinal lymph nodes (ILN).
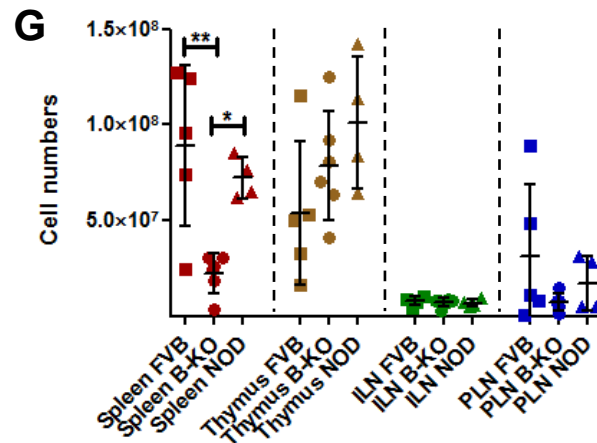
Fig.1.8 Diverse alterations of Treg numbers depending on the tissue environment. Frequency within CD4SP population (A) and absolute number (B) of GITR^{hi}CD25$^+$CD4SP cells of FVB-RAG-GFP, NODuMTKO-RAG-GFP (B-KO) and NOD-RAG-GFP; spleen, thymus, ILN$_s$ and PLN$_s$. Frequency within CD4SP population (C) and absolute number (D) of GITR^{hi}CD25$^+$GFP$^+$CD4SP cells of FVB-RAG-GFP, NODuMTKO-RAG-GFP (B-KO) and NOD-RAG-GFP; spleen, thymus, ILN$_s$ and PLN$_s$. Frequency within CD4SP population (E) and absolute number (F) of GITR^{hi}CD25$^+$GFP$^-$CD4SP cells of FVB-RAG-GFP, NODuMTKO-RAG-GFP (B-KO) and NOD-RAG-GFP; spleen, thymus, ILN$_s$ and PLN$_s$. (G) Absolute cell number of spleen, thymus, ILN$_s$ and PLN$_s$. (mice age; 9-11 wks old, all female, n= 4-6 per group pooled from two independent experiments with identical protocol). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *p<0.05, **p<0.01, ***p<0.001, ns= not significant, error bars = standard deviation.
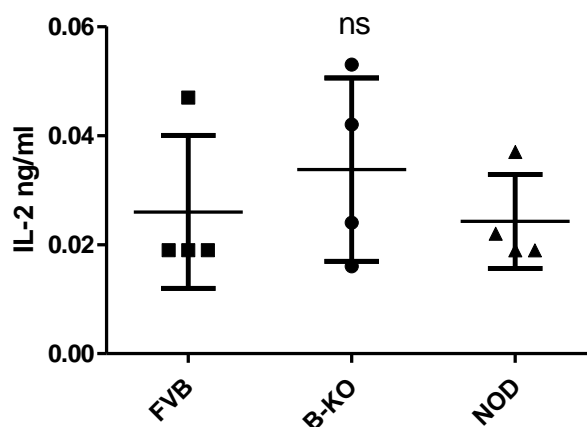
Fig.1.9 No change in IL-2 levels. IL-2 levels of thymic supernatants measured using ELISA with 2pg/mL sensitivity (mice age; 8-11 wks old, all female, n=12 from a single experiment). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, ns= not significant, error bars = standard deviation.

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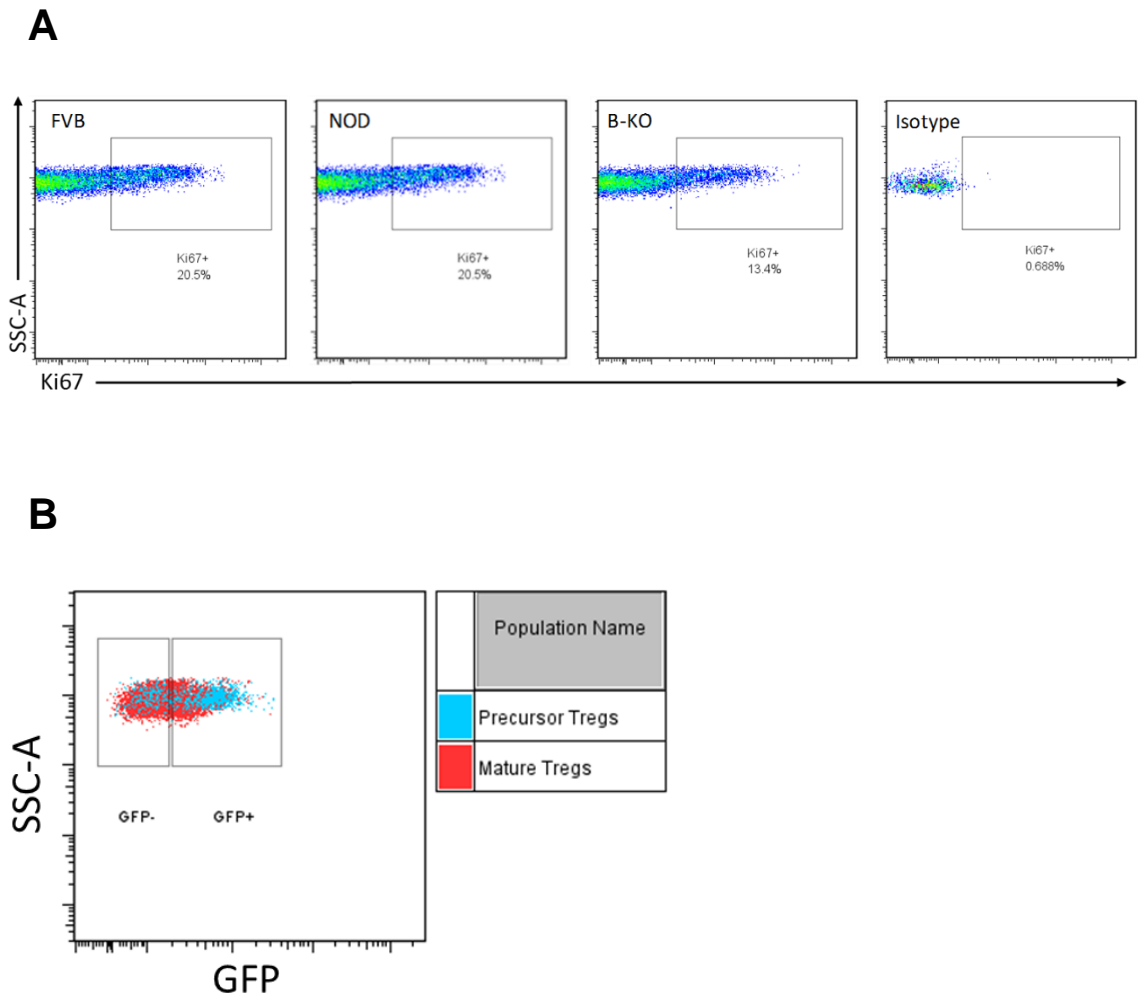
Fig.1.10 Gating strategy for measuring the frequency and absolute number of Tregs expressing Ki67. (A) Representative flow plots showing the Ki67 expression gated on CD4SPGITR^{hi}CD25$^+$Foxp3$^+$ thymocytes of FVB-RAG-GFP, NOD-RAG-GFP and NODuMTKO-RAG-GFP (B-KO) mice. (B) Representative flow plot of FVB-RAG-GFP mice showing GFP expression of precursor and mature Tregs.

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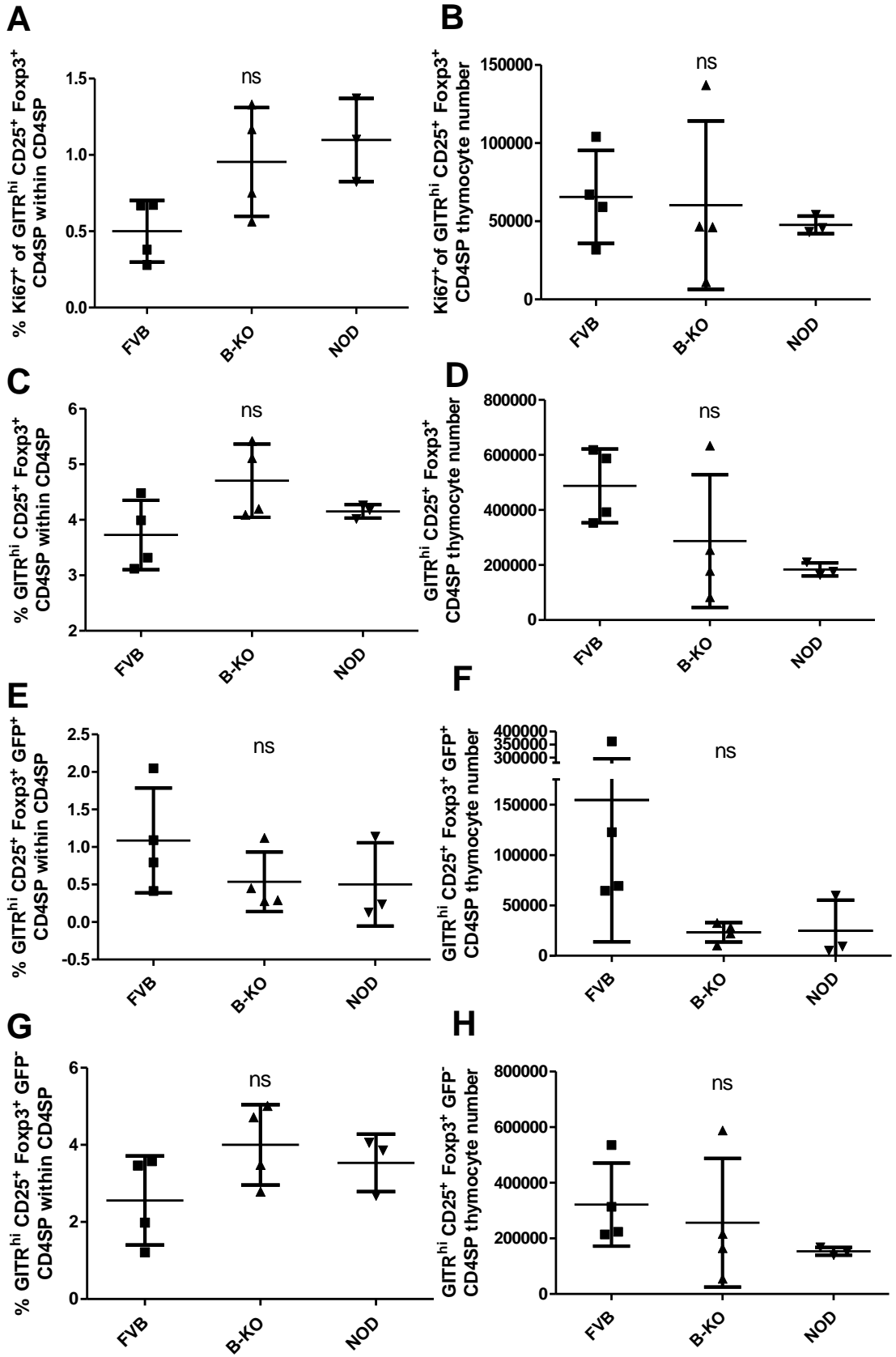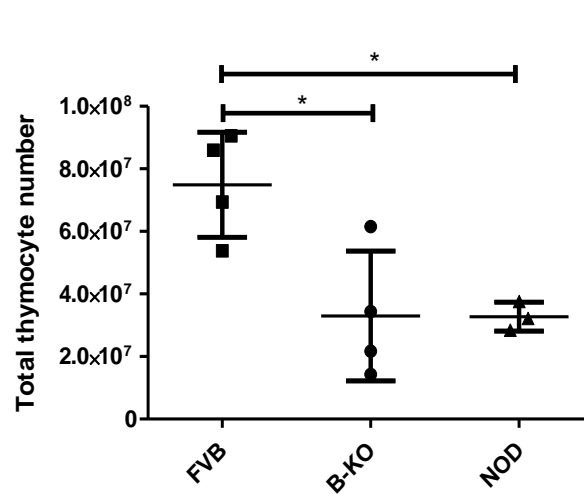
Fig.1.11 Trend towards an increase of mature Treg proliferation in the thymus of B-KO and NOD mice. (A) Absolute number of total thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (B) and absolute number (C) of GITR^{hi}CD25$^+$Foxp3$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (D) and absolute number (E) of GITR^{hi}CD25$^+$Foxp3$^+$GFP$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (F) and absolute number (G) of GITR^{hi}CD25$^+$Foxp3$^+$GFP$^-$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (H) and absolute number (I) of GITR^{hi}CD25$^+$Foxp3$^+$Ki67$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 11 wks old, all female, n= 3-4 per group from a single experiment). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *p<0.05, ns= not significant, error bars = standard deviation.

Fig.1.12 Decrease in newly developed precursor Tregs in B-KO and NOD mice is not due to proliferation abnormalities. Frequency within CD4SP population (A) and absolute number (B) of GITR^{hi}CD25$^+$Foxp3$^-$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (C) and absolute number (D) of GITR^{hi}CD25$^+$Foxp3$^-$GFP$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (E) and absolute number (F) of GITR^{hi}CD25$^+$Foxp3$^-$GFP$^-$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (G) and absolute number (H) of GITR^{hi}CD25$^+$Foxp3$^-$Ki67$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 11 wks old, all female, n= 3-4 per group from a single experiment (Fig.1.11)). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *p<0.05, **p<0.01, ***p<0.001, ns= not significant, error bars = standard deviation.

Fig.1.13 Abnormal increase of Treg proliferation in the spleen of B-KO mouse. (A) Absolute number of total splenocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (B) and absolute number (C) of GITR^{hi}CD25$^+$Foxp3$^+$CD4SP splenocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (D) and absolute number (E) of GITR^{hi}CD25$^+$Foxp3$^+$Ki67$^+$CD4SP splenocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 11 wks old, all female, n= 3-4 per group from a single experiment (Fig.1.11). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *p<0.05, **p<0.01, ***p<0.001, ns= not significant, error bars = standard deviation.

Fig.1.14 PLN Treg decrease in B-KO mice not due to proliferation abnormalities (A) Absolute number of total PLN cell number, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (B) and absolute number (C) of $GITR^{hi}CD25^{+}Foxp3^{+}CD4SP$ PLN_s, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (D) and absolute number (E) of $GITR^{hi}CD25^{+}Foxp3^{+}Ki67^{+}CD4SP$ thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 11 wks old, all female, n= 3-4 per group from a single experiment (Fig.1.11)). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *p<0.05, ns= not significant, error bars = standard deviation.
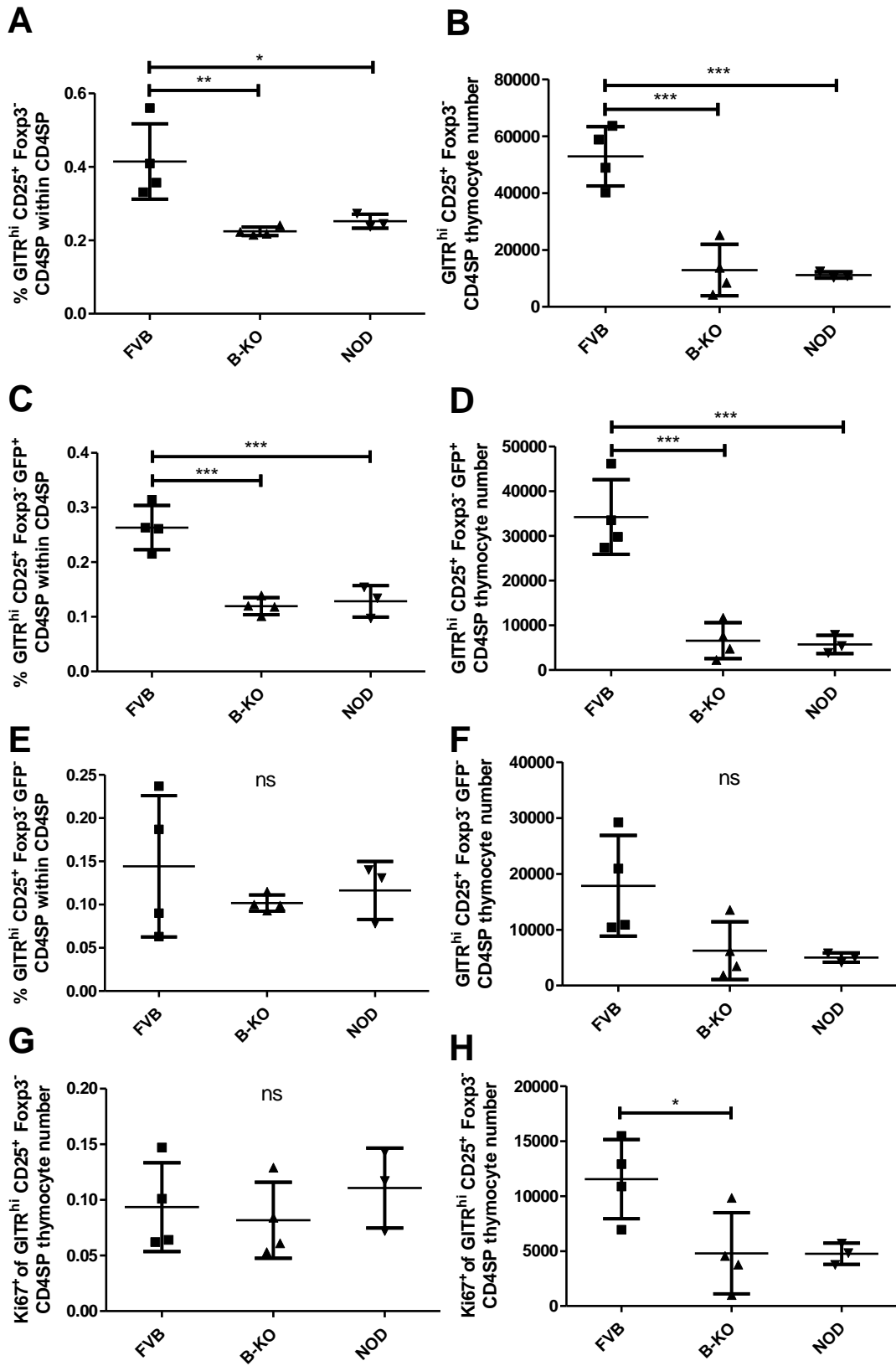
Fig.1.15 Treg development not compromised in younger mice (A) Absolute number of total thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (B) and absolute number (C) of GITR^{hi}CD25$^+$Foxp3$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (D) and absolute number (E) of GITR^{hi}CD25$^+$Foxp3$^+$GFP$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (F) and absolute number (G) of GITR^{hi}CD25$^+$Foxp3$^+$GFP$^-$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (H) and absolute number (I) of GITR^{hi}CD25$^+$Foxp3$^+$Ki67$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 4-6 wks old, all female, n= 3-7 per group from five independent experiments with identical protocol). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *p<0.05, ns= not significant, error bars = standard deviation.

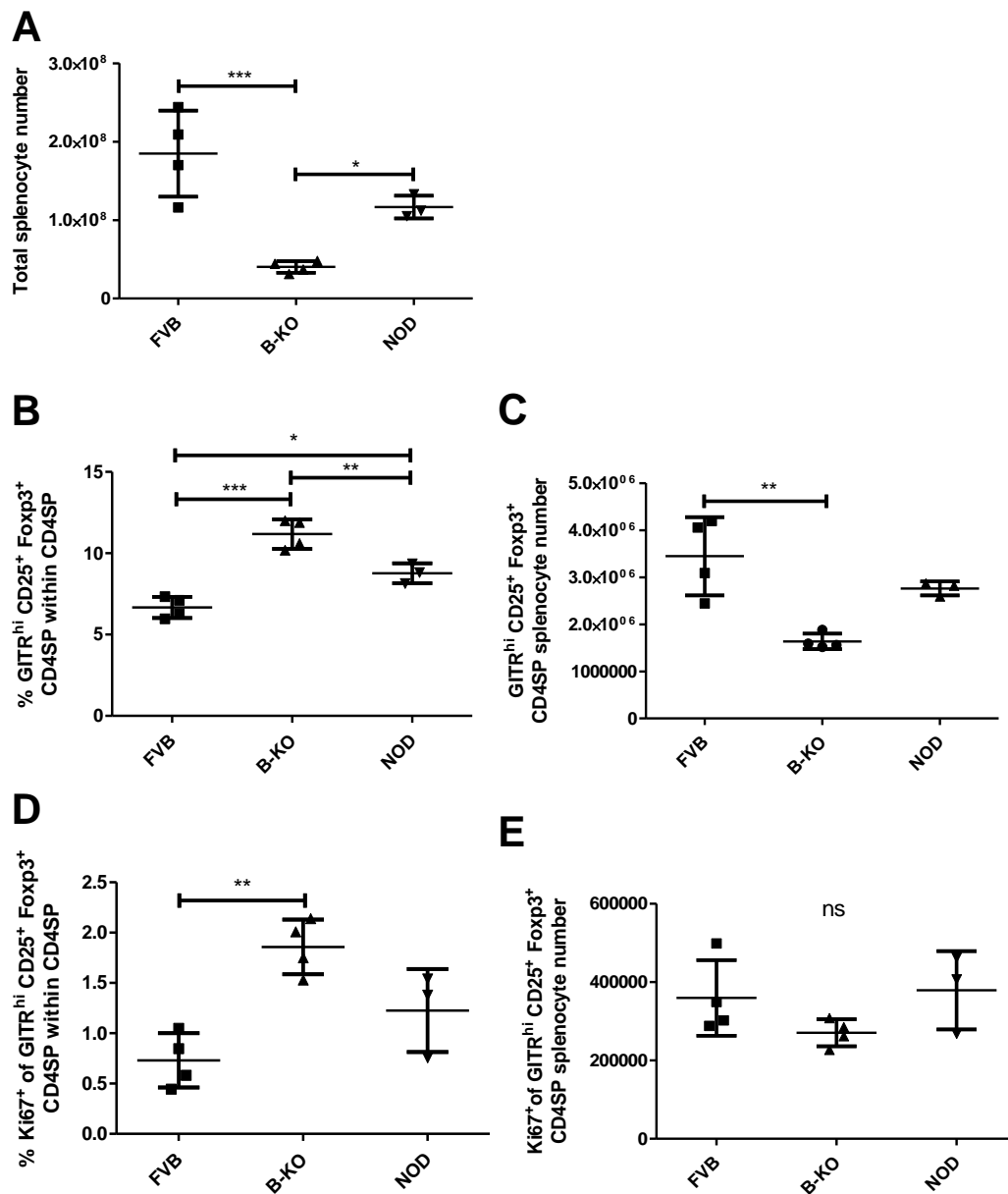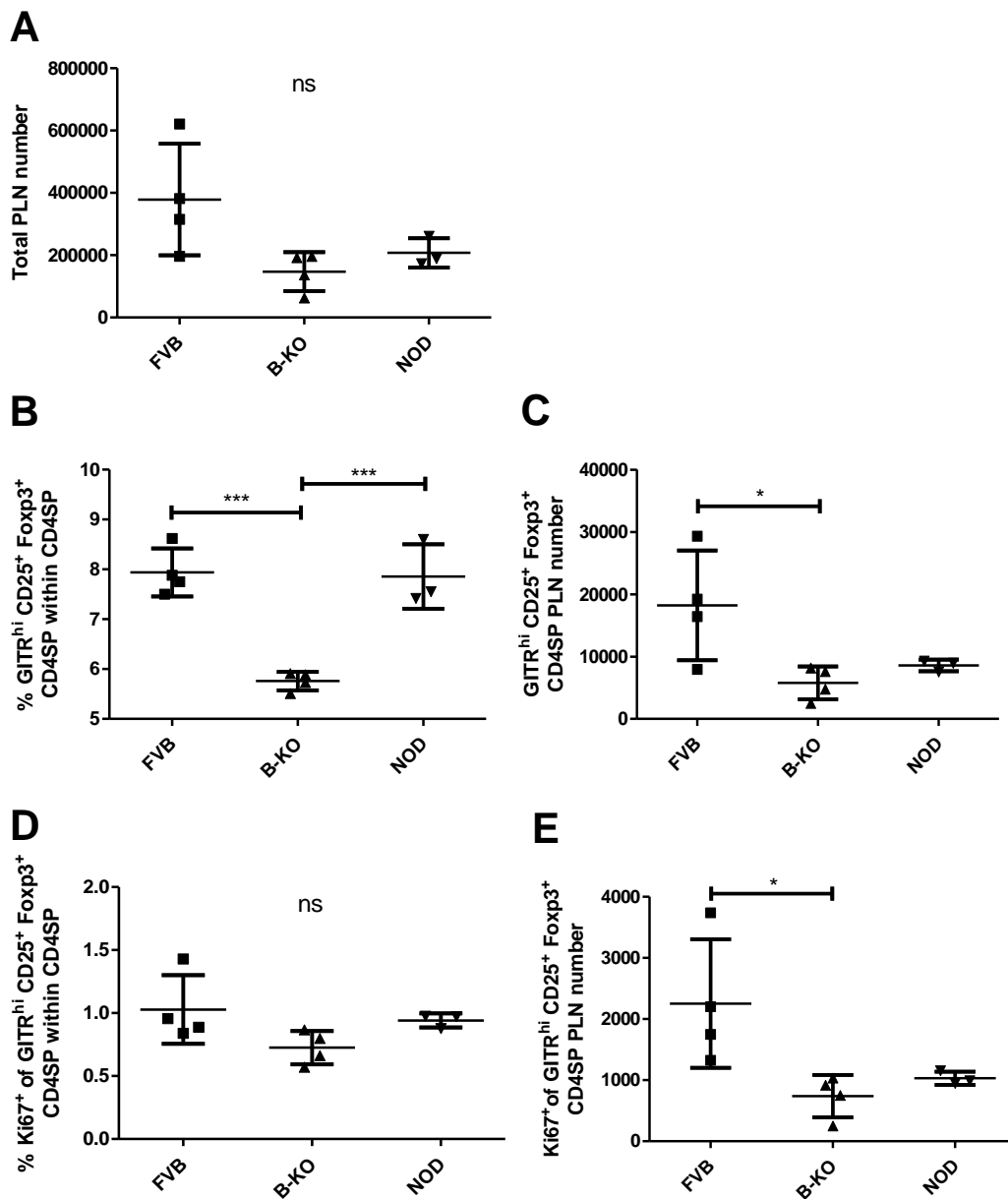**Fig.1.16 Increase in precursor Treg proliferation of younger B-KO mice.**
Frequency within CD4SP population (A) and absolute number (B) of GITR^{hi}CD25$^+$Foxp3$^-$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (C) and absolute number (D) of GITR^{hi}CD25$^+$Foxp3$^-$GFP$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (E) and absolute number (F) of GITR^{hi}CD25$^+$Foxp3$^-$GFP$^-$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (G) and absolute number (H) of GITR^{hi}CD25$^+$Foxp3$^-$Ki67$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 4-6 wks old, all female, n= 3-7 per group from five independent experiments with identical protocol (Fig.1.15)). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns= not significant, error bars = standard deviation.
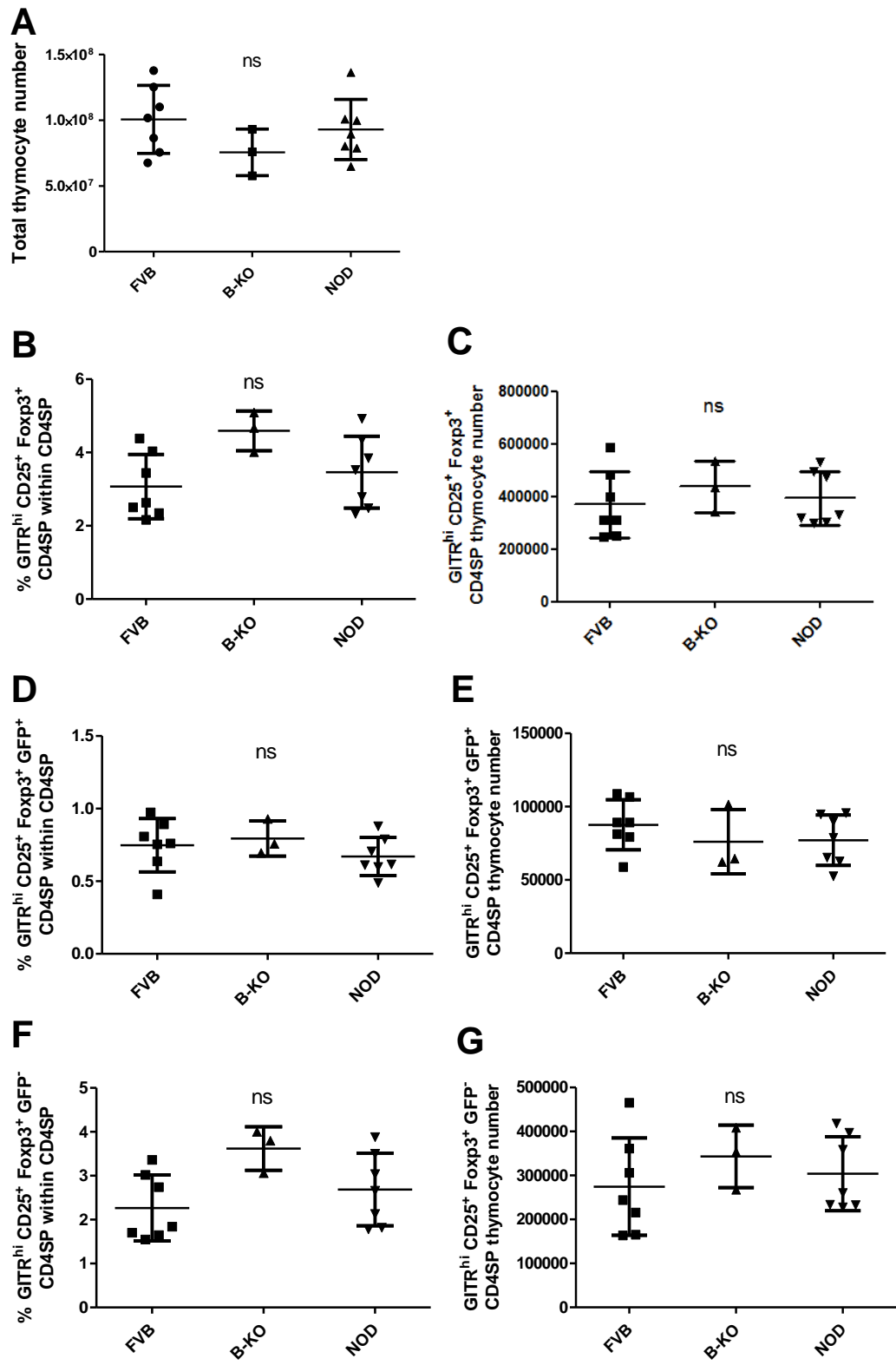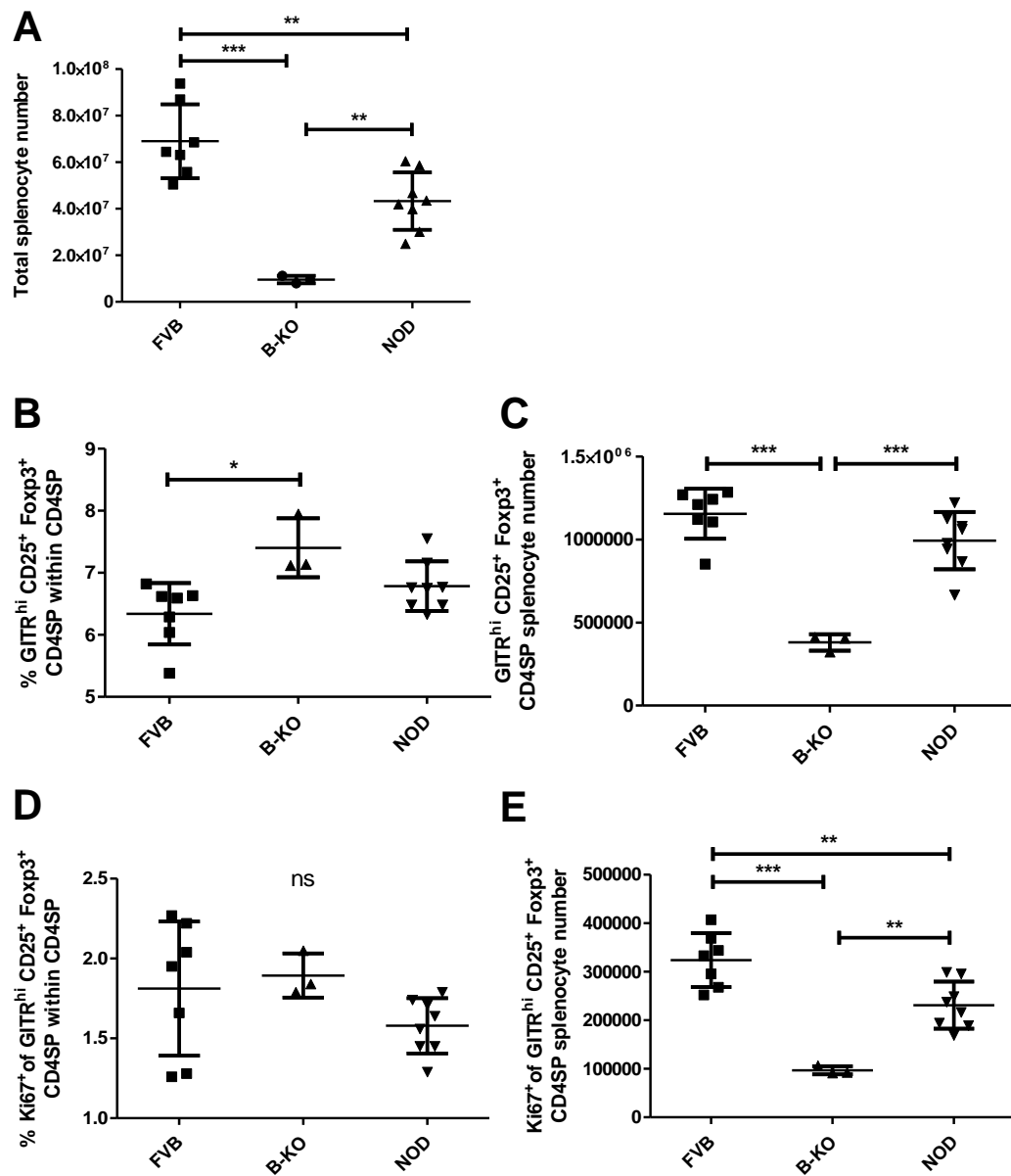
Fig.1.17 Normal splenic Treg proliferation of younger B-KO mice. (A)
Absolute number of total splenocytes, from FVB-RAG-GFP, NODuMTKO-RAG-
GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (B) and
absolute number (C) of GITR^{hi}CD25$^+$Foxp3$^+$CD4SP splenocytes, from FVB-RAG-
GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within
CD4SP population (D) and absolute number (E) of
GITR^{hi}CD25$^+$Foxp3$^+$Ki67$^+$CD4SP splenocytes, from FVB-RAG-GFP,
NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 4-6 wks old, all
female, n= 3-7 per group from a single experiment (Fig.1.15). Data presented as
mean and scatter plot; p values were determined using one-way ANOVA and
post hoc Tukey's test, *p<0.05, **p<0.01, ***p<0.001, ns= not significant, error
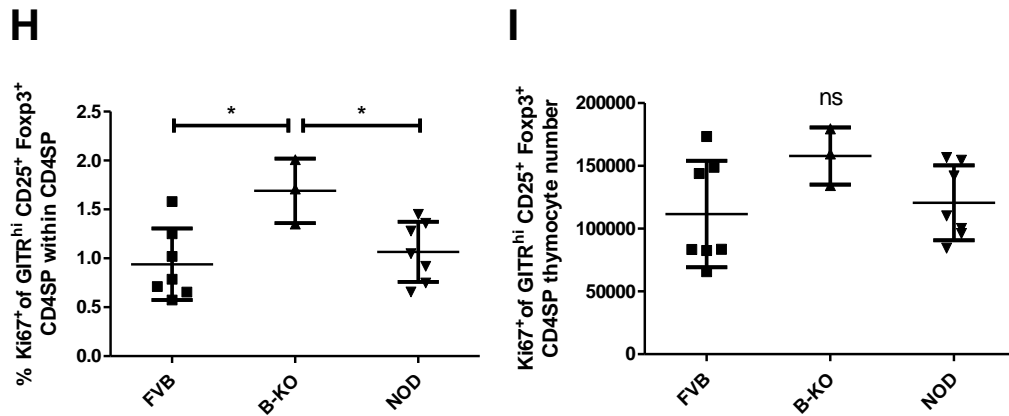bars = standard deviation.

Fig.1.18 No Treg alterations of PLNs in younger mice (A) Absolute number of total PLN cell number, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (B) and absolute number (C) of GITR^{hi}CD25$^+$Foxp3$^+$CD4SP PLNs, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. Frequency within CD4SP population (D) and absolute number (E) of GITR^{hi}CD25$^+$Foxp3$^+$Ki67$^+$CD4SP thymocytes, from FVB-RAG-GFP, NODuMTKO-RAG-GFP and NOD-RAG-GFP mice. (mice age; 4-6 wks old, all female, n= 3-7 per group from a single experiment (Fig.1.15)). Data presented as mean and scatter plot; p values were determined using one-way ANOVA and post hoc Tukey's test, ns= not significant, error bars = standard deviation.
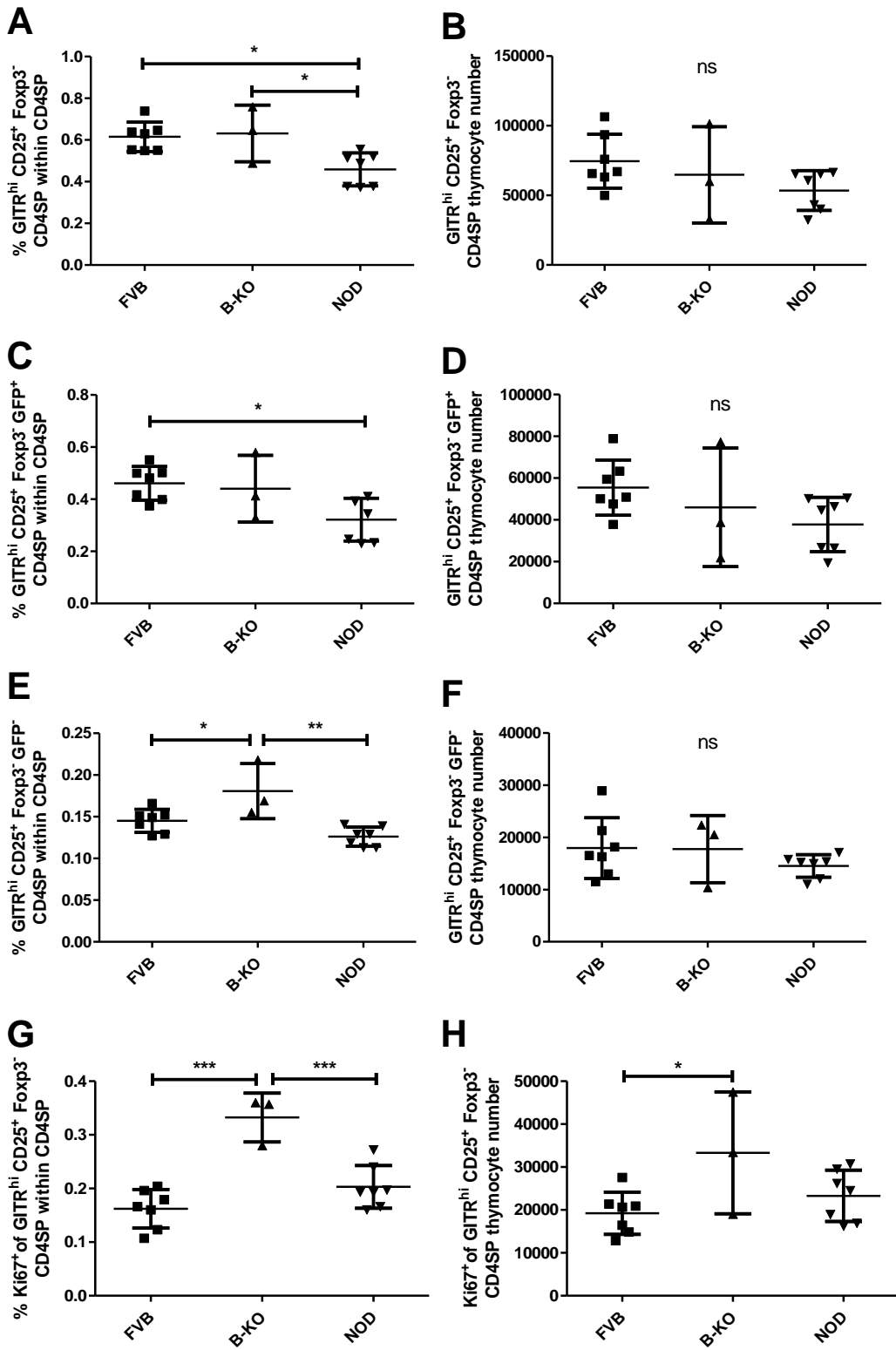
Discussion

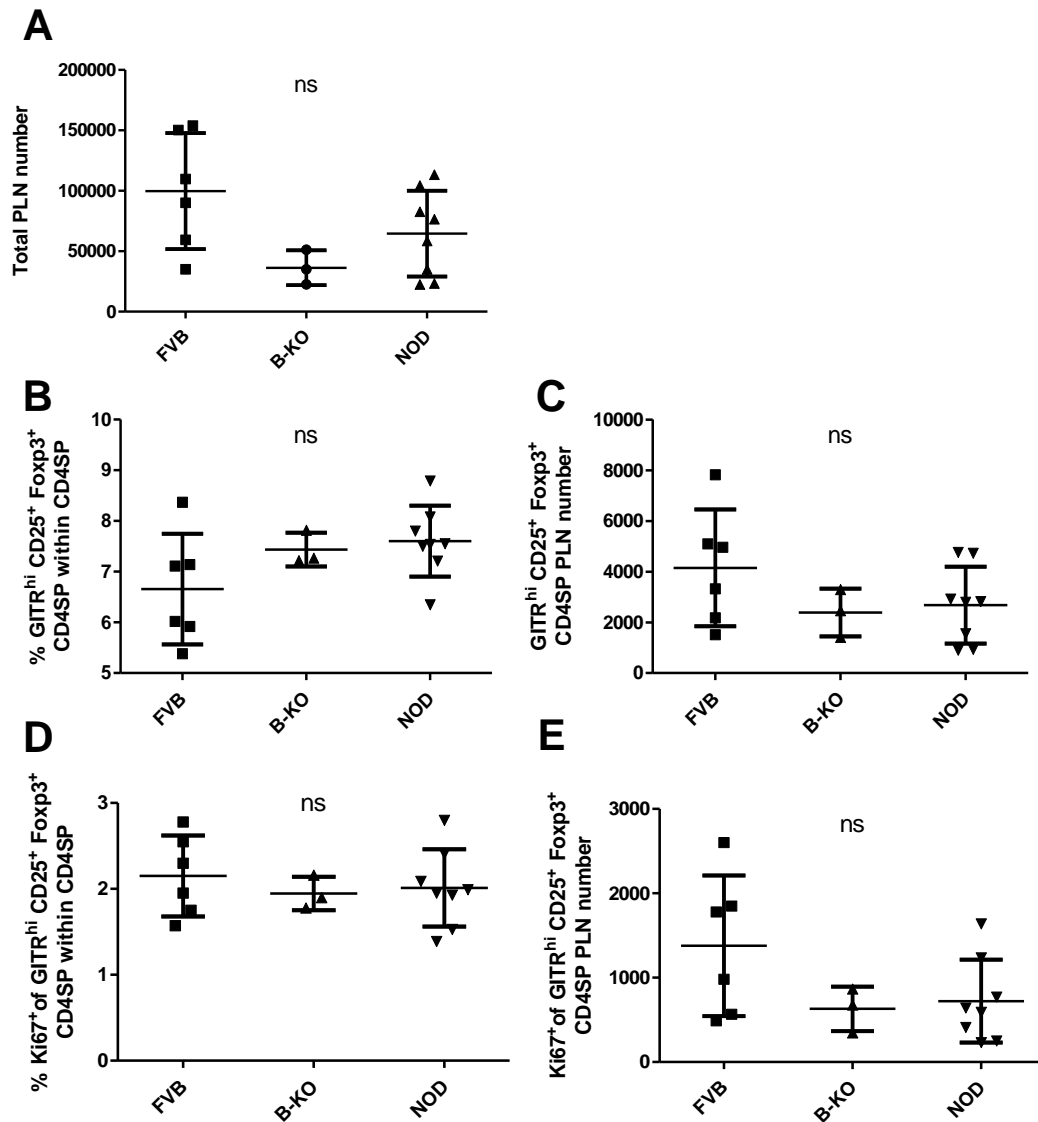Type 1 Diabetes (T1D) involves the failure of central tolerance in which autoreactive lymphocytes developing in the thymus somehow skip the elimination process, resulting in peripheral autoreactive T cells that escaped the thymus. It is an autoimmune disorder in which the insulin-producing β cells in the pancreas are destroyed by autoreactive T cells resulting in hyperglycaemia leading to short and long-term health conditions. Intriguingly, there is a great debate whether the paucity and/or defectiveness of Treg cells contribute or perpetuate the β cell destruction. Therefore, due to this contradictory body of findings regarding Treg numbers using diabetes-prone models, it is important to establish a pattern of Treg populations for a better understanding, as they seem to be a necessity in preventing autoimmune diseases such as T1D. This project aimed to revisit and investigate the development and particularly the homeostatic proliferation of Tregs in the thymus and the homeostatic proliferation in peripheral lymphoid organs using for the first time RAG-GFP expressing NOD and NOD μMTKO (B-KO) mice. Our investigations sought to observe Tregs in pre-diabetic NOD mice at 2 distinct periods of the disease progression; during 'Regulation' 8-14 weeks of age (old) and 'Sensitisation' 3-6 weeks of age (young). Initially using older mice, results showed an overall increase in thymic Tregs elucidated by an increase in both precursor and mature Tregs (hereafter called pTregs and mTregs, respectively) in comparison to B6 mice, signifying a developmental change in thymic Treg population. In addition, flow cytometric analysis also revealed a decrease in newly developed (ND) Tregs and an increase in resident Tregs identified as GFP$^+$ and GFP$^-$ respectively, in comparison to FVB-RAG-GFP mice. Interleukin-2 (IL-2) did not seem to be responsible for these alterations as there was no difference among the strains. However, based on Ki67 expression to assess Treg proliferation, intriguingly NOD thymic mTregs displayed a non-significant trend towards increase in proliferative status in comparison to FVB-RAG-GFP mice. Furthermore, we emphasize the importance of observing both the frequency and absolute number of Tregs as frequency is only reflective of other populations whereas absolute numbers are definitive. Moreover, we

speculate that B cells may affect homeostatic proliferation of Tregs in an age-dependent manner. In regards to peripheral Tregs residing in the spleen and pancreatic lymph nodes (PLNs), we observed a significant increase in frequency that was not however observed in the absolute numbers, of splenic Tregs and no alteration in PLN Tregs of NOD-RAG-GFP mice in comparison to FVB-RAG-GFP mice was documented despite age.

4.1 Homeostatic proliferation alterations in thymic Treg populations of NOD mice

4.1.1 Increase in thymic Treg numbers of NOD mice in comparison to B6

Tregs have been established to play an essential role in the maintenance of self-tolerance by controlling self-reactive T cells (Sakaguchi 2004). In regards to thymic Treg proportions using diabetes-prone mouse models, there have been controversial findings trying to establish in part the reasons of lack of peripheral tolerance leading to pancreatic β cell destruction in T1D. In our investigation using older NOD mice in comparison to slightly younger B6 mice as a control that were available at the time, we documented a trend towards increase in frequency and a significant increase in the absolute number of thymic Tregs (Fig.1.2A and B). Ideally the age of the mice should be the same, nonetheless both strains were still considered to be in the same phase of diabetes development that compromised for the slight age difference. Complementing our results, Feuerer M. *et al.* demonstrated in vitro using fetal thymus organ cultures of NOD mice, their capability of generating Tregs without defects in their thymic selection process in comparison to B6.H-2^{g7} mice, a NOD MHC class II-matched diabetes resistant mouse model (Feuerer et al. 2007). In addition, in agreement with our findings (Fig.1.2) more recently a relatively elevated frequency and absolute number of Tregs in NOD thymi was also displayed by Tellier J. *et al.* with a similar approach to ours using flow cytometry to analyze the co-expression of CD4, CD25 and Foxp3 of B6 and NOD mice (Tellier et al. 2013). However, Tellier J. *et al.* argues that it is very unlikely that this quantitative increase of Tregs in NOD mice is associated with their susceptibility to diabetes as both their control B6 mice and NOD mice developed diabetes with the same kinetics (Tellier et al. 2013). Furthermore, Zhen Y. *et al.* and colleagues used streptozotocin (STZ)-

induced diabetic mice to study the effects of long-term hyperglycemia on CD4$^+$CD25$^+$ Tregs in vivo (Zhen et al. 2011). STZ is a naturally occurring chemical that is toxic to beta cells and is used to produce animal models for T1D. Part of their results showed a significant increase in the frequencies of CD25$^+$ and Foxp3$^+$ nTregs in the thymi of mice with diabetes among all CD4 single positive (CD4SP) cells at 1 up to 4 months from the onset of diabetes (Zhen et al. 2011). Our results complement their findings as we also observed an increase in thymic Treg frequency at the pre-diabetic stage (Fig.1.4.). This gives rise to the question whether thymic Tregs in diabetes-prone mice remain enhanced in frequency throughout the disease progression or whether there is a certain time point where thymic Treg numbers are enhanced in diabetes-prone mice. Altogether, these studies supported our initial in vivo finding of increased thymic Treg populations, documenting an overall increase in thymic Tregs in NOD mice at the pre-diabetic stage in comparison to B6 mice (Fig.1.2 and 1.4). On the other hand, others have shown a significant decrease in thymic Tregs in NOD mice defined as CD4$^+$CD25$^+$ via flow cytometric analysis in comparison to BABL/c mice as a control, which could potentially explain their inability to maintain peripheral tolerance according to them (Wu et al. 2002). However, this decrease of thymic Tregs in NOD mice at 3 weeks of age may be accounted by the reduced thymus size in comparison to the control that resulted in a reduction of all thymocyte populations and not just Tregs (Wu et al. 2002). Additionally, an important question to ask is whether the control mice strains used in various studies including ours, represent realistic clinical findings that could be translated in patients later on as there are variations between control mouse strains. In contrast to both concepts of thymic Tregs either increasing or decreasing in diabetes-prone mice in comparison to controls, D'Alise A.M. *et al.* supports the notion of no deficit of thymic Treg frequencies in NOD mice where Tregs were identified based on CD4 and Foxp3 co-expression (D'Alise et al. 2008). It is possible that the use of different diabetes-prone mice models and controls in combination with different approaches, in vitro or in vivo investigations, as well as detection of Tregs using different marker panels, could explain the controversy of the findings between the studies mentioned above. Nonetheless, our findings in agreement with others stated above, support the notion of an increase in thymic Tregs in old NOD mice compared to B6 mice (Fig.1.2 and 1.4). Previously, it was demonstrated that in the absence of TGF-β receptor on T cells specifically,

resulted in the majority of T cells spontaneously differentiating in type 1 and type 2 cytokine secreting cells (Gorelik and Flavell et al. 2000). Additionally, it is known that T cell differentiation is highly regulated in part by the cytokine environment available at the time of antigen recognition (Murphy and Reiner et al. 2002, Zhu et al. 2009). For instance, Dang EV. *et al.* demonstrated a balance between Treg and Th cell 17 differentiation, regulated by a key metabolic sensor, hypoxia-inducible factor 1 (Dang et al. 2011). Therefore, it is possible that T cells may have differentiated at an abnormal rate in NOD mice towards Treg lineage due to impaired signalling pathways or antigen presentation (Bousso and Deguine 2011, Brincks et al. 2013). Although this increase could not distinguish between pTregs and mTregs due to lack of forkhead box P3 (Foxp3) marker (Hori 2003), another potential reason that could explain this result is possibly the retention and/or accumulation of re-circulated peripheral Tregs back to the thymus (McCaughtry et al. 2007). In addition, our documented increase (Fig.1.2 and 1.4) could also be accounted by an uncontrollable Treg expansion in the thymus by a mechanism similar to Tregs limiting activated T cell expansion (Bosco et al. 2006). However, these observations are paradoxical as Tregs are established to play key roles in the maintenance of immunologic self-tolerance (Sakaguchi et al. 2006) and therefore one would expect inhibition of T1D progress with enhanced Treg populations. However, it has been previously suggested that Tregs have reduced ability to suppress autoreactive T cells cells in β cells with age, that could explain this oddity (Gregori et al. 2003, You et al. 2005).

4.1.2 Increase in thymic Tregs due to an increase in both precursor and mature Tregs signify a developmental fault

Foxp3 intracellular transcription factor has been shown to be essential for Treg development and functionality (Fontenot et al. 2003, Hori 2003, Khattri et al. 2003). Therefore, Foxp3 represents a more specific marker for Treg identification in contrast to previous vastly used markers such as CD25, CD45RBlo, CTLA-4, CD62L, CD103 and GITR (Fehérvari and Sakaguchi 2004). Hence the use of Foxp3 in experiments in order to differentiate between Treg subpopulations such as pTregs and mTregs (Lio and Hsieh 2008, Burchill et al. 2008), as well as from activated, effector and memory T cells (Hori 2003, Zhan et al. 2007), is crucial for

a more precise analysis of Treg development and homeostasis. Tracking the co-expression of CD4, CD25, GITR and Foxp3 simultaneously, allowed distinction of pTregs from mTregs as previously described (Lio and Hsieh 2008, Burchill et al. 2008). Many studies predominantly identified Tregs with the individual co-expression of either CD25, GITR, CTLA-4 and/or Foxp3 on CD4SP cells, neglecting a separate analysis of pTregs from mTregs in the thymus, in order to investigate either the proportions or functionality of Tregs (Gregori et al. 2003). To our knowledge, no previous study has directly addressed homeostatic changes in thymic Tregs between pTregs and mTregs in NOD mice at the pre-diabetic stage. Interestingly, it appeared that there was a trend towards increase in pTregs and a significant increase in mTregs of older NOD mice compared to B6 control mice (Fig.1.4) (which could potentially account for the documented increase in total Tregs (pTregs and mTregs) in our findings (Fig.1.2 and 1.4) and others (Tellier et al. 2013). Therefore, based on these results without verifying evidence from others, we speculate that these alterations may have been due to developmental abnormalities at earlier stages of Treg development, resulting in more pTregs and subsequently more mTregs. Interestingly, preliminary data observing GITR expression at the double negative (DN) stages of thymocyte development, showed an elevated expression of GITR at the DN3 and DN4 stages of development in NOD mice in comparison to B6 (data not shown). Previous study demonstrated a critical role of GITR in dominant immunological self-tolerance maintained by $CD4^+CD25^+$ Tregs as signalling through GITR could override Treg-mediated suppression (Shimizu et al. 2002). Alteration of surface molecules in DN stages was not explored in depth in our investigation. However, it is possible that the increase in NOD pTregs could be a result of higher or lower expression of certain crucial cell surface molecules for development and/or functionality. Another plausible reason which could explain these results is a higher proliferation status of pTregs, that is disputed by further on findings.

4.1.3 Decrease in newly developed Tregs of NOD-RAG-GFP reporter mice indicates possible compromise in Treg development

Back in 1999, RJ. Monroe *et al*. and colleagues created a novel reporter mouse in which the endogenous RAG2 coding exon was replaced by a RAG2:GFP fusion gene in order to investigate further the developmental stages and

physiological factors that lead to modulation of RAG expression (Monroe et al. 1999). Alternatively, others constructed bacterial artificial chromosomes (BACs) modified by homologous recombination to encode GFP as a reporter instead of RAG2. BACs were then carried by transgenic mice in order to examine the regulation of RAG expression (Yu et al. 1999). In regards to Tregs, groups using RAG-GFP reporter mice have investigated Treg production by tracking thymic output of Tregs searching for recent thymic emigrants (RTEs) in the periphery based on GFP expression (Chougnet et al. 2010). In addition, our lab previously examined whether the CD40-CD154 pathway played a role in the thymic development of Tregs while looking at thymic resident and ND Tregs defined as GFP[-] and GFP[+] respectively (Cuss and Green 2012). Alternatively, bromodeoxyuridine (BrdU) was used to detect RTEs after 2 days of continuous administration (Tough 1994, McCaughtry et al. 2007). BrdU is a synthetic analogue of thymidine nucleoside and acts as a marker of DNA synthesis as it is incorporated in cells undergoing DNA replication which ultimately allows identificiation of actively dividing cells (Lin and Riggs et al. 1972, Duque and Rakik et al. 2011). Therefore, RTEs which have a higher proliferation status as they have just migrated from the thymus to the periphery, can be distinguished from other cells (McCaughtry et al. 2007). In our investigation using for the first time NOD-RAG-GFP mice in comparison to FVB-RAG-GFP mice, we documented a significant decrease in both the frequency and absolute number of ND Tregs of NOD mice (Fig.1.6C and D) in contrast to a significant increase in frequency and a non-significant trend towards increase in absolute number of resident thymic Tregs (Fig.1.6E and F). The decrease observed in ND Tregs is most unlikely to be accounted by a faster transition to the resident phenotypic characterisation, as GFP intensity declines at a constant rate with a half-life ~56 h in vivo as previously shown (McCaughtry et al. 2007). Despite the fact that there was no significant difference in total Tregs between FVB-RAG-GFP and NOD-RAG-GFP mice (Fig.1.6A and B) as previously shown in B6 and NOD mice (Fig.1.2A and B), NOD-RAG-GFP mice seem to have a constant Treg frequency irrespective of RAG-GFP expression. One major cause for non-significance in our results is the small group size used. However, our pilot experiments now allow us to calculate a level of variance within experimental groups, therefore future experiments can be adequately powered to detect significant changes. Others have previously documented a similar concern where gene-targeted mice in the

B6 background frequently bred into the polyomavirus middle T mouse model of breast cancer in the FVB strain, showed altered phenotypes (Davie et al. 2007). It is therefore essential to use animal models that would recapitulate clinical findings however, FVB mice were the only strain available harbouring RAG-GFP expression to be used as a comparison at the time. In addition, this non-significance could also be accounted by an insufficient number of mice used in order to detect statistical significance. Furthermore, there was a significant increase in the frequency and a non-significant trend towards increase in absolute number of resident Tregs. In agreement with others, a significant proportion of thymic Tregs is comprised of thymic resident Tregs (McCaughtry et al. 2007). Some studies suggested that these resident thymic Tregs may originate from peripheral Tregs re-circulating back to the thymus, as demonstrated in GK mice that lack generation of peripheral CD4 T cells due to the transgenic expression of anti-CD4 monoclonal antibodies that depletes peripheral CD4 T cells (Zhan et al. 2007) or using lymphopenic mice (Bosco et al. 2006). Another explanation is a possible retention or increased thymic Treg cell division (McCaughtry et al. 2007). Although these results cannot distinguish between pTregs and mTregs, the decrease of ND Tregs still signifies an abnormality in Treg development. Therefore, it is of great importance for subsequent investigations to include separate analysis of these two Treg subpopulations for a more precise and insightful picture on Treg development and homeostasis in NOD mice as they have unique homeostatic properties. Concluding, based on our findings we speculate that due to a defect in Treg development occurring either at a certain point or throughout the pre-diabetic stage of NOD mice that may or may not be true at the diabetic stage, could result in the malfunction of Tregs in an age-dependent manner as previously suggested leading to β cell destruction. (Gregori et al. 2003, You et al. 2005).

4.1.4 B cells may be involved in thymic Treg development and/or homeostasis in a disease progression stage-dependent manner

B cells are a distinct lymphocyte population that was initially discovered by Emil von Behring and Shibasaburo Kitasato in 1890 (Behring et al 1890). Since then extensive investigations on B cells elucidated their crucial role in humoral antibody-mediated immunity (Cooper 2015). Concurrently, further studies

indicated the ability of B cells to act as professional APCs able to generate or regulate in part immune responses by activating or tolerizing T cells in a variety of autoimmune diseases such as systemic

 lupus erithematosus (Mamula et al. 1994, Chan et al. 1999), rheumatoid arthritis (Takemura et al. 2001), T1D (Falcone et al. 1998, Greeley et al. 2001, Silveira et al. 2002, Wong et al. 2004) and multiple sclerosis (Lyons et al. 1999). In regards to T1D, B cells similarly to T cells seemed to infiltrate pancreatic islets of NOD mice and in combination with the presence of autoantibodies against the islet antigens in these mice, lead to the hypothesis that B cells are vital in the disease progression (Signore et al. 1989). Subsequently, indeed it was demonstrated by DV. Serreze *et al.* that B cells were essential in the initiation of T cell-mediated autoimmune diabetes using NOD.Igμ^{null} mice that lacked functional B lymphocytes (Serreze 1996). Similarly, others have shown protection of B cell-deficient or or anti-μ Ab-treated NOD mice from diabetes (Forsgren et al. 1991, Yang et al. 1997, Noorchashm et al. 1997). On the other hand, in contrast to the notion of mature B cells being essential in the pathogenesis of diabetes in NOD mice, M. Yang *et al.* suggested that this was not the case at least in some mice according to their findings (Yang et al. 1997). However, recently our group has documented that the NOD thymus is abnormal by having a significant increase in thymic B cells as mice progress to diabetes. These thymic B cells secrete autoantibodies that target insulin expressing thymic stroma cells inducing their apoptosis and as a consequence may impair negative selection of autoreactive T cells (Pinto *et al*, submitted). Nonetheless, the influence these thymic B cells have on Treg development is unknown. Intriguingly, more recently FT. Lu *et al.* and colleagues suggested a role of thymic B cells in regulating the number of thymic Treg cell population using B6 mice (Lu et al. 2015). To our knowledge, in regards to thymic Treg development and B cells in diabetes-prone mice have not yet been investigated. Therefore, in addition to NOD-RAG-GFP mice, using B cell deficient NOD mice expressing RAG-GFP (B-KO), we sought to observe any correlation between B cells and the development/homeostasis of thymic Tregs and their subpopulations initially at the pre-diabetic stage while looking at Ki67 expression, a nuclear protein commonly used as a marker for cellular division (Scholzen and Gerdes 2000, Akimova et al. 2011). The commonality that brings NOD-RAG-GFP and B-KO mouse strains together is their NOD genetic background thus, any changes observed between them imply a direct or indirect

impact by B cells. In our investigations we observed a non-significant increase in the of proliferative status of thymic mTregs (Fig.1.11A) but not of pTregs of old NOD-RAG-GFP and B-KO mice (Fig.1.12G) compared to FVB-RAG-GFP mice. This raises the question whether these highly proliferative Tregs emigrated from the thymus to the periphery as previously suggested (McCaughtry et al. 2007), due to the ongoing infiltration of pancreatic β cells at that stage or whether these Tregs have a lower survival rate. Furthermore, the similarity of Treg patterns between older NOD-RAG-GFP and B-KO mice (Fig.1.11 and 1.12) implied no effect of B cells on Tregs at the regulation stage of disease progression (see section 1.6.1). It is also worth noting that in contrast to our previous findings indicating a non-significant trend towards increase of pTregs in NOD mice compared to B6 (Fig.1.4B and C), both B-KO and NOD-RAG-GFP mice showed a significant decrease in both frequency and absolute number of pTregs in comparison to FVB-RAG-GFP mice (Fig.1.12A and B). However, their frequency is constant throughout the experiments in contrast to FVB-RAG-GFP mice, and the decrease in their number could be accounted due to following a slightly different protocol for the experimental groups in Figure 1.12 that consisted more washes compared to the protocol followed for the experimental groups in Figure 1.4, therefore losing more cells by the end of the process. This decrease could also be accounted by the overall decrease in thymocyte number (Fig.1.11I). FT. Lu *et al.*, documented a significant decrease in frequency and number of thymic Tregs of µMT FoxP3-GFP mice that lacked functional B cells compared to FoxP3-GFP mice as their control (Lu et al. 2015). However, in our results despite the fact that NOD-RAG-GFP mice have functional B cells, their mTreg frequency and number is similar to B-KO mice (Fig.1.11C and D) therefore opposing the concept of B cells' effect on thymic Tregs that FT. Lu *et al.* suggested which is the contribution of B cells in thymic Treg maintenance. This contradiction could possibly be explained by the use of different mouse models with a significantly different health background; healthy versus pre-diabetic mice. Sequentially, in order to observe whether these alterations differed in any way in other stages of the disease (see section 1.6.1), we chose to observe the sensitisation stage in younger mice due to time constrictions and mice available at the time. We documented similar frequency of pTregs between B-KO and FVB-RAG-GFP young mice but not in NOD-RAG-GFP mice that had a significantly lower frequency in comparison to the other strains and a non-significant trend towards

decrease in their absolute number compared to the control (Fig.1.16A and B). Moreover, in contrast to older mice Treg proliferative status that showed no significant difference among the strains (Fig.1.11 and 1.12), in younger mice we observed significant increase in frequency and a non-significant trend towards increase in absolute number of mTreg Ki67 exression of B-KO mice in comparison to the other strains (Fig.1.15H and I) and similarly a significant increase in pTreg Ki67 expression of B-KO mice in comparison to the other strains, however these experiments should be repeated with more mice in order to be verified as only three B-KO mice were available at the time (Fig.1.16G and H). Therefore, we agree with the concept proposed by FT. Lu *et al.* of a non-redundant role of thymic B cells regulating the number of thymic Treg cell population, yet our results differ from their findings of a suggested decrease in Treg proportions of B cell deficient mice (Lu et al. 2015). However, due to the small number of mice used for our experiments, statistical significance in some cases (i.e. proliferative mTregs in young and old mice) was not conferred therefore, speculations mentioned in this section could be disputed or verified in future investigations. Furthermore, we want to emphasize the importance of analysing and observing both the frequency and number of Treg populations, as we documented significant differences in both GFP$^+$ and GFP$^-$ pTreg frequencies of young mice among the strains that was not supported by their absolute numbers (Fig.1.16C, D, E and F). Numerous studies have formed their conclusions based solely on either frequencies or absolute number patterns that may not necessarily be the same when obtained together as we observed. Therefore, future experiments looking at both frequencies and absolute number would provide more evident results. Nonetheless, overall based on our results we hypothesize an involvement of B cells in thymic Treg development and/or homeostatic proliferation depending on the stage of disease progression the mice are.

4.1.5 Thymic Treg alterations not due to changes in interleukin-2 levels

Another reason that could elucidate these thymic Treg alterations in NOD mice besides B cells and proliferation status, is their maintenance in the thymic microenvironment. Interleukin-2 receptor alpha chain (CD25) was discovered to be a constitutively expressed Treg marker that allowed distinction of Tregs from

other inactive T cell populations, but not from activated T cells as they express it as well (Gregori et al. 2003, Fontenot et al. 2005, Paschou 2011) in normal naive animals. Based on this discovery several studies later on uncovered an essential role of interleukin-2 (IL-2) in Treg function both in vivo and in vitro (Furtado et al. 2002, de la Rosa et al. 2004). IL-2 is a pleiotropic cytokine historically initially described as a T cell growth factor, subsequently demonstrated to promote Treg survival and function as well as controlling Treg development and homeostasis (Antony et al. 2006, Burchill et al. 2008, Grinberg-Bleyer et al. 2010, Sakaguchi 2011). For instance, IL-2 or CD25 deficient mice all developed autoimmune diseases with age as they had little or no Tregs in their periphery verifying their importance to Tregs survival, maintenance and/or development (Malek et al. 2002, Almeida et al. 2002). In addition, IL-2 levels were suggested to be closely linked to Treg homeostasis as a feedback mechanism in order to control T cell expansion during immune responses (Almeida et al. 2006). Furthermore, IL-2 was demonstrated to reverse temporarily established T1D in NOD mice via low dose of IL-2 injection (Grinberg-Bleyer et al. 2010). However, high doses of exogenous administration of IL-2 in vitro seemed to abrogate the suppressive activity of Tregs (Moon et al. 2015) which illustrates the complex immunological pathways in feedback mechanisms. On the other hand, it was previously suggested that in BB rats that spontaneously develop T1D similarly to NOD mice, IL-2 administration enhanced spontaneous development of T1D (Kolb et al. 1986) contradicting other findings. Therefore, we assessed if thymic IL-2 levels in FVB-RAG-GFP, NOD-RAG-GFP and B-KO mice could explain the thymic Treg alterations observed at the pre-diabetic stage. J. Yamanouchi *et al.* and colleagues aimed to test effects of the NOD allele of insulin-dependent-diabetes 3 (Idd3) encoding the IL-2 gene. They demonstrated ~50% decrease in IL-2 production by CD8$^+$ T cells in non-transgenic NOD mice (Yamanouchi et al. 2007). Although we did not assess IL-2 production by thymocytes and only observed the total IL-2 levels tracked using enzyme-linked immunosorbent assay (ELISA), present in thymi of each strain, our results in contrast to the notion of an IL-2 decrease in NOD mice, showed no significant alterations in thymic IL-2 levels among the strains. It is worth noting that these IL-2 levels account for both IL-2 present intracellularly and extracellularly of total thymic cells, therefore differences between IL-2 available in the extracellular matrix and IL-2 within thymocytes cannot be distinguished. Interestingly, although A. Antov *et al.* using

IL-2 deficient mice revealed a vital role of signal transducer and activator of transcription 5 (STAT5) transcription factor in CD4$^+$CD25$^+$ Treg homeostasis and hence maintenance of self-tolerance, in regards to thymic Treg levels they seemed to be similar in either STAT5 or IL-2 deficient mice in comparison to wild type, yet they were lacking Foxp3 marker in their experiments (Antov et al. 2003). However, most likely their results were skewed as they might have included in their results other T lymphocyte populations such as activated effector T cells, as previously suggested (Hori 2003, Zhan et al. 2007) due to the fact that they lacked Foxp3 marker in their experiments, making their conclusion less reliable. Additionally, another group utilizing sequencing assays revealed a mutation in *Stat5b* gene, part of the STAT protein family, situated in chromosome 11 that was previously mapped for T1D susceptibility in NOD mice (Davoodi-Semiromi et al. 2004). Furthermore, later on IL-2 mediated JAK-STAT5 signalling pathway was demonstrated to be essential for maintaining Foxp3 expression in thymic Tregs (Murawski et al. 2006) and the development of Foxp3$^+$ Tregs (Burchill et al. 2007). Since we did not observe any alterations in IL-2 levels among the strains that could explain our findings (Fig.1.9), one would assume that the JAK/STAT5 signalling pathway is not impaired. However, our experiments cannot verify this assumption and future experiments on JAK/STAT5 pathway in particular would elucidate whether this pathway accounts for the alterations observed in thymic Tregs in NOD mice. Therefore based on our results, we conclude that the alterations of thymic Tregs did not occur due to IL-2 level instabilities implying survival, induction and/or expansion were not compromised because of this. Nonetheless, as far as we know they could have been compromised by other factors not assessed by our investigations such as JAK/STAT5 signalling pathway (Murawski et al. 2006) or apoptosis (Shi et al. 1995). In summary, even though IL-2 has an essential role in Treg function, development and homeostasis, there seems to be no compromise among the thymi of pre-diabetic strains although IL-2 levels in other lymphoid organs and periods of the disease are yet to be investigated.

Overall, based on our attempts to investigate thymic Treg development and homeostasis we conclude that there is a fault in Treg development at earlier stages of T1D with B cells being involved as well in pTreg development. Lastly, IL-2 and proliferation status defined by Ki67 expression do not seem to account

for these thymic Treg alterations. Repetition of these experiments with more mice and a more suitable control for comparison would clarify a constant pattern, leading to new biological questions thus investigations enhancing our knowledge on Tregs in diabetic mice.

4.2 Peripheral Treg populations affected differently in NOD and B cell deficient NOD mice in a tissue-dependent manner

4.2.1 Tregs proliferation status of NOD-RAG-GFP mice show a non-significant trend in the spleen depending on disease progression

Failing peripheral immunoregulatory mechanisms potentially followed by compromised peripheral tolerance, are believed to contribute in T1D progression (Rapoport 1993, Salomon et al. 2000, Gregori et al. 2003). Therefore, a vast number of research has been conducted on peripheral Treg proportion and/or functionality in order to elucidate any abnormalities of peripheral Treg populations in diabetes-prone mice. Intriguingly, in regards to peripheral Treg proportion (frequencies and/or absolute numbers), there are discrepancies amongst findings. Hence we decided to revisit and re-investigate peripheral Treg populations using FVB-RAG-GFP, NOD-RAG-GFP and B-KO mice initially in the spleen at two distinct stages of T1D disease progression; sensitisation and regulation (see section 1.6.1).

The spleen is a secondary lymphoid organ found in most vertebrates and acts primarily as a blood filter organ (Steiniger and Barth 2000). In addition to that, the spleen has been demonstrated to play an essential role in B cell development as immature B cells have been illustrated to migrate from the bone marrow via the bloodstream to the spleen, where their development regulated by Notch signalling is completed (Kuroda et al. 2003, Mebius and Kraal 2005). Regarding our investigations, although the absolute numbers of splenic Tregs in NOD-RAG-GFP and FVB-RAG-GFP mice were similar in younger mice (Fig.1.17C), we still suspect that there was a non significant increase of splenic Tregs in old NOD-RAG-GFP mice in comparison to the control due to the fact that NOD-RAG-GFP

mice were significantly less in total splenocyte number compared to FVB-RAG-GFP mice (Fig.1.17A) and one would expect that all cell populations (including Tregs) within the spleen, would be proportionally less as well. This is also implied while looking at the frequency of splenic Tregs of younger mice (Fig.1.17B) where there is a non-significant trend towards increase in NOD-RAG-GFP mice compared to FVB-RAG-GFP mice. In addition, we did not document any significant alterations in splenic Tregs between NOD-RAG-GFP and FVB-RAG-GFP mice (Fig.1.13). Furthermore, splenic Tregs of old B-KO mice displayed a significant increase in proliferation status based on their frequency, but not in their absolute numbers, that was not observed in younger mice (Fig.1.13D and 1.17D). The total splenocyte number in both young and old B-KO mice (Fig.1.13D and 1.17D) was plummeted as B cells are a major portion of the spleen (Berzins et al. 2003, Mebius and Kraal 2005). Previously, in disagreement with our findings, AJ. Wu *et al.* suggested a significant decrease in the absolute number of splenic CD4$^+$CD8$^-$CD25$^+$ Tregs in NOD mice compared to BALB/c mice at 3, 8 and 15 weeks of age (Wu et al. 2002). One possible reason that could explain the contradictory body of findings is the use of different control mouse models and this concept was challenged by SP. Berzins *et al.* and indeed found significant alterations between BALB/c and B6 non-autoimmune control mice of pooled lymph nodes (Berzins et al. 2003). Correspondingly to the notion of deficient peripheral Tregs in diabetes-prone mice that could be associated with autoimmune disease development, P. Alard *et al.* also demonstrated lower frequencies of splenic CD4$^+$CD25$^+$ Tregs of 9 week old NOD mice compared to B6 control mice in contrast to our frequencies where we documented a significant increase in frequency of splenic Tregs of old NOD mice compared to the control that was not however projected by their absolute numbers (Fig.1.13B and C) (Alard et al. 2006). Based on our results, it is worth pointing out again the importance of inspecting both the frequency and the absolute number of populations as they are more definitive than the changes observed in frequencies that could well be an artefact of other lymphocyte population changes that have not been analyzed in our studies. However, in both investigations (Wu et al. 2002, Berzins et al. 2003) they lacked Foxp3 marker that is essential for distinguishing CD4$^+$CD25$^+$ Tregs from activated T cells (Fontenot et al. 2003) amongst other phenotypically similar T lymphocytes (Hori 2003, Zhan et al. 2007) that could skew their results. On the other hand, others support the notion of no

splenic Treg deficit in NOD mice in comparison to other non-autoimmune prone mouse strains at the pre-insulitis stage (Berzins et al. 2003) or throughout the different stages of T1D disease progression (Mellanby et al. 2007, D'Alise et al. 2008), questioning the growing hypothesis that the abnormal functional activity of Tregs could be an essential step in the development of T1D. At first glance based on absolute numbers (Fig.1.17C) our results on young NOD-RAG-GFP mice seem to be in agreement with the concept of no alterations at the prediabetic stage (Berzins et al. 2003, Mellanby et al. 2007). In regards to old B-KO mice, we documented a significant increase in the frequency of Ki67 expressing splenic Tregs that was not seen in their absolute number, in comparison to the other groups (Fig.1.13D and E). Similarly to our case, Y. Zhen *et al.* noted a universal decrease in the total splenocyte number, including CD4$^+$ T cells, that projected in a significant decrease in CD4$^+$CD25$^+$Foxp3$^+$ splenic Tregs of STZ-induced diabetic mice in comparison to their control (Zhen et al. 2011). Therefore, the similar numbers of Ki67 expressing splenic Tregs in old B-KO mice among the other two strains implies and supports the increase observed in their frequency. However, due to the small number of mice and the significant decreased spleen cellularity of old B-KO mice compared to the other strains, it is hard to distinguish such a change in their splenic Treg numbers although their frequencies significantly increased (Fig.1.13D). To our knowledge, there has not been extensive research between Treg homeostasis and B cells in the periphery of diabetes-prone mice. Nonetheless, our results imply a correlation between the homeostatic proliferation of splenic Tregs and B cells in an age-dependent manner as there were no alterations in younger mice. Whether this pattern persist in diabetic mice or whether these highly proliferative splenic Tregs in B-KO mice are functional is unknown.

4.2.2 There is no significant trend in Tregs residing in pancreatic lymph nodes of NOD mice despite age

The PLNs seem to be a key site for activation and tolerance induction of autoreactive T cells against β cells in T1D (Höglund et al. 1999). Hitherto, T1D progression was suggested to depend on a delicate balance between Tregs and effector Th cells in both the PLNs and the inflamed pancreas (Bluestone and Tang 2005). Although we did not investigate the numbers of effector Th cells,

initially we observed a nonsignificant increase in the absolute number of total Tregs of NOD-RAG-GFP mice compared to FVB-RAG-GFP mice as depicted in Figure 1.8, contradicting previous findings suggesting a decrease in PLN Tregs in NOD mice (Pop et al. 2005, Nti et al. 2012). Intriguingly, previously chronic inflammation has been shown to increase Treg frequencies in the PLNs of T1D-mouse models prior to diabetes development and these Tregs were hyper-suppressors of the autoreactive response (Green et al. 2002). However, due to a change in our protocol using the same strains as before FVB-RAG-GFP, NOD-RAG-GFP and B-KO, in order to introduce Ki67 in the markers panel, dissimilarly to the spleen there were some alterations in our findings in sequential experiments as depicted in Figure 1.14 and 1.18, regarding PLN Tregs probably due to their smaller cellularity resulting in more obvious changes. For example, looking at the non-significant trends of the experimental groups, in contrast to before (Fig.1.8B), there was no difference in PLN Treg number of old NOD-RAG-GFP mice (Fig.1.14C) compared to the other groups. Furthermore, we documented no alterations in PLN Tregs among the strains at the earlier-stages of insulitis of younger mice (Fig.1.18). In addition, we also noticed a universal decrease in PLN cellularity (Fig.1.14A and 1.18A) possibly due to the extra washing steps required for the Ki67 protocol. In disagreement with our findings, according to SM. Pop *et al*. they demonstrated a decline in the frequency of Foxp3$^+$CD25$^+$CD62L^{hi} Treg cells residing in the PLNs of NOD mice in an age-dependent manner compared to B6 mice (Pop et al. 2005). In addition, more recently BK. Nti *et al*. using FACS analysis and real-time qRT-PCR, claimed a profound drop of PLN CD4$^+$Foxp3$^+$ Tregs and Foxp3 transcripts to 0% in 5 week-old NOD mice in comparison to autoimmune-free B10 mice (Nti et al. 2012). Although there was a nonsignificant difference in the PLN Treg numbers among the strains, there was a displayed non-significant trend towards decrease in the absolute number of Tregs of NOD-RAG-GFP and B-KO mice compared to the control at the pre-diabetic stage (Fig.1.14 and 1.18) that agrees with the notion of SM. Pop *et al*. and BK. Nti *et al*. of a decrease in PLN Tregs of NOD mice. However, we rationalised those findings due to a nonsignificant decrease in total PLN numbers (i.e. cellularity) and not due to genuine PLN Treg decline in pre-diabetic NOD mice as there were no alterations in their frequencies (Fig.1.14 and 1.18). Additionally, the small number of mice used could overshadow the genuine pattern either towards an increase or decrease of PLN Tregs in NOD mice at the

pre-diabetic stage, therefore repetition of these experiments with more mice is vital in order to either verify or dispute the patterns we observed. On the other hand, similarly to our findings there were no alterations of PLN Tregs in NOD mice in an age-dependent manner as depicted by RJ. Mellanby *et al.* (Mellanby et al. 2007). Therefore, based on our current results we support the notion of no compromise in Treg proportions in an age-dependent manner of NOD mice. Although we did not investigate whether the function of these Tregs was impaired as they age-declined, it is possible as demonstrated previously in other peripheral Tregs, a defective function in suppressing the proliferation of effector T cells (You et al. 2005, Mellanby et al. 2007). In regards to B cell deficient NOD mice, the patterns observed in PLN Treg numbers were similar to NOD mice despite age with only one distinct decrease in both their frequency and number of old B-KO mice compared to FVB-RAG-GFP (Fig.1.14B and C). This may be accounted by a decrease in their proliferation based on their absolute number in comparison to control, but not reflected by their frequency (Fig.1.14D and E). However, there seems to be no effect on Treg homeostatic proliferation in the pancreatic lymph node environment by B cells of young mice (Fig.1.18). These results in combination with our previous thymic investigations, support a concept that during autoimmunity, distinct anatomic sites play different independent roles in the disease progression (Ochando et al. 2005).

4.3 Conclusions and future directions

Investigations on potential effects of B cells on Treg development in the thymus have not been investigated extensively in addition to exploring the homeostasis of ND and mature Tregs in the thymus in type 1 diabetic RAG-GFP mice. Hence, we sought to observe the development and homeostasis of Tregs in primary lymphoid organs and their homeostasis in secondary lymphoid organs using for the first time RAG-GFP mice. Based on our results, we hypothesize an early developmental fault in thymic Tregs of NOD mice that emphasize the argument that efficacious prevention of T1D will require detection of incidences occurring at earlier events in the process. Furthermore, documented alterations in the thymus do not seem to be accounted by compromised IL-2 levels implying normal survival based on IL-2. In regards to the periphery, we support the notion of an increase in splenic Tregs in NOD and B-KO mice with B cells affecting splenic Treg homeostatic proliferation

in an age-dependent manner. However, based on our current results, overall there seemed to be no alterations in PLNs among the strains despite age, signifying a potential functional defect in PLN Tregs rather than a decrease in their number rationalizing T1D progression. Therefore, in addition to repeating the above experiments in order to verify these future studies investigating the numbers of Tregs should be coupled with investigations on Treg functionality among different tissues as defining Treg populations at distinct anatomic compartments might signify the exact sites where impairments take place. In addition, future experiments should include investigations on the ability of thymic Tregs to egress the thymus and migrate to peripheral lymphoid organs by looking at S1P1 molecule in order to elucidate the increase in thymic NOD resident Tregs. Also, IL-2 levels should be investigated in other secondary lymphoid organs which might explain the increase in splenic Tregs. In addition, it is worth investigating if there are any spatial interactions between B cells and Tregs in the spleen or even the thymus based on colocalisation observed using tissue fluorescence analysed on confocal microscope. Furthermore, cell apoptosis assays of Tregs again in primary and secondary lymphoid organs are vital for a more defined Treg homeostasis in T1D mice.

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