Forward genetic and cellular studies of intestinal immune regulation: the roles of Carma1, Interleukin-10 and Gimap5

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Abstract: The differentiation and functional specialisation of $CD4^+$ T cells into distinct subsets allows inflammatory responses to be targeted to diverse types of insults. One subset of $CD4^+$ T cells, $Foxp3^+$ regulatory T (T_{reg}) cells, acts to restrain excessive effector T cell responses and can develop in either the thymus or peripheral tissues. Here, a mouse with a germline point mutation in *Carma1*, an essential component of the signalling cascade linking the T cell receptor to NF-κB activation, is described. These mice were used to demonstrate a cell-intrinsic requirement for Carma1 in the development of thymic T_{reg} cells that could be bypassed in peripheral $CD4^+$ T cells. Interestingly, Carma1-deficient T_{reg} cells accumulated both at the site of persistent viral infection and in the intestine of healthy mice, which harbours a high load of resident bacteria, suggesting that chronic inflammation can drive peripheral T_{reg} cell induction or accumulation. Subsequent work showed that T_{reg} cells in colon of healthy wild-type mice are unique in that they constitutively produce the immunosuppressive cytokine interleukin 10 (IL-10), whereas in the small intestine both $Foxp3^+$ T_{reg} cells and $Foxp3^-$ $CD4^+$ T cells that resemble Tr1 cells produce IL-10. Production of IL-10 from both T cell subsets required the presence of intestinal bacteria, because IL-10 expression was abrogated by broad-spectrum antibiotic treatment. Additionally, IL-10 expression was enhanced in the colon and caecum upon colonization with *Helicobacter hepaticus,* a pathogen that can break tolerance of intestinal bacteria in *Il10*$^{-/-}$ mice. Finally, the characterisation of a mouse homozygous for a germline point mutation in *Gimap5* demonstrated that this gene was required for the survival of T, B and NK lymphocytes. Additionally, these mice exhibited spontaneous hepatic extramedullary haematopoiesis, with an abundance of $Lin^-Sca\text{-}1^+Thy\text{-}1^{high}$ innate lymphoid cells in the liver, and became colitic when infected by *H. hepaticus*. Adoptively transferred lymphocytes prevented disease in these mice, suggesting that regulatory lymphocytes need Gimap5 to survive and function. Collectively, these studies identify novel genetic and microbial factors involved in maintaining intestinal homeostasis.

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Table of Contents

List of Figures

List of Tables

Abbreviations

αGalCer, alpha-galactosylceramide
Aire, autoimmune regulator
ANOVA, Analysis of Variance
APECED, autoimmune polyendocrinopathy-candidiasis-ectoderma dystrophy
Atg5, autophagy-related 5
Bcl10, B cell CLL/lymphoma 10
BCR, B cell receptor
Blimp-1, B lymphocyte induced maturation protein 1
BMDC, bone marrow-derived dendritic cell
CARD, caspase recruitment domain
Carma1, CARD-MAGUK1
Ccr4/7, chemokine (C-C) motif receptor 4
CFA, Complete Freund's adjuvant
CFSE, carboxyl fluorescent succinimidyl ester
CLP, common lymphocyte progenitor
CTL, cytotoxic T lymphocyte
Ctla-4, cytotoxic T-lymphocyte antigen 4
DC, dendritic cell
DN, double negative
DNA, deoxyribose nucleic acid
ELISA, enzyme-linked immunosorbent assay
ENU, *N*-ethyl-*N*-nitrosourea
Erk, extracellular signal-regulated kinase
FCS, foetal calf serum
Flt3, FMS-like tyrosine kinase 3
Foxp3, forkhead box P3
GALT, gut-associated lymphoid tissue
GI, gastrointestinal
Gimap5, GTPase of the immunity-associated protein 5
Gitr, glucocorticoid-induced tumour necrosis factor receptor
*GMP***,** granulocyte/macrophage progenitor
GUK, guanylate kinase
HIV, human immunodeficiency virus
HRP, horseradish peroxidase
HSC, haematopoietic stem cell
IAN, immune associated nucleotide
IκBα, NF-κB inhibitor alpha
IBD, inflammatory bowel disease
ICOS, inducible costimulator
IEC, intestinal epithelial cell
IFN, interferon
Ig, immunoglobulin
IKK, inhibitor of NF-κB kinase
IL, interleukin
IMDM, Isocove's Modified Dulbecco's Media
IPEX syndrome, immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome
iT$_{reg}$ cell, induced regulatory T cell
Jnk, Jun N-terminal kinase
LIP, lymphopaenia induced proliferation
Lod, logarithm (base 10) of odds
LP, lamina propria
LPS, lipopolysaccharide
MACS, magnetic activated cell sorter
Malt1, mucosa associated lymphoid tissue lymphoma translocation gene 1
MCMV, mouse cytomegalovirus
MEP, megakaryocyte/erythroid progenitors
MHC, major histocompatibility complex
mLN, mesenteric lymph node
mTor, mechanistic target of rapamycin
NF-κB, nuclear factor κB
NLR, Nucleotide-binding domain, Leucine-rich repeat containing protein
iNKT cell, Vα14^{+} TCR expressing invariant natural killer T cell
NORS, no regular secondary structure
Ox40, tumour necrosis factor superfamily, member 4
PBS, phosphate buffered saline
PI3K, phosphoinositide 3-kinase
Pkc, protein kinase C
PMA, phorbol myristate acetate
Prdm, PR domain containing
PSA, polysaccharide A
RA, retinoic acid
Rag, recombination-activating gene
RBC, red blood cell
SCID, severe combined immunodeficiency
SFB, segmented filamentous bacteria
SH3, Src homology 3
Smad, SMA-related mothers against decapentaplegic homolog
SP, single positive
SPF, specific pathogen free
Stat, signal transducer and activator of transcription
Tak1, TGF-β-activated kinase 1
TCR, T cell receptor
TGF-β, transforming growth factor beta
T$_{H}$, T helper
TLR, Toll-like receptor
TNF-α, tumour necrosis factor alpha
Tr1 cell, T regulatory type 1 cell
T$_{reg}$ cell, regulatory T cell

Chapter I. General Introduction

1.1 Overview

The studies described in this thesis cover a number of topics in immunology that all relate to aspects of how lymphocytes maintain self-tolerance. Many relevant introductory points to each study are brought up at the beginning of each chapter. However, a common theme in these studies relates to how tolerance of resident intestinal bacteria is ensured in the gut. Here, maintenance of immune homeostasis in the intestine is considered in some detail as a paradigm to introduce the pathways and strategies used by the immune system to maintain self-tolerance.

The benefit derived from harbouring symbiotic organisms is a force that has shaped evolution (Dale and Moran, 2006) and, in mammals, nowhere is this more apparent than in the gastrointestinal (GI) tract. The intestinal microbial flora, which is largely composed of resident bacteria that most densely populate the colon, benefits the host by extracting dietary nutrients and preventing colonization by opportunistic pathogens (Duerkop et al., 2009). Tolerance of endogenous microbes can be advantageous, but must be achieved while minimizing the risk of systemic infection. The GI tract forms the largest mammalian epithelial surface, so this constitutes a substantial challenge. Pathogenic and commensal bacteria are diverse and derive from intermingled phylogenies, making it difficult, if not impossible, for the host to distinguish between them at a molecular level. Instead, intestinal physiology has evolved to sequester most bacteria in the lumen, in a layer of mucus and immunoglobulin A (IgA), and to reinforce the gut with multiple layers of defence, consisting of barrier, innate and adaptive components that limit microbial flora-driven inflammation.

1.2 Multiple mechanisms enforce intestinal homeostasis

The epithelial layer of the GI tract largely consists of intestinal epithelial cells (IECs) connected by tight junctions, as well as mucus-secreting goblet cells and antimicrobial-peptide-producing Paneth cells (Artis, 2008). Interspersed throughout the epithelium are gut-

associated lymphoid tissues (GALT), including Peyer's patches in the small intestine and isolated lymphoid follicles in the colon, which contain IgA-secreting plasma cells. Together, these varied cell populations support a mucus layer, containing IgA and antimicrobial peptides, which dramatically reduces the bacterial load at the barrier between the epithelium and lumen.

Although relatively devoid of live bacteria, the intestinal epithelium may be constantly exposed to immunostimulatory molecules, such as lipopolysaccharide (LPS), that diffuse through the mucus layer. To prevent continual immune activation, IECs exclude innate immune pathogen sensing receptors, such as the sensor of bacterial flagellin Toll-like receptor 5 (TLR5), from the luminal interface (Gewirtz et al., 2001). Additionally, IECs, intestinal macrophages and dendritic cells (DCs) in the intestinal draining lymph are hyporesponsive to LPS (Cerovic et al., 2009; Lotz et al., 2006). Innate immune recognition of invasive pathogens at the intestinal epithelial interface may instead rely on intracellular sensors, such as endosomal TLRs, cytoplasmic nucleic acid sensors and Nucleotide-binding domain, Leucine-rich repeat containing proteins (NLRs). Upon detection of pathogens, inflammatory cytokines are secreted that recruit DCs, monocytes and neutrophils to the intestine. In the absence of infection, intestinal DCs remain quiescent and promote tolerance by migrating to the GALT where they present luminal-derived antigens to lymphocytes (Coombes and Powrie, 2008).

T cells provide a third layer of intestinal defence that limits infections by pathogens that enter through the GI tract. Innate-like lymphocytes, principally intraepithelial γδ T cells, provide signals that enhance barrier function (Chen et al., 2002). Conventional $CD4^+$ T cells and $CD8^+$ T cells are also found in the intestine; however, these cells present the inherent risk of reacting toward dietary- or microbial flora-derived antigens. Indeed, antigen-experienced T cells in the mesenteric lymph node (mLN) of healthy mice are capable of inducing intestinal inflammation upon adoptive transfer into immunodeficient recipients (Asseman et al., 2003).

Inappropriate innate and adaptive immune responses in the GI tract are normally restrained by regulatory lymphocytes (Maloy et al., 2003; Read et al., 2000). Although regulatory activity has been ascribed to several types of intestinal lymphocytes, compelling genetic and functional evidence suggests that $CD4^+Foxp3^+$ regulatory T (T_{reg}) cells and interleukin 10 (IL-10)-producing T cells carry out nonredundant functions. Notably, mice lacking forkhead box p3 (Foxp3) develop fatal multiorgan inflammation that can be suppressed by adoptively transferred $Foxp3^+$ T_{reg} cells (Fontenot et al., 2003). Mice engineered to lack expression of specific regulatory cytokines in T cells, such as IL-10 or transforming growth factor β (TGF-β), succumb to wasting disease and colitis when disease-triggering bacteria are present in the intestinal flora (Li et al., 2007; Roers et al., 2004; Rubtsov et al., 2008). In humans, intestinal inflammation often occurs in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome (Powell et al., 1982), which is caused by germline mutations in *FOXP3* (Ziegler, 2006). Importantly, both microbial flora and innate immune dependent signals modulate the activity of T_{reg} cells in the intestine, allowing T_{reg} cells to reinforce barrier and innate immune functions in the intestine. Here, the developmental origins of effector and T_{reg} cells and their roles in maintaining intestinal homeostasis are described.

1.3 Functional specialisation of effector and regulatory $CD4^+$ T cell subsets

The differentiation and functional specialisation of effector $CD4^+$ T cells allow for effective immune responses to diverse insults and occur in response to combinations of antigen receptor, costimulatory, cytokine, metabolic and environmental signals. These cues trigger chromatin remodeling and the induction or repression of specific transcription factors after T cell activation (Wilson et al., 2009). These events can result in the differentiation of T helper type 1 (T_H1) cells poised to control intracellular virus, bacteria or parasite infections, T_H2 cells that clear intracellular helminths and promote antibody responses and T_H17 cells that may prevent fungal infections (Figure 1.1). T_{reg} cells act to dampen immunopathology and are also subject to functional specialisation (Koch et al., 2009; Zheng et al., 2009).

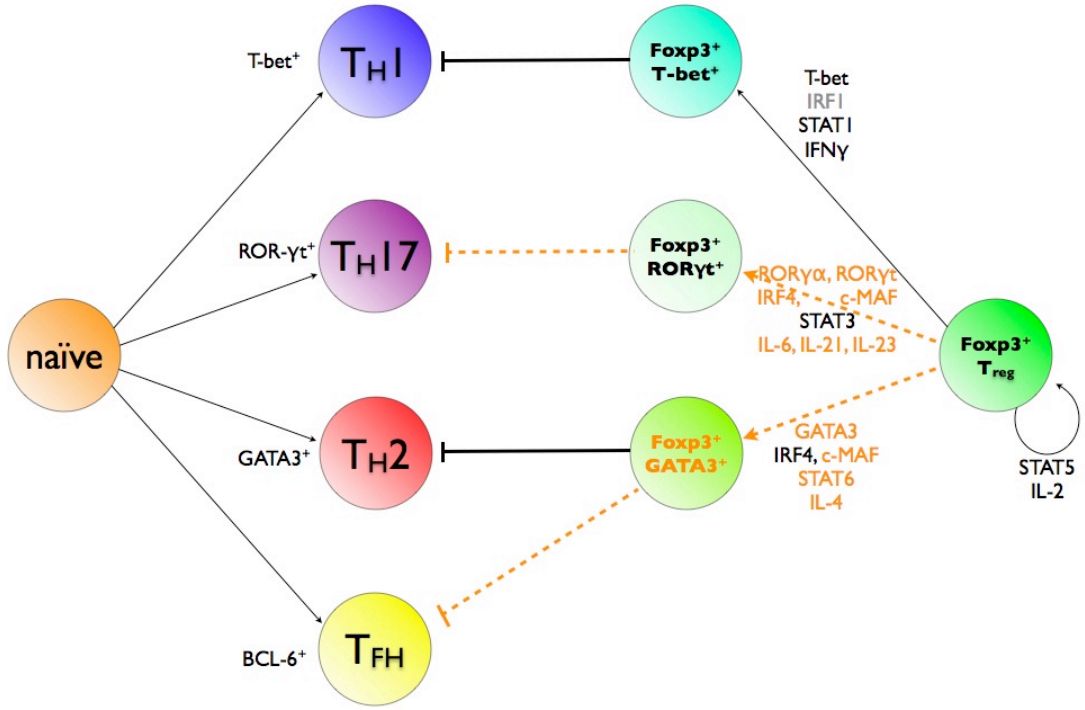

Figure 1.1. Effector CD4$^+$ T cells and T_{reg} cells undergo polarization in the periphery after exiting the thymus.

This process may lead to the development of T_{reg} cell subsets specialized to regulate specific effector T cell subsets. 'Master transcription factors' required for the development of effector T cell subsets are indicated (black text). Several factors central to effector T cell differentiation are also critical for T_{reg} cell polarization (black). Additional factors important for effector T cell differentiation have not yet been investigated in the context of T_{reg} cell differentiation (orange). Also, regulation does need act directly on effector CD4$^+$ T cells but may occur through interactions with cells of the innate immune system or other lymphocytes.

In the intestine, all of these subsets can develop under different types of inflammatory conditions.

The 'master transcription factor' theory holds that each $CD4^+$ T cell subset is controlled by a distinct dominant transcription factor. Thus, differentiation of the effector T_H1, T_H2, T_H17 or follicular helper T cell (T_{FH}) subsets requires the expression of T-bet, GATA-3, ROR-γt, or Bcl6, respectively, whereas T_{reg} cells require Foxp3 expression. Interleukin 10 (IL-10)-producing $Foxp3^-$ Tr1-like cells are an additional subset for which no 'master transcription factors' has yet been identified. However, empirical data suggest a much more complicated situation exists *in vivo*, where a multitude of transcription factors interact physically and transcriptionally to polarize a T cell. For example, Foxp3 can physically interact with coexpressed ROR-γt to inhibit its function (Zhou et al., 2008) and GATA-3 can directly bind the Foxp3 promoter to prevent its activation (Mantel et al., 2007). In addition, some transcription factors can help drive the polarization of multiple lineages in a context-dependent manner. For example, Irf4 is required for both T_H2 and T_H17 differentiation (Brustle et al., 2007), and c-Maf drives both T_H17 and T_{FH} responses (Bauquet et al., 2009). Despite these complexities, 'hallmark' $CD4^+$ T cell lineages are found in a wide variety of infections and autoimmune conditions. The diversity of $CD4^+$ T cell responses, plasticity of T cell subsets and different transcriptional states are the subject of intense current debate. An emerging theory is that $Foxp3^+$ T_{reg} cells are 'instructed' by inflammatory molecules in specific tissues to mount the appropriate type of regulatory response (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009).

1.4 Characteristics of $Foxp3^+$ T_{reg} cells that develop in the thymus

The majority of $CD4^+Foxp3^+$ T_{reg} cells probably originate in the thymus, where their development requires signals that activate nuclear factor-κB (NF-κB) via the protein kinase c θ (Pkcθ) → Carma1 → c-Rel signalling pathway and limit activation of the phosphoinositide

3-kinase (PI3K) → Akt → mechanistic target of rapamycin (mTor) pathway (Feuerer et al., 2009; Hori, 2010). The T cell receptor (TCR) is a dominant source of these signals. The expression of a TCR repertoire in T_{reg} cells that is largely distinct from conventional T cells has been interpreted to indicate that thymic T_{reg} cells bind self-peptides with moderate affinity (Feuerer et al., 2009; Josefowicz and Rudensky, 2009). After TCR stimulation, T_{reg} cell precursors must receive costimulatory signals through CD28 and encounter the common-γ chain cytokines IL-2 or IL-15 in order to activate signal transducer and activator of transcription 5 (Stat5) and induce Foxp3 expression (Burchill et al., 2008; Lio and Hsieh, 2008). IL-2, as well as TGF-β, is also important in the periphery where they help maintain $Foxp3^+$ T_{reg} cells.

The affinity of TCR binding to major histocompatibility complex (MHC) molecules alone does not determine whether a thymocyte will express Foxp3. Interestingly, a study with transgenic mice expressing TCRs common to T_{reg} cells demonstrates that a very small thymic niche exists for each clone with a given TCR specificity (Bautista et al., 2009). Limiting the thymic T_{reg} cell niche by the availability of self-antigens would allow many clones with diverse specificities to develop. Both thymic DCs and epithelial cells are capable of presenting antigens that induce Foxp3 expression (Wirnsberger et al., 2009). DCs in the thymus could potentially include cells that acquire antigen in the peripheral tissues, such as the gut, and then migrate back to the thymus, although this possibility has not yet been extensively tested. Intriguingly, presentation of the full spectrum of $CD4^+$ T cell-selecting ligands requires nonhaematopoietic thymic stromal cells to be able to undergo autophagy (Nedjic et al., 2008). Thymi lacking *autophagy-related 5* (*Atg5*) expression aberrantly select $CD4^+$ T cells, indicating that part of the normal T cell repertoire might recognize antigens that depend upon autophagy for their processing and presentation. When $Atg5^{-/-}$ thymi are transplanted into autophagy-sufficient athymic nude mice, recipient mice develop colitis despite the presence of normal T_{reg} cell numbers in the thymus and intestine. This finding suggests the possibility that the intestine is enriched in autophagy-dependent antigens, which

normally activate protective T_{reg} cells. In recipients of *Atg5*$^{-/-}$ thymi, such T_{reg} cells would be absent and autophagy-dependent antigens might instead activate colitogenic T cells that escape deletion in the autophagy-deficient thymus.

After exiting the thymus, some T_{reg} cells migrate to the GI tract where they can recognize intestinal antigens and prevent inappropriate immune responses. Such a mechanism has been demonstrated via the T-cell-transfer model of colitis in which adoptively transferred naive $CD4^+CD45RB^{high}$ T cells cause intestinal inflammation in severe combined immunodeficiency (SCID) recipient mice (Powrie et al., 1993). The intestinal flora is required to drive inflammation, as indicated by the fact that colitis is attenuated in germ-free SCID mice receiving naive T cells (Aranda et al., 1997). Several factors may underlie this phenomenon, including the absence of microbial flora-derived antigens and the impaired formation of the GALT in germ-free mice. In mice housed in specific-pathogen-free (SPF) conditions, cotransferred T_{reg} cells prevent colitis (Read et al., 2000). By some reports, T_{reg} cells isolated from germ-free mice are also able to prevent intestinal inflammation (Annacker et al., 2000; Singh et al., 2001), although another study has produced conflicting results (Strauch et al., 2005). These differences may reflect the adaptation of T_{reg} cells to both self- and exogenous-antigens presented in the intestine. For example, certain intestinal bacteria, such as *Helicobacter hepaticus*, promote enhanced suppressive capacity of regulatory T cells in both the $CD4^+CD45RB^{lo}CD25^+$ and $CD4^+CD45RB^{lo}CD25^-$ compartments (Kullberg et al., 2002). Indeed, in a setting where germ-free T_{reg} cells function, T_{reg} cells isolated from SPF-housed mice are better suppressors than those from germ-free mice (Singh et al., 2001). Thus, additional flora-dependent shaping of the T_{reg} cell pool can occur in the peripheral lymphoid organs that allows for efficient regulation of the GI tract.

1.5 The intestinal T_{reg} cell niche: $Foxp3^+$ T_{reg} cells

Generating additional T_{reg} cells extrathymically is one way that regulation could be enhanced in the intestine. Naive $CD4^+$ T cells can differentiate into $Foxp3^+$ induced regulatory T cells

(iT_{reg} cells) when activated by transient TCR stimulation (Sauer et al., 2008) or TCR stimulation in the presence of TGF-β and IL-2 (Chen et al., 2003) and the absence of inflammatory cytokines that promote effector T cell differentiation (Maynard and Weaver, 2009). *In vivo*, transfusion of antigen under nonimmunogenic conditions, homeostatic proliferation or chronic inflammation can generate iT_{reg} cells (Curotto de Lafaille and Lafaille, 2009). Although thymic T_{reg} cells and iT_{reg} cells may express different gene transcripts, such as *Helios*, and epigenetic markers (Hill et al., 2007; Wei et al., 2009), they are currently difficult to distinguish on a single-cell basis. Therefore, it is currently only possible to approximate the contribution of iT_{reg} cells to the total intestinal T_{reg} cell pool. One approach involves adoptively transferring naive $CD4^+CD45RB^{hi}Foxp3^-$ T cells into *recombination activating gene 2*-deficient (*Rag2*$^{-/-}$) recipients and observing when and where cells begin to express Foxp3. Normally, naive T cell transfer into *Rag2*$^{-/-}$ recipients gives rise to very few $Foxp3^+$ T_{reg} cells and favours the accumulation of colitogenic T_H1 and T_H17 effector cells (Leppkes et al., 2009; Powrie et al., 1994). However, when naive T cells are transferred into *Rag2*$^{-/-}$ recipient mice lacking IL-23 (IL-12p19), which do not develop intestinal inflammation, preferential induction of Foxp3 expression occurs among T cells that migrate to the colon lamina propria (LP) and mLN (Izcue et al., 2008). These iT_{reg} cells function and contribute to the prevention of colitis, as indicated by the fact that intestinal inflammation occurs when Foxp3-deficient naive T cells, which cannot give rise to iT_{reg} cells, are used as donors. Interestingly, naive T cells transferred into *Rag2*$^{-/-}$ recipients that are treated with IL-6R blocking antibody also generate a higher frequency of $Foxp3^+$ T_{reg} cells in both the GI tract and spleen, demonstrating a general role for inflammatory Stat3-activating cytokines in limiting Foxp3 induction (Izcue et al., 2008). Lymphopaenia-induced proliferation (LIP) is physiologically relevant, because it occurs in the neonatal setting in the absence of intestinal inflammation (Min et al., 2003) and could contribute to an early T_{reg} cell population in the developing immune system.

To assess iT_{reg} cell generation in an adult mouse, congenically marked $CD4^+Foxp3^-$ T cells can be transferred into lymphocyte-replete recipients. However, in this setting, few transferred $CD4^+Foxp3^-$ T cells become $Foxp3^+$ and are estimated to comprise only ~ 4 – 7 % of the normal T_{reg} cell pool (Lathrop et al., 2008). Importantly, $Foxp3^+$ iT_{reg} cells become twice as frequent in the mLN than the spleen or peripheral lymph nodes, suggesting that iT_{reg} cell generation occurs more frequently in the GALT. An alternative approach with Carma1-deficient mice (described in detail in Chapter III), which are devoid of thymic T_{reg} cells (Barnes et al., 2009; Medoff et al., 2009; Molinero et al., 2009) but can generate iT_{reg} cells (Barnes et al., 2009), has yielded similar results. In these mice, T_{reg} cells in the spleen and peripheral lymph nodes are only ~ 3 – 4 % as frequent as T_{reg} cells in wild-type mice, but this frequency increases to ~ 8 % and ~ 40 % in the mLN and colon LP, respectively. Because transferred T cells have to compete with pre-existing T cells for cytokines needed to induce Foxp3 (Lathrop et al., 2008) and Carma1-deficient $CD4^+$ T cells have a higher TCR signalling threshold for Foxp3 induction (Barnes et al., 2009), these represent conservative estimates of the normal iT_{reg} cell population. Additionally, mice housed in specific pathogen free (SPF) conditions are not exposed to persistent infections or chronic inflammation, which could further promote iT_{reg} cell generation (Curotto de Lafaille and Lafaille, 2009). However, it is reasonable to assume that the majority of T_{reg} cells in the GI tract of healthy mice represent $Foxp3^+$ T_{reg} cells exported from the thymus. Taken together, these studies indicate that the noninflamed GI tract is a permissive site for the accumulation of iT_{reg} cells that act together with thymus-derived T_{reg} cells to reinforce intestinal homeostasis.

In order to suppress immune responses toward exogenous dietary and flora antigens, the GI tract could be enriched for reactive thymic-derived T_{reg} cells or iT_{reg} cells could be generated from conventional $CD4^+$ T cells. T_{reg} cells in the mLN express rearranged TCR alleles with sequences that are distinct from those expressed by T_{reg} cells in the peripheral lymph nodes and show little overlap with the TCRs expressed by naive or memory T cells in the mLN (Lathrop et al., 2008). This observation suggests that intestinal antigens, either self-antigens

expressed by cells in the GI tract or exogenous-antigens derived from the lumen, do shape the intestinal T_{reg} cell pool. In support of the latter possibility, several studies have demonstrated that orally fed antigen can expand antigen-specific regulatory T cell populations (Chen et al., 1994). Although oral tolerance might also involve the expansion of reactive thymus-derived T_{reg} cells, work from the Powrie and other labs has demonstrated that this phenomenon involves the induction of Foxp3$^+$ iT_{reg} cells from the conventional T cell pool and predominantly occurs in the GALT (Coombes et al., 2007; Mucida et al., 2005; Sun et al., 2007). Orally induced T_{reg} cells are functional and act both locally in the gut and systemically. For example, orally induced ovalbumin-specific Foxp3$^+$ T_{reg} cells can suppress lung inflammation in a model of asthma driven by T_H2 effector cells (Curotto de Lafaille et al., 2008). Different T_{reg} cell effector mechanisms are required to prevent inappropriate immune responses to dietary- versus microbial flora-derived antigens, with the production of IL-10 being dispensable for oral tolerance but essential for maintaining tolerance of the intestinal microbial flora (Fowler and Powrie, 2002). In humans with inflammatory bowel disease (IBD), oral tolerance is impaired (Kraus et al., 2004), although further research is needed to determine whether this finding represents genetic defects that predispose individuals to developing IBD (Kraus et al., 2006) or a secondary consequence of intestinal inflammation.

1.6 The intestinal T_{reg} cell niche: IL-10-producing T cells

Although the generally immunosuppressive cytokine IL-10 is an important effector molecule expressed by Foxp3$^+$ T_{reg} cells, *Il10*$^{-/-}$ mice do not develop the lymphoproliferative autoimmune disease observed in neonatal Foxp3-deficient mice. Instead, they are highly susceptible to intestinal inflammation that can be triggered after weaning by the presence of common intestinal bacteria, such as *H. hepaticus*, in the context of a "normal" SPF-flora (Kullberg et al., 1998). Other genera of bacteria, for example segmented filamentous bacteria, can act as triggering microbes in alternate colitis models (Stepankova et al., 2007), and the flora can also contain bacteria that offset the presence of disease-triggering micro-

organisms by promoting IL-10 expression (Mazmanian et al., 2008). The composition and density of the intestinal flora varies greatly throughout the GI tract, so it might be fitting that the need for IL-10 varies similarly. Thus, whereas Foxp3$^+$ T_{reg} cells represent a constitutive regulatory presence, IL-10 acts as an inducible immunoregulatory factor that can be called into action when and where inflammatory conditions demand. IL-10 acts in part by activating Stat3. The finding that mice lacking Stat3 in the myeloid compartment develop colitis similar to *Il10*$^{-/-}$ mice suggests that myeloid cells are essential targets of IL-10 signalling in the intestine (Takeda et al., 1999). Exogenous IL-10 can limit the endoplasmic reticulum stress response in an IEC cell line (Shkoda et al., 2007), so IL-10 might also contribute to the maintenance of intestinal barrier function.

Mice that co-express Foxp3 and IL-10 reporter constructs allow IL-10 expression in Foxp3$^+$ T_{reg} cells and other T cell subsets to be monitored at the single-cell level (Kamanaka et al., 2006; Maynard et al., 2007). Consistent with a requirement for T cell-derived IL-10 in maintaining intestinal homeostasis (Roers et al., 2004), a substantial fraction (~ 10 – 30 %) of tissue-resident intestinal CD4$^+$ T cells can produce IL-10 (Kamanaka et al., 2006; Maynard et al., 2007; Uhlig et al., 2006). In the colonic LP, nearly all of the IL-10-producing T cells are Foxp3$^+$ T_{reg} cells. These cells have a nonredundant function, as shown by the fact that *Helicobacter*-infected mice with a specific deletion of IL-10 in Foxp3$^+$ cells develop typhlitis (inflammation of the caecum) and mild colitis (Rubtsov et al., 2008). However, the colitis observed in these mice is less severe than in *Il10*$^{-/-}$ mice, suggesting that other sources of IL-10 are also functionally important. Among intraepithelial lymphocytes in the small intestine, most IL-10-producing CD4$^+$ T cells are Foxp3$^-$ and do not secrete effector T cell cytokines or IL-2 (Maynard et al., 2007), reminiscent of previously described IL-10-T_{reg} or T regulatory type 1 (Tr1) cells (Vieira et al., 2004). It remains debatable whether these cells represent a distinct subset of CD4$^+$ T cells, or an 'exhausted' effector cell. In the small intestine LP, both Foxp3$^+$ T_{reg} and Tr1-like cells produce IL-10. This intestinal regulatory T cell subset

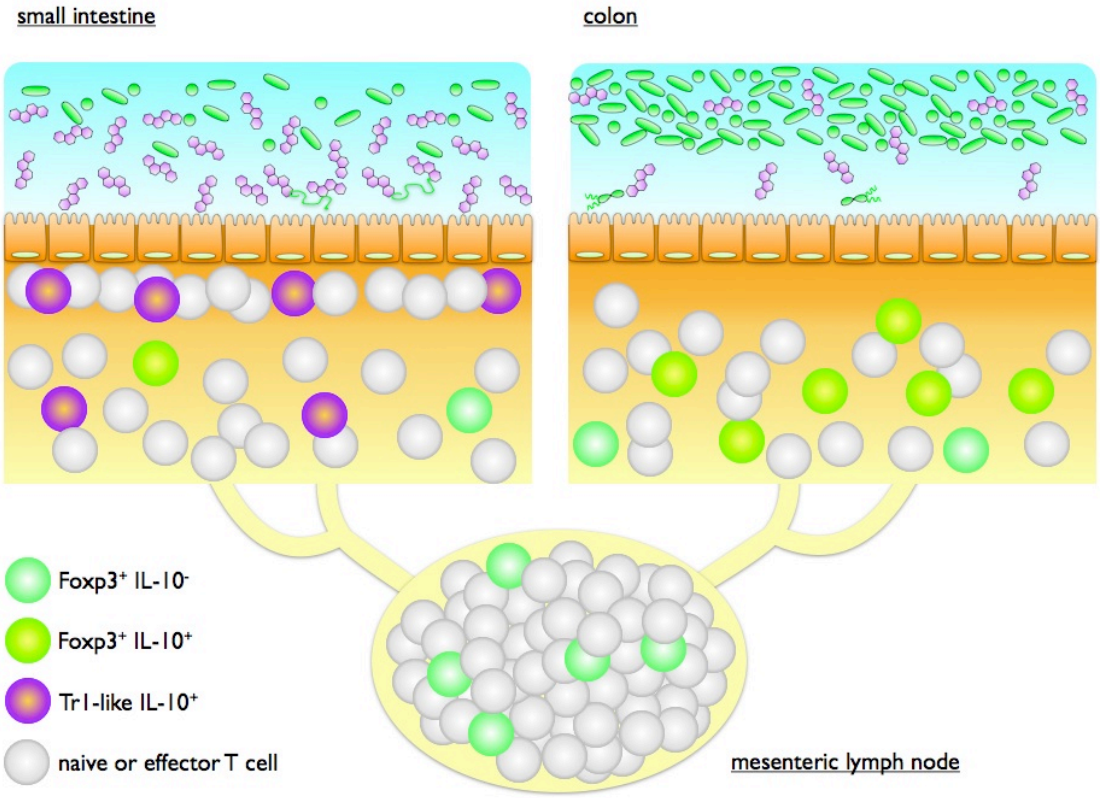

Figure 1.2. The anatomy of intestinal regulatory T cell populations.

$CD4^+$ T cell populations with regulatory functions in the intestine include $Foxp3^+$ T_{reg} cells and $Foxp3^-$ Tr1-like cells. In the colon, which harbours a dense resident bacterial community, most of the $Foxp3^+$ T_{reg} cells in the lamina propria produce IL-10 and Tr1-like cells are less frequent. Fewer bacteria colonize the small intestine, where the bulk of dietary nutrients are absorbed. Here, a high frequency of Tr1-like cells patrol the intraepithelial layer, whereas both Tr1-like cells and $Foxp3^+$ T_{reg} cells populate the lamina propria. In the draining mLN, fewer IL-10-producing cells are found than in the intestinal tissue and the major regulatory population is the $Foxp3^+$ T_{reg} cell. Some intraepithelial lymphocytes are present in the colon, but the frequency of $Foxp3^+$ T_{reg} and Tr1-like cells in this location has not been reported.

compartmentalisation suggests that the GI tract contains several distinct immunological microenvironments that differentially promote IL-10 production (Figure 1.2).

The signals that turn on IL-10 expression in T cells are distinct from those that induce Foxp3 expression. Both $Foxp3^+$ T_{reg} cells and Tr1-like cells can express IL-10 (Asseman et al., 1999; Vieira et al., 2004). Effector T cells can also co-express IL-10 along with IFN-γ, IL-4 or IL-17 in infectious contexts (O'Garra and Vieira, 2007), and this has been shown, *in vitro*, to require extracellular signal-regulated kinase 1 (Erk1) and Erk2 activation (Saraiva et al., 2009). The best-characterized pathway for inducing IL-10 expression occurs in response to IL-27, a member of the IL-12 family of cytokines that has both effector and regulatory properties. Exposure to IL-27 can induce IL-10 expression in $CD4^+$ T cells by triggering a sequence of events that include upregulation of the transcription factor c-Maf and subsequent induction of IL-21, which acts as a growth factor for IL-10-producing T cells (Pot et al., 2009; Spolski et al., 2009). IL-27-deficient mice are not susceptible to flora-triggered colitis, like IL-10-deficient mice, in part because IL-27 also promotes effector T cell responses. However, *Il27r*$^{-/-}$*Il10*$^{-/-}$ double-deficient mice still develop colitis, although with slower kinetics than *Il10*$^{-/-}$ mice (Villarino et al., 2008). These findings suggest that other pathways for both effector T cell responses and IL-10 induction operate in the intestine. For example, in the presence of TGF-β, IL-6 can drive IL-10 production independently of IL-21 signalling (Spolski et al., 2009). IL-6 is produced in large amounts during intestinal inflammation, but its contribution to the induction of IL-10 in the steady state is less obvious and merits investigation. Furthermore, although IL-27 and TGF-β can induce iT_{reg} cells that express IL-10 in vitro (Stumhofer et al., 2007), the signals that regulate IL-10 expression in $Foxp3^+$ T_{reg} cells *in vivo* remain elusive (Maynard et al., 2007; Maynard et al., 2009).

Mouse models have identified several transcription factors as potential regulators of IL-10 expression, including two that have been implicated in IL-21 signalling, B lymphocyte induced maturation protein 1 (Blimp-1) and c-Maf. Mice lacking haematopoietic or T cell

expression of Blimp-1, which is encoded by *PR domain containing 1* (*Prdm1*) and highly expressed by both activated T cells and $CD4^+CD25^+$ T cells, develop spontaneous colitis (Kallies et al., 2006; Martins et al., 2006). Colitis may occur because of excessive effector T cell responses, because Blimp-1 is needed to limit IL-2 expression and polarization toward a T_H1 cell effector phenotype. Blimp-1 may also be required for regulatory T cell function. Whereas mRNA encoding Blimp-1 is not expressed in large amounts among resting $Foxp3^+$ T_{reg} cells (Hill et al., 2007), the promoter of *Prdm1* does contain a Foxp3 binding site (Zheng et al., 2007) making it unclear whether activated T cells or perhaps activated $Foxp3^+$ T_{reg} cells are the major source of Blimp-1 in the $CD4^+CD25^+$ T cell pool. Although T_{reg} cells from Blimp-1-deficient mice can protect in the T-cell-transfer model of colitis (Kallies et al., 2006), both the $CD25^+$ and $CD25^-$ $CD4^+$ T cell populations show a reduced frequency of IL-10^+ cells compared to wild-type mice (Martins et al., 2006); however, this reduction could be a secondary effect of ongoing inflammation. Another candidate transcription factor for promoting IL-10 expression, c-Maf, can be induced by IL-27 and IL-21 (Pot et al., 2009) or signalling through the inducible costimulator (ICOS) molecule (Bauquet et al., 2009). ICOS-deficient $CD4^+$ T cells show defects in IL-10 expression (Pot et al., 2009), and many IL-10-producing $CD4^+$ T cells, including $Foxp3^+$ T_{reg} cells, coexpress ICOS (Huehn et al., 2004; Ito et al., 2008). Intriguingly, preliminary reports suggest that ICOS-deficient T_{reg} cells fail to control T-cell-transfer-induced colitis (Zheng et al., 2009). Transcription factors activated by TGF-β signalling are also likely to be important in generating intestinal IL-10-producing T cells (Kitani et al., 2003; Maynard et al., 2007).

1.7 The intestinal T_{reg} cell niche: TGF-β

TGF-β is a pleiotropic cytokine, important for the maintenance and effector function of both $Foxp3^+$ T_{reg} and Tr1-like cells in the intestine (Chen et al., 1994; Li et al., 2006; Marie et al., 2005; Marie et al., 2006; Maynard et al., 2007; Powrie et al., 1996). Unlike the susceptibility to colitis observed in *Il10*$^{-/-}$ mice, *Tgfb1*$^{-/-}$ mice succumb to a T cell-dependent

lymphoproliferative autoimmune disease by several weeks of age (Diebold et al., 1995). This difference has made studying the role of TGF-β in the GI tract especially challenging, necessitating the use of both adoptive transfer and conditional gene-targeting approaches. In the absence of signalling through TGF-βRII, T cells become partially activated and prone to differentiate into autoreactive effector T cells (Gorelik and Flavell, 2000; Li et al., 2006; Marie et al., 2006). In the T-cell-transfer model of colitis, transferred naive T cells expressing a dominant-negative TGF-βRII molecule cause intestinal inflammation that cannot be suppressed by T_{reg} cells (Fahlén et al., 2005). Furthermore, $CD4^+$ T cells from mice that overexpress *Mothers against decapentaplegic homolog 7* (*Smad7*), an endogenous inhibitor of TGF-β signalling through Smad2 and Smad3, are similarly resistant to suppression (Fantini et al., 2009). Therefore, TGF-β signalling into T cells is essential to limit colitogenic effector T cell responses.

Regulatory T cells are one source of intestinal TGF-β. Studies from the Powrie lab and others demonstrated that T_{reg} cells from *DO11.10.Tgfb1*$^{-/-}$ mice (Fahlén et al., 2005) or *Tgfb1*$^{-/-}$ neonates (Kullberg et al., 2005) function in the T-cell-transfer model of colitis. However, another study using the same model reported that TGF-β1-deficient T_{reg} cells do not limit weight loss or intestinal inflammation (Li et al., 2007). Differences in the intestinal flora, genetic background, T_{reg} cell TCR repertoire or ongoing inflammation in the donor mice might underlie these conflicting results. Interestingly, TGF-β1-deficient T_{reg} cells cotransferred with TGF-β1-deficient naive T cells cause worse disease than TGF-β1-deficient T_{reg} cells cotransferred with wild-type naive T cells (Li et al., 2007). Furthermore, Tr1-like cells can suppress T cell proliferation in vitro by producing TGF-β and IL-10 (Maynard et al., 2007). These findings are consistent with the existence of T_{reg} cell-dependent and -independent sources of TGF-β in the $CD4^+$ T cell pool that contribute to intestinal homeostasis.

Although the intestine harbours a high concentration of TGF-β, the majority is thought to exist in an inactive form. Integrins have a key role in activating TGF-β, including $\alpha_v\beta_6$ integrin expressed by IECs and $\alpha_v\beta_8$ integrin expressed by DCs (Lacy-Hulbert et al., 2007; Munger et al., 1999; Travis et al., 2007). *Helicobacter*-infected mice lacking β_8-integrin expression in $CD11c^+$ cells develop spontaneous colitis, demonstrating an essential role for DCs expressing TGF-β-activating integrins in the intestine (Travis et al., 2007). In addition to being a source of TGF-β, T cells might also express molecules important for TGF-β activation, thereby promoting both T_{reg} cell induction and maintenance (Andersson et al., 2008). Thrombospondin is one such molecule (Crawford et al., 1998) and it is notable that a population of latency-associated peptide-expressing $CD4^+CD45RB^{lo}CD25^-$ T cells that are enriched for thrombospondin expression and can prevent intestinal inflammation in the T-cell-transfer colitis model (Oida et al., 2003). Another important molecule is the proprotein convertase Furin, which can activate TGF-β along with a number of other substrates. Mice engineered to lack *Furin* expression in T cells have normal to elevated numbers of $Foxp3^+$ T_{reg} cells, but show impaired TGF-β-dependent processes in the GI tract and develop colitis by six months of age (Pesu et al., 2008). In summary, $CD4^+$ T cells, DCs and IECs all collaborate to activate TGF-β and maintain T cell tolerance in the intestine.

1.8 The intestinal T_{reg} cell niche: conditioning factors

TGF-β is one of many factors that control the size and composition of the intestinal T_{reg} cell niche. The frequency of peripheral T_{reg} cells is reduced in the absence of TGF-βRII signalling (Li et al., 2006; Marie et al., 2006), but remains normal in mice with a T cell-specific deletion of TGF-β1 (Li et al., 2007). Although it is possible that other TGF-β isoforms compensate for the absence of TGF-β1, non-T cell sources of TGF-β likely contribute to the maintenance of the intestinal T_{reg} cell compartment. Apoptotic cells represent an important potential source of bioactive TGF-β (Chen et al., 2001), capable of enhancing TGF-β production from immature DCs and macrophages, and, in turn, generating

iT_{reg} cells (Perruche et al., 2008). Because epithelial cells have a high rate of turnover, apoptosis of IECs may be an important process in establishing the TGF-β-rich intestinal environment that supports a high frequency of T_{reg} cells. In support of this idea, mice lacking expression of α_v integrins in haematopoietic cells show defects in the phagocytosis of apoptotic cells and develop spontaneous colitis (Lacy-Hulbert et al., 2007). Like β_8 integrin-deficient mice (Travis et al., 2007), these mice have a specific reduction of $Foxp3^+$ T_{reg} cells in the colon LP, but not the spleen or lymph nodes (Lacy-Hulbert et al., 2007). $\alpha_v\beta_8$ integrin can activate TGF-β, so it is not yet clear whether the intestinal inflammation and T_{reg} cell deficiency in α_v integrin-deficient mice reflect an absence of bioactive TGF-β or impaired apoptotic cell uptake. It is also possible that these processes are intertwined (Perruche et al., 2008). Apart from sustaining T_{reg} cells, apoptotic cell-derived TGF-β can instead support effector T cell responses during inflammatory conditions. For example, during *Citrobacter rodentium* infection, the apoptosis of IECs accelerates, resulting in the production of large amounts of TGF-β in an inflammatory milieu that contributes to the T_H17 cell response in this acute model of intestinal inflammation (Torchinsky et al., 2009).

The intestinal flora also influences the balance of intestinal T_{reg} and T_H17 cells by generating immunomodulatory metabolites. A comparison of serum from germ-free and conventionalized mice by mass spectrometry found that the flora influences the concentration of ~ 10 % of common circulating metabolites (Wikoff et al., 2009). Affected molecules include the aromatic amino acids—phenylalanine, tryptophan and tyrosine—and their derivatives, including the signalling molecule serotonin. Interestingly, these classes of molecules directly influence the differentiation of T_H17 cells *in vitro* (Veldhoen et al., 2009). In the intestinal lumen, resident bacteria secrete adenosine 5′-triphosphate (ATP), which similarly favours the expansion of T_H17 cells (Atarashi et al., 2008). Accumulation of T_H17 cells can come at the expense of the maintenance of $Foxp3^+$ T_{reg} cells. For example, mice colonized with an intestinal flora that supports high frequencies of T_H17 cells in the small

intestine LP have correspondingly lower frequencies of Foxp3$^+$ T_{reg} cells among CD4$^+$ T cells (Ivanov et al., 2008). In the presence of an intestinal flora that favours T_H17 cell accumulation, the induction of Tr1-like cells might represent a backup or alternative regulatory system (Figure 1.3).

The flora also contains bacterial molecules that promote regulation. Polysaccharide A (PSA), a carbohydrate expressed by the human commensal bacterium *Bacteroides fragilis*, is sufficient to ameliorate T cell-driven colitis in an IL-10-dependent manner (Mazmanian et al., 2008). Previous studies demonstrated that PSA could be presented to CD4$^+$ T cells by MHC class II molecules and favours T_H1 cell effector responses, which are normally associated with intestinal inflammation (Mazmanian et al., 2005). Whether PSA also induces protective Tr1-like or T_{reg} cell responses remains an important unanswered question. Another intestinal bacterium, *Faecalibacterium prausnitzii*, has been shown to induce IL-10 expression in circulating T cells (Sokol et al., 2008). Discovering the molecules and mechanisms underlying the many "probiotic" properties of the intestinal flora is an area of great therapeutic interest.

In addition to affecting the ratio of T_{reg} cells to effector T cells, dietary- and microbial flora-derived molecules influence the frequency of intestinal Foxp3$^+$ T_{reg} and Tr1-like cells. Deoxyribose nucleic acids (DNA) in the intestinal lumen, presumably released from dying bacteria, contain TLR9-activating motifs (Hall et al., 2008). *In vitro*, stimulation of DC and T cell cocultures with TLR9 ligands induces inflammatory cytokines, including interferon γ (IFN-γ), IL-4 and IL-6, which inhibit the generation of iT_{reg} cells. The increased frequency of Foxp3$^+$ T_{reg} cells in the small intestine LP of *Tlr9*$^{-/-}$ mice suggests that such a mechanism may operate *in vivo*. TLR9-mediated activation of the IL-6 and IL-21 signalling pathways also induces IL-10 expression among Foxp3$^-$ CD4$^+$ T cells *in vitro* (Maynard et al., 2009). Whether TLR9 signalling exerts similar effects *in vivo*, promoting Tr1-like cell responses, remains to be determined. Retinoic acid (RA), a vitamin A metabolite, can act as an opposing

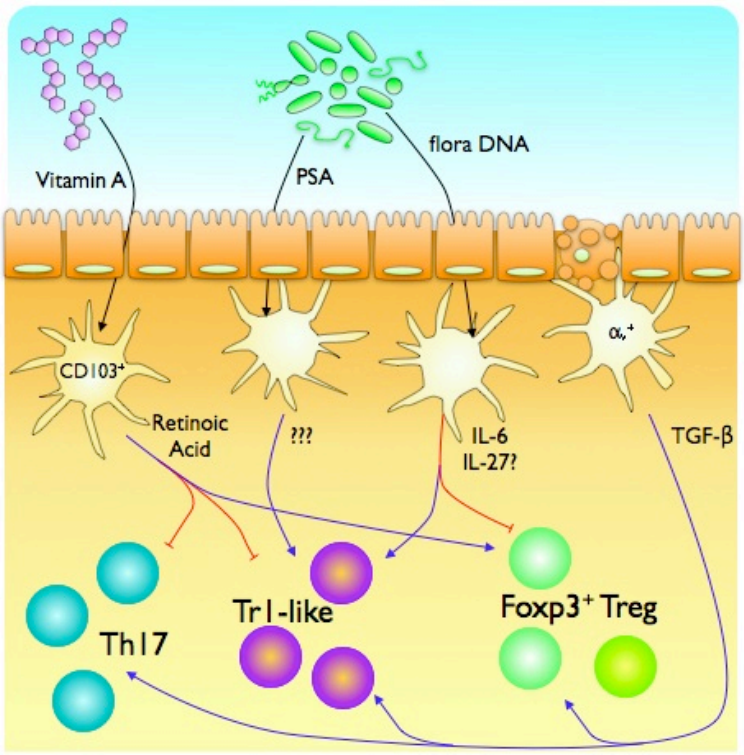

Figure 1.3. Distinct stimuli promote intestinal T_H17, Tr1-like and Foxp3$^+$ T_{reg} cells.

Of the many factors unique to the intestinal environment, several stimuli that affect the differentiation of T cell subsets have been identified. Retinoic acid (RA), a metabolite of dietary vitamin A, can promote iT_{reg} cell generation and inhibit T_H17 and Tr1-like cell responses in part by reducing IL-21 transcription and IL-6R expression in T cells. PSA, a molecule expressed by the bacterium *Bacteroides fragilis*, can ameliorate intestinal inflammation by an IL-10-dependent mechanism, which might involve the expansion of Tr1-like cells. Bacterial DNA from the flora can activate TLR9, thereby limiting T_{reg} cell accumulation and potentially favouring Tr1-like cell responses via the production of IL-6 and perhaps IL-27, which can induce the IL-10-promoting cytokine IL-21. All three T cell subsets utilize TGF-β for their maintenance. Apoptotic IECs are one potential source of TGF-β, which might be activated by both myeloid cells expressing α_v integrins and T cells expressing TGF-β-activating molecules.

influence, favouring the generation of Foxp3$^+$ iT$_{reg}$ cells (Coombes et al., 2007; Mucida et al., 2007; Sun et al., 2007) and limiting the induction of IL-10 expression (Maynard et al., 2009). RA might inhibit IL-10 expression by reducing the T cell surface expression of IL-6Rα and transcription of IL-21 in activated T cells (Hill et al., 2008). Mice raised on a vitamin A-deficient diet show an abundance of IL-10-producing Tr1-like cells throughout the intestine, yet retain a relatively normal frequency of IL-10-producing Foxp3$^+$ T$_{reg}$ cells (Maynard et al., 2009). Therefore, both TLR9 ligands and RA reciprocally influence the accumulation of Tr1-like and Foxp3$^+$ T$_{reg}$ cells in the intestine (Figure 1.3), but other factors appear to regulate IL-10 expression in Foxp3$^+$ T$_{reg}$ cells.

Identifying cells that deliver IL-10- or Foxp3-inducing signals is an area of intense investigation. Intestinal DCs expressing $\alpha_E\beta_7$ integrin (CD103) and CD11b$^+$ intestinal macrophages are enriched in their capacity to store and produce RA and are thus potent generators of iT$_{reg}$ cells *ex vivo* (Coombes et al., 2007; Denning et al., 2007; Sun et al., 2007). CD103$^+$ DCs comprise ~ 30-50 % of intestinal DCs and are rather homogenously distributed throughout the colon LP, small intestine LP and mLN of healthy mice (Annacker et al., 2005; Sun et al., 2007). Yet, the composition of Foxp3$^+$IL-10$^-$, Foxp3$^+$IL-10$^+$ and Tr1-like cells varies greatly between intestinal compartments (Figure 1.2) (Maynard et al., 2007). Currently available data cannot explain the heterogeneous distribution of T$_{reg}$ and Tr1-like cells in the intestine. However, it is notable that these populations in the small intestine LP are particularly sensitive to changes in the flora induced by vancomycin treatment, TLR9 deficiency or RA depletion (Hall et al., 2008; Ivanov et al., 2008; Maynard et al., 2009). The colon LP contains a large population of IL-10-producing Foxp3$^+$ T$_{reg}$ cells and comparatively fewer Tr1-like cells that are less sensitive to such perturbations. It may be that RA and microbial flora-derived DNA are dominant forces that shape the differentiation of CD4$^+$ T cells in the small intestine. Because the colon harbours the highest concentration of resident bacteria in the GI tract, additional, partially overlapping pathways may operate to maintain intestinal homeostasis.

1.9 Modes of regulation in the lymph nodes and tissues

Of the many mouse models of colitis, the T-cell-transfer model is unique in its ability to probe the regulatory mechanisms utilized by T_{reg} and Tr1-like cells (Strober et al., 2002). Although the effector functions of T_{reg} cells have been recently reviewed (Shevach, 2009), their mode of action in the GI tract is distinct. Somewhat surprisingly, T_{reg} cells lacking β_7 integrin, an important molecule for trafficking to the intestinal LP and intraepithelial tissues, function in the T-cell-transfer colitis model (Denning et al., 2005). By contrast, chemokine (C-C) motif receptor 4 (Ccr4)- and Ccr7-deficient T_{reg} cells are impaired in their ability to repopulate the mLN and fail to prevent colitis (Schneider et al., 2007; Yuan et al., 2007). Together, these findings suggest that T_{reg} cells can prevent colitogenic T cell responses via actions in the mLN. One potential mechanism involves limiting the duration of contact between T cells and DCs, perhaps because of the high expression of cytotoxic T lymphocyte antigen 4 (Ctla-4) on T_{reg} cells, thus reducing the likelihood that a naive T cell would become activated (Tadokoro et al., 2006). T_{reg} cells may accomplish this by forming aggregates around DCs, preventing access by other T cells (Onishi et al., 2008). In this context, an intestinal T_{reg} cell population with a TCR repertoire able to recognize dominant intestinal antigens would be able to out compete naive or effector T cells with similar antigen specificities for access to DCs, thereby preventing the priming or proliferation of colitogenic T cells.

Large numbers of adoptively transferred T_{reg} cells can also cure established colitis induced by the transfer of naive T cells into *Rag2*$^{-/-}$ recipients (Mottet et al., 2003). In this setting, T_{reg} cells migrate into the inflamed LP and secrete IL-10 (Uhlig et al., 2006). The requirement for IL-10 in the cure setting, but not classic T-cell-transfer model (Asseman et al., 2003; Powrie et al., 1993), is of particular note. In colitic mice, changes in the flora can be observed, including an increase in segmented filamentous bacteria in close proximity to the epithelium (Stepankova et al., 2007). It is also worth noting that the prevention of T-cell-transfer colitis

requires T_{reg} cell-derived IL-10 when recipient mice are infected with *H. hepaticus* (Kullberg et al., 2002). Among common microbes in the murine intestinal flora, *H. hepaticus* is somewhat unique in that it can partially penetrate the mucus layer and approach the epithelium in the caecal crypts (Chan et al., 2005). Through a flora-centric lens, these requirements for IL-10 might reflect the need to temper exuberant innate immune responses toward bacteria that become associated with the epithelium, either as a result of mucus-secreting goblet cell depletion during the course of colitis or penetration of the mucus layer by certain microbes. IL-35 is another T_{reg} cell effector cytokine reported to be important for resolving established inflammation (Collison et al., 2007), although it acts through indirect effects on other T cells *in vitro* (Collison et al., 2009) and demands further mechanistic study *in vivo*.

An emerging concept in T_{reg} cell biology is that cytokines and signals that promote the expression of transcription factors classically associated with effector T cell subsets can also induce expression of the same transcription factors in subsets of T_{reg} cells, allowing their proliferation and acquisition of particular regulatory functions (Koch et al., 2009; Zheng et al., 2009). Such a strategy could tailor T_{reg} cells to best respond to different types of inflammation. This paradigm also appears to apply to intestinal T_{reg} cells, because deletion of Stat3 in $Foxp3^+$ T_{reg} cells leads to T_H17 cell-dependent intestinal inflammation (Chaudhry et al., 2009). Further studies are required to identify how Stat3 orchestrates the function of T_{reg} cells in the intestine.

1.10 Gaining therapeutic insights from murine colitis models

Despite its utility in dissecting intestinal effector and regulatory pathways, one limitation of the T-cell-transfer model is the introduction of lymphopaenia-induced proliferation as an additional variable predisposing mice to autoimmunity (reviewed in (Coombes et al., 2005)). Despite this complication, a strong correlation exists between genes involved in T cell-transfer colitis and those linked to human IBD-susceptibility alleles by genome-wide

association studies, such as *ICOSLG*, *IL10*, *IL12B*, *IL23R*, *STAT3* and multiple autophagy-related genes (Barrett et al., 2008; Franke et al., 2008). Therefore, the T-cell-transfer model seems to have general utility for discovering factors involved in IBD pathogenesis, including environmental factors or genes that genome-wide association studies might not identify because of low frequency risk alleles in human populations. One potential insight offered by the T-cell-transfer model is that the GI tract might support sustained proliferation and competition between effector and regulatory T cells. Costimulatory molecules have a central role in controlling T_{reg} cell proliferation and accumulation that has been reviewed extensively elsewhere (Bour-Jordan and Bluestone, 2009). Intriguingly, T_{reg} cells require expression of the costimulatory molecule Tumour necrosis factor superfamily, member 4 (Ox40) in order to efficiently repopulate the peripheral lymphoid organs and colon of *Rag2*$^{-/-}$ recipients. However, in mixed Ox40-deficient and wild-type bone marrow chimaeras, accumulation of Ox40-deficient T_{reg} cells occurred normally in the secondary lymphoid organs, but was selectively reduced in the colon LP (Griseri et al., 2010). Thus, genes required for lymphopaenia-induced proliferation may also be particularly important for the maintenance of T_{reg} cells in the intestine. It is notable that the role of costimulation in the generation of Tr1-like cells remains largely unexplored.

Enhancing Tr1-like or T_{reg} cell function represents a potential therapeutic strategy for treating human IBD, which most often presents in patients as patchy, recurring inflammation involving the ileum and colon (Crohn's disease) or continuous inflammation along the length of the colon (ulcerative colitis). In both diseases, increased numbers and frequencies of Foxp3^{+} T_{reg} cells are observed in the intestine (Uhlig et al., 2006). T_{reg} cells in human IBD retain some functionality as they secrete IL-10 in the colon LP, although it is possible that inflammatory conditions dampen IL-10-independent functions of T_{reg} cells. Importantly, effector T cells in human IBD express large amounts of the intracellular inhibitor of TGF-β signalling, SMAD7, rendering them resistant to T_{reg} cell-mediated suppression (Fantini et al., 2009; Monteleone et al., 2001). In order for T_{reg} cells to restore intestinal homeostasis, innate

immune activation might first need to be controlled in order to remove stimuli that render effector T cells unresponsive to TGF-β signalling. Although it is suspected that bacteria in the microbial flora are the major stimuli that trigger innate immune activation, the human microbial flora can contain viruses, fungi, protozoan parasites and worms that could also be involved in IBD pathogenesis (Artis, 2008).

The most effective IBD therapies currently used in the clinic might have underappreciated roles in enhancing regulatory activity. Steroid regimens serve as an initial therapeutic option for IBD and have systemic anti-inflammatory properties. *In vitro*, combinations of the steroid dexamethasone and vitamin D_3 potently induce Tr1-like cells (Vieira et al., 2004), and it is possible that similar effects occur in the GI tract after treatments with steroid regimens. Tumour necrosis factor α (TNF-α) neutralization represents an alternative therapeutic strategy that, in addition to reducing innate immune activation, may enhance the function of T_{reg} cells (Valencia et al., 2006). Blockade of IL-12p40, a component of IL-12 and IL-23, reduces innate immune activation and effector T cell responses, but might also result in the induction of iT_{reg} cells in the GI tract (Izcue et al., 2008). Designing therapeutic strategies that target innate immune activity or intestinal barrier function, but also leave behind an enhanced population of Tr1-like or T_{reg} cells, might improve the prospects for long-term remission of IBD symptoms and restore the symbiotic relationship of the host with the intestinal flora. Indeed, one unique therapeutic approach confers regulatory function on the flora itself by expressing an *Il10* transgene in the intestinal bacterium *Lactococcus lactis* (Braat et al., 2006).

Finally, insights into intestinal immune regulation gained from murine colitis models have potential applications beyond understanding the aetiology of IBD. In human tumour immunotherapy, one promising approach involves CTLA-4 blockade, which acts in part by reducing T_{reg} cell function in order to enhance antitumor immune responses (Peggs et al., 2006). As predicted from mouse models (Read et al., 2000), colitis is a problematic,

sometimes lethal side effect of this strategy. In bone marrow transplantation, a procedure utilized to treat a number of haematological conditions, MHC incompatibility can result in graft-versus-host disease (GVHD). Colitis is a common manifestation of GVHD and is associated with impaired accumulation of $FOXP3^+$ T_{reg} cells in the intestine (Rieger et al., 2006). During acute human immunodeficiency virus (HIV) infection, rapid depletion of intestinal $CD4^+$ T cells that express the HIV coreceptor CCR5 occurs in the GI tract (Brenchley and Douek, 2008). In the simian immunodeficiency virus macaque model of HIV infection, this phenomenon includes the loss of intestinal $FOXP3^+$ T_{reg} cells and the absence of regulatory function among total intestinal $CD4^+$ T cells by fourteen days after infection (Chase et al., 2007). Consequently, the remaining intestinal $CD4^+$ T cells proliferate and acquire effector function without restraint, resulting in intestinal inflammation that likely contributes to further viral dissemination. Incorporating targeted therapies that reduce immune activation and reinforce immune regulation in the intestine, such as blockade of the IL-23 signalling pathway, into the treatment of these devastating conditions is worth considering.

1.11 Future Perspectives

Here, the immunological niches, cell types and molecules unique to the intestine that promote immune regulation have been discussed. In humans, intestinal inflammation can be initiated at a specific location in the GI tract, but then spread in the absence of effective intervention. Although this spreading suggests a degree of communication between different niches in the gut, less is known about how events in one part of the GI tract affect another. For example, interesting studies have implicated the liver in inducing tolerogenic T cells (Crispe, 2009) that could potentially play an underappreciated role in maintaining intestinal homeostasis. Apart from anatomical differences, the availability of dietary- and microbial flora-derived molecules also varies throughout the GI tract. In the light of emerging evidence that T_{reg} cells are both affected by and influence organ-specific metabolism (Cobbold et al., 2009; Lumeng

et al., 2009), understanding the cellular targets and pathways activated by these molecules in the GI tract promises to be a fertile area for further research.

1.12 Aims of this thesis

The studies described here stemmed from a screen for genes involved in T cell activation, however the phenotype of mice with mutations in two genes important for T cell activation also showed defects in tolerance pathways. The first aim was to identify how a mutation that abolished the activity of Carma1 impaired the development of $Foxp3^+$ T_{reg} cells in the thymus. A second, related aim was identify factors important for activated $Foxp3^+$ T_{reg} and Tr1-like cells in the intestine to produce the immunoregulatory cytokine IL-10. The final aim was to dissect the spontaneous immunopathology that occurred in mice that lacked a GTPase, Gimap5, important for lymphocyte survival.

Chapter II. Material and Methods

2.1 Mice

All experiments carried out at The Scripps Research Institute (TSRI) were in accord with the guidelines of the National Institutes of Health (Bethesda, USA) and approved by the Institutional Animal Care and Use Committee of TSRI. Experiments performed at the University of Oxford were in accord with the UK Animal Procedures Act of 1986 and approved by a project license from the UK Home Office.

2.1.1 ENU mutagenesis

N-ethyl-*N*-nitrosurea (ENU) is a chemical mutagen (Figure 2.1) that randomly alkylates thymine residues in the DNA of both somatic and germline cells. If the alkylated residue is not repaired, the substitution of an adenine for a thymine residue can occur in the parallel strand of DNA, resulting in the introduction of a germline point mutation. In addition to A→T transversions (44% of total ENU-induced mutations), ENU can also induce A→G transitions (38%), G→A transitions (8%), A→T transitions (5%) and G→C transversions (3%) type mutations in mouse germline cells. When these germline mutations are translated into protein coding sequence, ENU-induced mutations comprise 64% missense, 10% nonsense and 26% splicing error type mutations (Justice et al., 1999). Mice with germline ENU-induced mutations can be bred to yield offspring that can be then subjected to phenotypic screens.

2.1.1.1 Generation of G_3-ENU mice

To screen for recessive mutations (i.e. loss of function alleles), G_3-ENU mice were generated that had an average of 3-4 homozygous point mutations in their genome that resulted in a change in protein coding sequence (Beutler et al., 2007) (Figure 2.2). C57BL/6 mice (The Jackson Laboratory) were used as G_0 male sires and received three injections at weekly intervals of 100 mg/kg ENU (SIGMA) *intra peritoneally*. This treatment causes the death

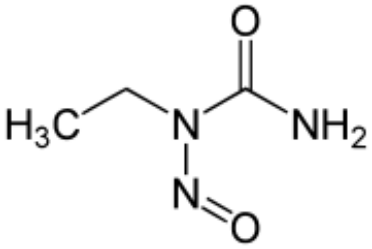

Figure 2.1. The chemical structure of ENU.

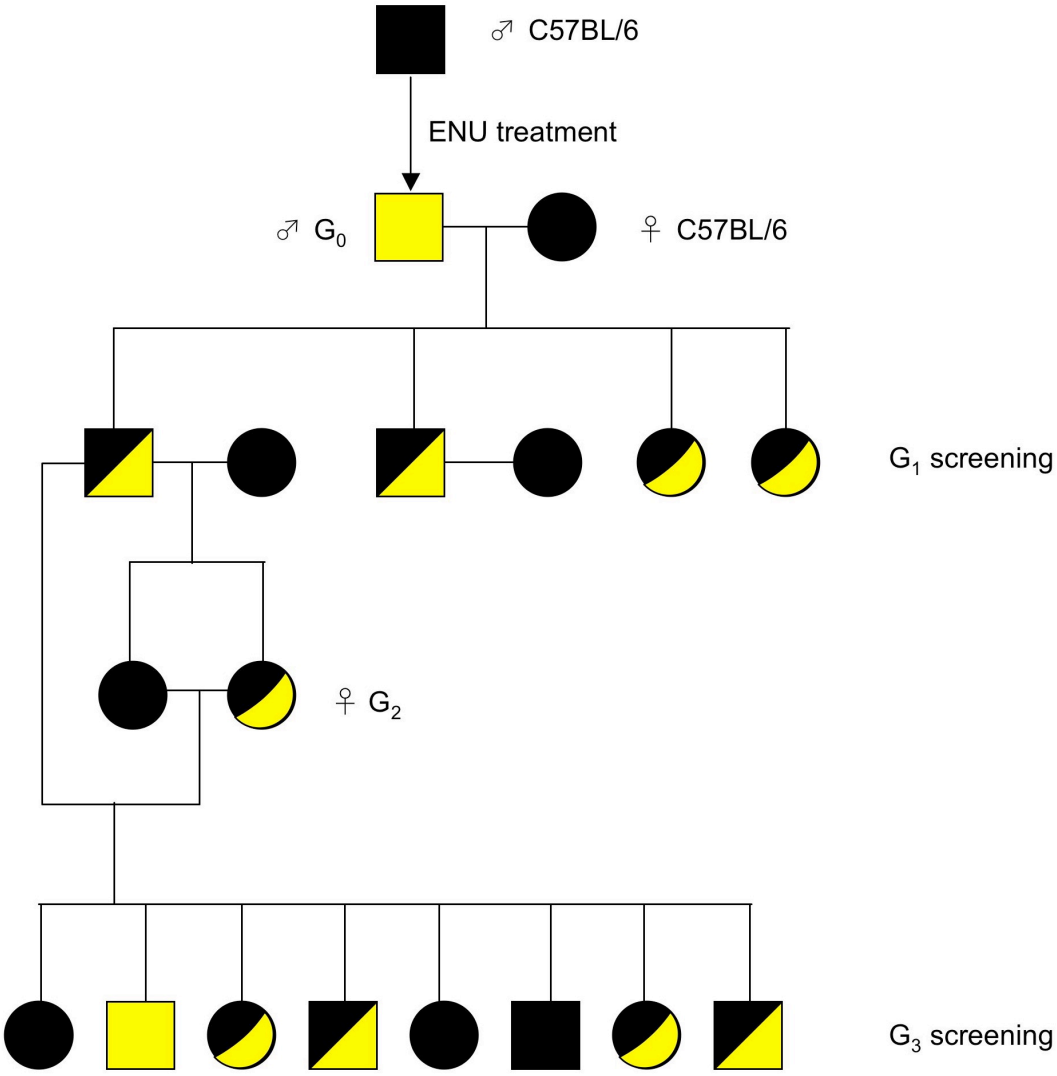

Figure 2.2. A diagram of the breeding scheme used to generate G_3-ENU mice.
G_0 mice were treated with ENU and then allowed to recover for 12 weeks before being bred to a wild-type female to yield G_1 offspring. G_1 mice can be screened for dominant acting mutations. Male G_1 mice bred to female G_2 daughters yield a G_3 generation that can be screened for recessive mutations. On average, each mouse screened has four homozygous mutations that occur in coding regions of the genome.

of most spermatogoneal stem cells. On average 10-100 stem cells survive and give rise to sperm cells that carry ENU-induced mutations. Crossing G_0 males with C57BL/6 wild-type females yields G_1 offspring that are heterozygous for all ENU-induced mutations present in the germline of sperm cells from the G_0 father. G_1 male offspring are then crossed with C57BL/6 wild-type females to yield G_2 offspring that carry 50% of the ENU-induced mutations present in the G_1 male as heterozygous alleles. Crossing G_1 males with G_2 female daughters gives rise to a G_3 generation. Any ENU-induced mutation present in a G_1 parent has a 1/8 chance of being homozygous in a G_3 mouse. Screening six G_3 mice per G_1 pedigree allows 49.3% of mutations present in the genome of a G_1 mouse to be rendered homozygous. Because screening more mice per pedigree results in an asymptotic decline in the percentage of alleles rendered homozygous, six mice per pedigree were subjected to any one phenotypic screen.

2.1.1.2 Screening G_3-ENU mutant mice for defects in $CD8^+$ T cell and NK cell function

A broad screen was designed to identify mutations that impaired the CTL response, encompassing antigen processing and presentation, the priming, proliferation and cytotoxic capacity of $CD8^+$ T cells, as well as the ability of NK cells to kill MHC class I-deficient target cells. Screening first involved immunising G_3-ENU mice with 1 x 10^8 γ-irradiated (1,500 rad) *act-mOVA* splenocytes. This immunogen is known to generate OVA-specific $CD8^+$ T cell effector responses that can be robustly detected as early as seven days post-immunisation (Janssen et al., 2006; Krebs et al., 2009). On day seven post-immunisation, G_3-ENU mice were injected *intra venously* with three populations of CFSE-labelled cells. One population was labelled with a high concentration of CFSE (5 μM) and then pulsed with the OVA-derived MHC class I epitope SIINFEKL (10 nM) for two hours at 37º C in IMDM with 10% FCS. Another population of splenocytes from *Tap1*$^{-/-}$ mice had reduced MHC class I expression and were labelled with an intermediate concentration of (1 μM) CFSE. As a

reference population, C57BL/6 wild-type splenocytes were labelled with a low concentration of (200 nM) CFSE.

CFSE labelling was carried out by washing cells three times in PBS with 0.1% FCS, then incubating in the dark at 37º C with CFSE for 10 minutes in PBS with 0.1% FCS. After 10 minutes, 20% of the total incubation volume of 4° C FCS was added to quench the CFSE labelling. The three populations of cells were combined, washed with PBS (no FCS) three times and then resuspended at a concentration of 5 x 10^8 cells/mL. 200 μL of CFSE-labelled splenocytes (1 x 10^8) were injected into each mouse *intravenously*.

After 48 hours, ~200 μL of blood was drown from the retro-orbital plexus and then pelleted at 7,500 g. Serum was removed by aspiration and blood cells were resuspended in 1 mL of RBC lysis buffer (SIGMA, USA) for five minutes. Cells were washed with PBS, and then fixed overnight with BD Perm/Fix buffer (BD Bioscience, USA). The presence or absence of each CFSE-labelled cell population was determined by flow cytometry. In a C57BL/6 wild-type immunised mouse, the control population ($CFSE^{low}$) would remain in circulation, but the SIINFEKL-pulsed population ($CFSE^{high}$) would be killed by $CD8^+$ T cells and the *Tap1*$^{-/-}$ population ($CFSE^{int}$) would be killed by NK cells. If a mutation impaired the $CD8^+$ T cell response, both the control and SIINFEKL-pulsed populations would be recovered. If a mutation impaired NK cell function, both the control and *Tap1*$^{-/-}$ populations would be recovered (Figure 2.3). If a mutation impaired the function of both $CD8^+$ T cells and NK cells, all three populations would be recovered.

2.1.1.3 Generating homozygous stocks of mutant mice

Each G_3-ENU mouse identified to have impaired $CD8^+$ T cell or NK cell responses was crossed with a C57BL/6 wild-type mouse. $CD8^+$ T cell and NK cell function were then tested in F_1 offspring to determine whether the mutation acted in a dominant fashion. If no

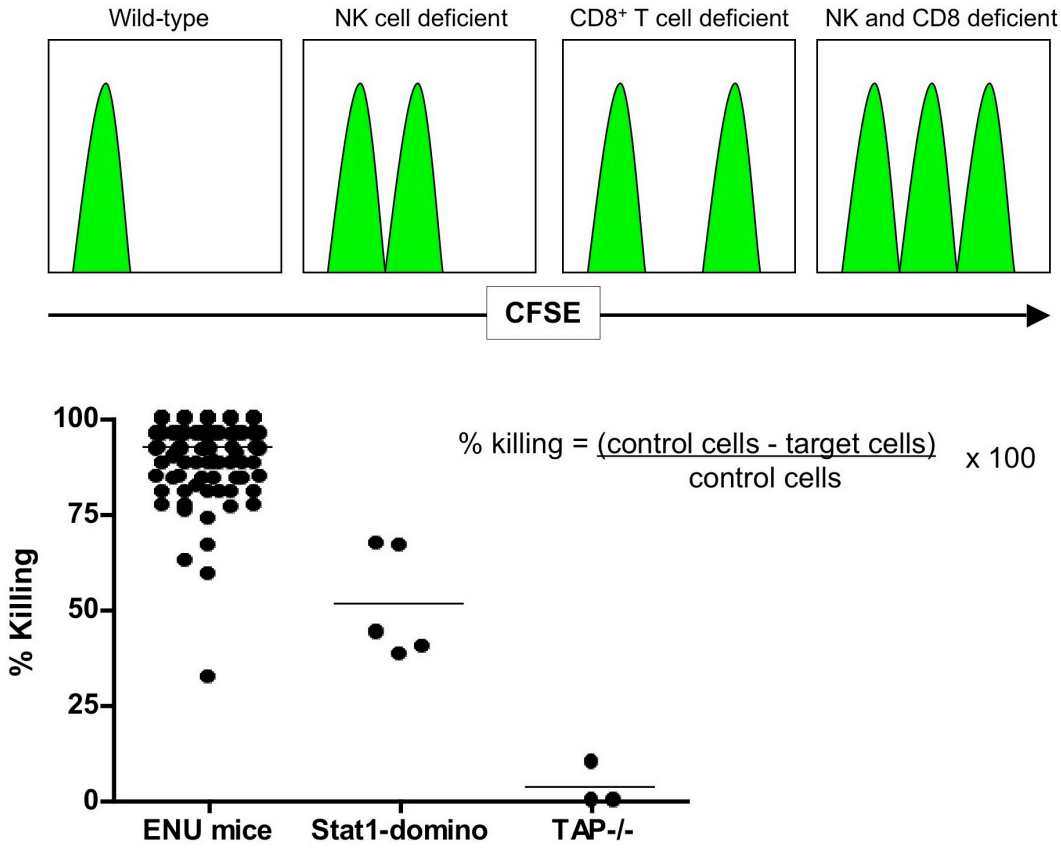

Figure 2.3. Assessing NK and CD8$^+$ T cell function by target cell killing.
Cartoons representing recovered target cell populations are shown above. For mice with the indicated phenotypes, different populations of target cells would be recovered. A representative batch of G_3-ENU mice that was screened is shown. Mice without CD8$^+$ T cell or NK cell function (*Tap1*$^{-/-}$) or with intermediate activity of these cell types (*Stat1*$^{dom/dom}$) were regularly included as internal controls. The percent killing of CD8$^+$ T cell targets is shown in the scatter plot and was calculated using the indicated formula based on the total cell number of control and target cells recovered. From this cohort of mice, one G_3-ENU mouse was selected for further breeding and confirmation.

dominant phenotype was observed, F_1 mice were backcrossed to the G_3-ENU mutant founder mouse. F_2 offspring were then tested for $CD8^+$ T cell responses and NK cell cytotoxicity. If ~50% of F_2 mice showed an immunodeficient phenotype, the mutation would be considered to be recessive and transmissible. Male and female mice showing the immunodeficient phenotype were bred to create a homozygous stock. Mutants with reduced viability (such as *sphinx* mice) were maintained either by crossing homozygous males with heterozygous females or by intercrossing heterozygous mice.

2.1.1.4 Positionally cloning recessive ENU-induced mutations

To link an ENU-induced mutation to a genomic region, backcrossed mice were subjected to whole-genome linkage analysis. Homozygous mutant stock mice (C57BL/6 background) were crossed to a mapping strain (C3H/HeN) to yield F_1 offspring. F_1 mice were backcrossed to homozygous stock to yield a F_2 generation that were each homozygous for C57BL/6 alleles across 50% of their genome and heterozygous (one C57BL/6 and one C3H/HeN allele) across the other 50%. Because the mutation arose on a C57BL/6 background, any chromosomal region with heterozygous alleles in a F_2 mouse showing the immunodeficient phenotype could be considered to be unlinked to the causative mutation, whereas regions with homozygous C57BL/6 alleles could be linked to it. The opposite is true for F_2 mice with a wild-type phenotype–in this case, the mutation must be linked to markers heterozygous for C57BL/6 and C3H/Hen alleles. Genotyping 15-30 backcrossed F_2 mice is generally sufficient to link an ENU-induced mutation to a chromosome. The degree of confidence in genetic linkage can be calculated by generating a logarithm of odds (LOD) score. A LOD score (Z) reflects the ratio of the probability of a linkage to a probability of non-linkage for each genomic marker (Lathrop and Lalouel, 1984). An LOD score of 4.0 or more a multiple markers on a chromosome is generally considered robust enough to assign a particular mutation to a chromosome.

Once a mutation was mapped to a chromosome, a "critical region" was defined by examining many genetic markers (microsatellite or SNP) on the chromosome of interest. Based on crossovers on the chromosome of interest, the mutation confined to a region with a distal and proximal boundary based on genetic marker genotypes. To narrow the critical region, additional backcrossed or intercrossed F_2 mice were genotyped for the chromosome of interest. For mice with informative crossovers, the phenotype was also determined. Once the critical region was narrowed to about 1.0 centimorgans (a genomic region for which a recombination event would occur on average in 1% of mice), either candidate genes or all predicted coding base pairs (based on Ensembl annotations and in house gene prediction and repeat masking software) were sequenced. Generally, high quality sequence could be obtained for most coding base pairs. Comparing homozygous mutant DNA sequence to the DNA sequence of wild-type C57BL/6 mice allowed ENU-induced mutations to be identified.

To confirm that a mutation caused a phenotype, several approaches could be taken. First, mRNA expression and protein abundance could be measured for the gene or gene product of interest. In the case of *tut* mice, the phenotype matched the phenotype of four other *Carma1*$^{-/-}$ mice, so no further confirmation was carried out. For *sphinx* mice, putative knockout mice (Nitta and Takahama, Japan) were crossed with *sphinx* mice to test for genetic complementation. An intermediate phenotype was observed in *Gimap5*$^{sph/-}$ mice, suggesting that either complementation did not occur, or that the *sphinx* mutation had dominant effects. Later it was determined that the mouse was a partial knockout of *Gimap5* and the *sphinx* phenotype mirrored another strain of *Gimap5*$^{-/-}$ mice and so the *sphinx* mutation was predicted to act in a recessive manner and to abolish Gimap5 protein activity.

2.1.2 Inbred, knockout and transgenic mice

Bim$^{-/-}$, *C57BL/6J*, *Cd4*$^{-/-}$, *Cd8*$^{-/-}$, *DBA/2J*, *Fas*$^{lpr/lpr}$, *FVB/NJ, Jnk2*$^{-/-}$, *NODShiLtJ* mice were obtained from The Jackson Laboratory (Bar Harbour, ME). *β2m*$^{-/-}$, *C3H/HeN*, *HY-TCR* transgenic, *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ mice were obtained from Taconic Farms (Germantown,

NY). *BALB/c* mice were maintained in an internal colony at TSRI. *Act-mOVA.Kb*$^{-/-}$, *Pkcθ*$^{-/-}$ and *Jα18*$^{-/-}$ mice were housed at the La Jolla Institute for Allergy and Immunology. *Foxp3-hCD2* 'knock-in', *IL-10*$^{gfp/gfp}$ 'tiger', *Il21r*$^{-/-}$ and *Myd88*$^{-/-}$ mice and *129.SvEv*, *129.SvEv-Foxp3-gfp* 'fusion protein' and *129.SvEv.Rag2*$^{-/-}$ mice were maintained in an internal colony at the University of Oxford in the Pathology Department. All knockout mice were crossed onto the *C57BL/6J* genetic background unless otherwise indicated.

2.2 Antibodies and biomolecules

2.2.1 Flow cytometry

The following anti-mouse antibodies were used in this study for flow cytometry: B220, CD11b, CD21, CD23, CD25, CD3, CD4, CD44, CD45RB, CD5, CD62L, CD69, CD8, Foxp3, γδ TCR, Gitr, (anti-human) CD2, HY TCR, ICOS, IFN-γ, IL-13, IL-4, IL-7Rα, NK1.1, PD-1, Sca-1, Thy-1.2, TNF-α, $V_{\alpha}2$ TCR (eBioscience); Ctla-4, IgD, IgM, TCR-V_{β} chains, TCRβ (BD Bioscience). FITC-conjugated anti-mouse NKp46 antibody was provided by Eric Vivier and generated in-house. PE-conjugated CD1d-tetramers loaded with αGalCer were provided by Isaac Engel and Mitchell Kronenberg; PE-conjugated anti-mouse m38 and m45 tetramers were provided by Beatrice Bollinger, Geraldine O'Hara and Paul Klenermann.

An Annexin V staining kit was used to measure phosphatidyl serine exposure as an indicator of apoptosis (BD bioscience).

Intracellular cytokine and intracellular Foxp3 staining was carried out as follows, keeping all cells and solutions at 4º C. After staining for cell-surface molecules, cells were fixed in 200 μL Fixation/Permeabilisation buffer (eBioscience) overnight in a 96-well round bottom plate. Cells were washed in 200 μL 1x Permeabilisation buffer three times. Then cells were resuspended in 50 μL of 1x Permeabilisation buffer with 1/500 diluted CD16/32 (Fc receptor)

blocking antibody and left on ice for ten minutes. 50 μL of anti-mouse Foxp3-PE antibody, 1/100 diluted, was added directly to wells that contained resuspended cells and blocking antibody. At least one hour later, cells were washed three times with 1x Permeabilisation buffer, then washed 2x with PBS with 2% FCS before analysis on a flow cytometer.

To generate neutralising antibodies for use *in vivo*, hybridoma cell lines that produced indicated monoclonal antibodies were grown in house and antibody was purified on a HPLC column. The following clones were used: anti-IL-6R (15A7), anti-IL-10R (IB1.2), anti-IL-12p40, anti-IFN-γ and anti-TGF-β. Zymogenetics provided neutralising antibodies for IL-21.

2.2.2 Immunoblotting

These antibodies were used for immunoblotting: Bcl10 (Santa Cruz); β-actin, pAkt (S473), pAkt (T306), Akt, Card11/Carma1, pErk, Erk, pGSK3b, pIκBα, IκBα, pJnk, Jnk, p-p38, p38, pPdk1, pPTEN, Tak1 (Cell Signaling). Anti-Gimap5 monoclonal antibody (clone MAC421) was provided by Geoffrey Butcher and is thought to target an epitope between residues 1 – 10 at the NH_2 terminus of mouse Gimap5 (C. Carter, G.W. Butcher, T. Nitta, K. Yano and Y. Takahama, unpublished observations). Purified CD3ε (145–2C11) and CD28 (37.51) antibodies (eBioscience) were used at indicated concentrations for T cell activation. F(ab')2–anti-IgM (Jackson ImmunoResearch) and anti-CD40 (eBioscience) were used for B cell activation. Neutralising anti-IL-2 antibodies (JES6-1) were used for *in vitro* T_{reg} cell studies (eBioscience).

2.2.3 Biomolecules

For *in vitro* lymphocyte activation studies, recombinant IFN-α, IFN-γ, IL-2, IL-4, human TGF-β1 and TNF-α (R&D Systems, Minneapolis, MN), LPS (Axxora, San Diego, CA) and PMA/ionomycin (Sigma-Aldrich, St. Louis, MO) were used. For *i*NKT cell activation, αGalCer was obtained from Kirin Brewery (Japan).

2.3 *In vivo* assays

2.3.1 *In vivo* NK cell and $CD8^+$ T cell cytotoxicity assay

G_3-ENU mice were immunised with 1 x 10^7 γ-irradiated (1500 rad) *act-mOVA* splenocytes. Seven days later, 1 x 10^7 CFSE-labelled cells were injected *intravenously*, consisting of a mixture of three different splenocyte populations (C57BL/6, $\beta 2m^{-/-}$ and SIINFEKL-peptide pulsed C57BL/6 splenocytes) at a ratio of 1:1:1. After 48 hours, blood was drawn from the retro-orbital plexus, red blood cells (RBCs) were lysed and the presence or absence of each CFSE-labelled cell population was determined by flow cytometry.

2.3.2 Secondary expansion of $CD8^+$ T cells

To assess the magnitude of the $CD8^+$ T cell response, mice were immunised with 1×10^7 γ-irradiated (1,500 rad) *act-mOVA* splenocytes. Seven days later, 5×10^6 splenocytes were isolated and cultured with 10 nM SIINFEKL peptide in Isocove's Modified Dulbecco's Media (IMDM) media supplemented with 10% Foetal calf serum (FCS) (Atlanta Bio). Five days later, cells were restimulated with 100 nM SIINFEKL peptide in the presence of brefeldin A. The production of IFN-γ was measured by intracellular cytokine staining.

2.3.3 *i*NKT cell activation.

*i*NKT cells were activated by injecting mice *intravenously* with 2 μg of αGalCer. Serum was collected 90 minutes later, and cytokine concentrations in the serum were measured by ELISA (eBioscience). Per-cell cytokine production was assessed by intracellular staining of *i*NKT cells followed by analysis by flow cytometry.

2.3.4 Serum anti-chromatin IgG2a measurement

ELISA plates were coated with mouse chromatin overnight at a concentration of 3.5 μg/mL (provided by Argy Theofilopoulos). Serum was transferred into chromatin-coated wells, and any IgG2a molecules that bound chromatin were detected with an alkaline phosphatase-conjugated anti-mouse IgG2a antibody (Caltag).

2.3.5 Basal serum concentrations of Ig subclasses

ELISA plates were coated with $F(ab)'_2$ fragments of goat anti-mouse IgG or IgM molecules (Jackson ImmunoResearch) at a concentration of 5 μg/mL. Serum was then transferred into coated wells. Ig subclasses (IgG1, IgG2a, IgG2b, IgG3, IgM or total IgG) that were captured were detected using Ig subclass-specific alkaline phosphatase-conjugated antibodies (Caltag).

2.3.6 Measurement of MCMV-specific Ig.

To measure the concentration of MCMV specific Ig in the serum, 96-well ELISA plates were coated with virus and then blocked in 5% milk. Serum samples were diluted 1:200, then serially diluted threefold. Anti-horseradish peroxidase (HRP) conjugated goat anti-mouse antibodies that recognized specific Ig subclasses were used for detection.

2.3.7 Assessing T-dependent and -independent antibody responses

To generate T-dependent antibody responses, six to eight week old and sex-matched mice were immunized *intra peritoneally* with either 5 μg LPS (Axxora) and 50 μg of NP36-CGG (Biosearch Technologies) mixed 1:1 with alum (Pierce) or an emulsion of 500 μg ovalbumin (SIGMA) in Complete Freund's adjuvant (CFA) (SIGMA). To generate T-independent antibody responses, mice were immunized *intra peritoneally* with 50 μg NP50-Ficoll (Biosearch Technologies). Serum NP-specific or OVA-specific Ig was detected by ELISA. Briefly, 96-well round-bottom plates (Fisher Scientific) were coated with 5 μg/ml NP30-BSA (Biosearch Technologies) or ovalbumin (SIGMA). Serum samples from immunized mice were serially diluted in 1% milk and added to coated or uncoated (to determine background) wells. Plates were incubated with HRP-conjugated goat anti-mouse IgM, IgG or IgG1

(Southern Biotechnology Associates), developed with Peroxidase Substrate and Stop Solution (KPL), and measured for absorbance at 450 nm.

2.3.8 Bone marrow chimaeras.

Recipient mice were γ-irradiated (2 × 500 rads) and injected *intravenously* with single cell suspensions of 1×10^8 donor bone marrow cells or foetal liver cells. Eight to ten weeks later, (enough time for the haematopoietic system of recipient mice to be reconstituted) lymphoid tissues were harvested, homogenized, stained and analyzed by flow cytometry.

2.3.9 Antibiotic treatment

Mice received antibiotic cocktail of 1 g/L ampicillin (SIGMA), 500 mg/L vancomycin (ARCOS), 1 g/L neomycin sulfate (Fisher), 1 g/L gentamicin (SIGMA) and 1 g/L metronidazole (SIGMA) administered orally via drinking water for five weeks. For the first two weeks of treatment, mice also received a paste of powdered chow reconstituted with antibiotic treated water to limit weight loss and dehydration.

2.3.10 Complete blood counts

Blood samples were taken from the retro-orbital plexus of mice at indicated ages and analyzed using a Hemavet 950 veterinary haematology system (Fisher Scientific).

2.3.11 Blood chemistry

Concentrations of three panels of serum molecules were measured by the UCSD mouse phenotyping core facility (San Diego, CA).

2.3.12 *In vivo* cytokine neutralisation

To block cytokines during development, neonatal mice were injected with purified antibodies *intra peritoneally* when 14 and 21 days old. Batches of antibody were used that showed efficacy in other *in vivo* colitis experiments carried out in the Powrie lab.

2.3.13 *Helicobacter hepaticus* culture and feeding

H. hepaticus NCI-Frederick isolate 1A (ATCC) was grown under high CO_2 conditions (in sealed glass containers) at 37º C on blood agar (Oxoid) plates that were made with laked horse blood (Oxoid) and contained the following antibiotics: trimethoprim (5 μg/ml), vancomycin (10 μg/ml) and polymyxin B (25 IU/ml) (Oxoid). Cultures from blood agar plates were transferred into liquid cultures of TSB media containing the same concentration of antibiotics and 10% FCS (Gibco). An OD600 measurement of 0.05 was used to inoculate bacteria. Vented plastic Erlenmeyer flasks (Corning) were securely placed in the same glass containers under high CO_2 conditions and put on a shaking incubator at 150 rpm and maintained at 37º C for 24 hours. Bacteria were pelleted by spinning in an ultracentrifuge at 7,500 rpm for 15 minutes and then resuspended in PBS with 10% FCS. Bacterial viability was confirmed by staining resuspended bacteria with a fluorescent Live/Dead assay kit (Molecular Probes) and then visualising bacteria with a fluorescent microscope. Live bacteria appeared green and any dead bacteria were red. Generally liquid cultures had a viability of greater than 98% and bacteria were highly motile. 1 x 10^8 bacteria were fed to mice by oral gavage for three consecutive days to ensure that recipients became colonised with *H. hepaticus*.

2.3.14 Whole splenocyte transfer

Three- to four-week-old *Gimap5*$^{sph/sph}$ mice were injected *intra venously* with 1 x 10^8 splenocytes in 200 μL of complete IMDM media with 10% FCS. Red blood cells (RBC) in donor splenocytes were lysed prior to adoptive transfer with RBC lysis buffer (SIGMA).

2.3.15 Naïve and regulatory T cell transfer

To enrich $CD4^+$ T cells prior to sorting, single-cell suspensions of splenocytes and lymph node cells were incubated with supernatants from in-house grown rat anti-mouse B220, CD8, CD11b and MHC class II hybridoma cultures for 30 minutes. Cells were washed in PBS with

0.1% Bovine serum albumin (BSA) and then resuspended in the same media with sheep anti-rat coated magnetic beads (Dynal) at a 1:1 ratio of beads to cells. Cells and beads were placed on a rotor for 20 minutes at 4º C. Next, all cells that had bound magnetic beads were removed by placing the tube of cells on a magnet and removing the supernatant, which was enriched for $CD4^+$ T cells. Enriched cells were stained with antibodies to mouse CD4, TCRβ, CD45RB and CD25, along with the 7-AAD viability dye (eBioscience), prior to sorting on a MoFlow fluorescence assisted cell sorter (FACS) machine. Purity of sorted cell populations was usually at least 98%. 1×10^5 putative regulatory T cells and 4×10^5 naïve T cells were injected into recipient mice *intravenously*. Recipient mice were weighed each week to monitor the onset of systemic wasting disease and the presence of sticky faeces was taken as an indicator of the onset of intestinal inflammation.

2.3.16 Measuring the severity of intestinal inflammation

Small ~0.5 cm segments of proximal, mid and distal colon, and a longitudinally cut section of caecum were taken from 129.SvEv *Rag2-/-* mice that had previously been injected with naïve and putative regulatory T cells and fixed in a 3.6% formaldehyde saline solution. Tissues were embedded in Paraffin, cut using a microtome and stained with H&E by the Pathology histology facility (R. Stilton). In a blinded fashion, each colon or caecum section was scored according to these criteria: *epithelial hyperplasia and/or goblet cell depletion* (none = 0, mild (1.5x hyperplasia or 25% depletion) = 1, moderate (2-3x or 25-50%) = 2, severe (>3x or >50% = 3), *inflammation in the lamina propria* (none - 0, mild, a few leucocytes in the crypts = 1, moderate, a marked infiltrate and crypt broadening = 2, severe, dense infiltrate throughout = 3), *area affected* (none = 0, up to 25% = 1, 25-50% = 2, >50% = 3) and *markers of severe inflammation* (none = 0, submucosal inflammation or a few crypt abscesses = 1, many crypt abscesses or excessive submucosal inflammation or crypt branching = 2, many crypt abscesses and excessive submucosal inflammation or ulceration or extensive fibrosis = 3). For the colon, scores were added for each region (proximal, mid and distal) and then averaged. For the caecum, scores represent the sum of the four criteria.

2.3.17 Measuring the severity of systemic inflammation

From the same mice subjected to a colitis protocol, whole spleen was taken and weighed. Also, after confirming death, livers of mice were perfused by cutting the portal vein and then flushing 10 mL of PBS through the left ventricle of the heart. Lobes of perfused liver were fixed in 3.6% formaldehyde, embedded in paraffin and sectioned by microtome (Pathology histology core, R. Stilton).

2.3.18 Caecum and colon LP lymphocyte isolation

Caecums were longitudinally and laterally (i.e. if the caecum was imagined to be a shoe, the laces and heel would be cut) and colons were cut longitudinally. Luminal contents were removed by shaking tissues vigorously in tubes containing PBS and 0.1% BSA. Samples were then shaken again in PBS with 0.1% BSA in a fresh tube to remove remaining luminal contents. To remove epithelial cells, tissues were placed in 50 mL Falcon tubes with 10 mL of pre-warmed (37º C) complete RPMI with 5% FCS and 5mM EDTA (Gibco). Tubes were placed in a shaking incubator for 15 minutes at 37º C at 200 rpm. Supernatant was then aspirated and discarded, and the EDTA wash was repeated twice more. To neutralize EDTA activity, 10 mL of complete RPMI with 5% FCS and 15mM Hepes (PAA) was then added to each tube and incubated at 37º C for 10 minutes. Supernatant was discarded and replaced with 37º C complete RPMI with 5% FCS, 40 μg/mL bovine pancreas DNase (Roche) and 0.5 mg/mL type II collagenase (SIGMA) and then placed on the shaking incubator at 37º C and 150 rpm for one hour. Supernatants of digested tissues were passed through a 70 μm cell strainer (BD Falcon) and washed with 4º C RPMI with 5% FCS and 5 mM EDTA. Supernatants were centrifuged at 1350 rpm and resuspended in 3 mL of a 30% Percoll (GE) solution. Underlaying 4 mL of 40% and 3 mL of 75% Percoll layers beneath the 30% layer that contained LP cells created cell separation gradients. Gradients were centrifuged for twenty minutes at 1800 rpm without braking. Epithelial cells were aspirated from the 30/40% interface and discarded. The leukocyte fraction was collected from the 40/75% interface and

washed with PBS with 0.1% FCS to remove excess Percoll. Cells were pelleted and then stained for flow cytometric analysis.

2.3.19 Small intestine LP lymphocyte isolation

A similar protocol was used to isolate small intestine LP lymphocytes as was used for colonic LP cells with the following modifications. Prior to cutting the small intestine longitudinally, Peyer's patches were removed using forceps and a razor blade and visceral fat was removed using scissors. For the EDTA wash, only 1 mM EDTA was used.

2.3.20 Liver leukocyte isolation

After confirming death, the portal vein was cut and livers were perfused through the left ventricle of the heart with 10 mL PBS followed by 10 mL of RPMI with 5% FCS and 0.5 mg/mL type VIII collagenase (SIGMA) and 40 μg/mL bovine pancreas DNase (Roche). Livers were diced and placed in 50 mL Falcon tubes with 10 mL of collagenase solution in a shaking incubator at 150 rpm for 20 minutes. Leukocytes were isolated from supernatants of digested tissues on a Percoll gradient as described above.

2.3.21 MCMV infections

The mouse cytomegalovirus (MCMV) smith strain was isolated from the salivary glands of three-week-old infected BALB/c mice. 1×10^5 PFU of MCMV was injected *intra peritoneally* per mouse.

2.4 *In vitro* assays

2.4.1 mRNA isolation and qPCR

Sorted cells were pelleted and resuspended in lysis buffer (Ambion) and mRNA was purified using an RNaqueous Micro kit (Ambion). Concentration and purity of RNA was analysed on a Nanodrop spectrophotometer. Quantitative PCR measurements of *Il21r* and *Hprt*

abundance was carried out using Taqman primers labelled with FAM/TAMRA or VIC/TAMRA, respectively. Expression of *Il21r* was normalised to *Hprt* abundance. For measuring *Gimap5* mRNA abundance, cDNA was made from total mRNA of B cells, and *Gimap5* and *Actin* were amplified by standard PCR for the indicated number of cycles.

2.4.2 *Carma1$^{tut/tut}$* genotyping

Genomic DNA was obtained by digesting tail or ear biopsies in proteinase K and then isolating DNA by isopropanol precipitation. A section of genomic DNA encompassing the *tut* mutation was amplified using the following primer sequences: (forward) 5'-ATGCTTCTTCATTGGGTGGA -3' and (reverse) 5'- AATTACGGCAGCTCACCATC -3'. Standard PCR conditions were used and a single 444 bp band was amplified with the following conditions: 94º C 10 min, (94º C 30 sec, 55º C 60 sec, 72º 60 sec) x 35, 72º C 10 min, 4º C ∞. To distinguish between wild-type, heterozygous and homozygous mice, a restriction digest was carried out by incubating 10 μL PCR product, 17 μL H_2O, 3 μL NEB buffer 3 and 0.4 μL DdeI restriction enzyme at 37º C overnight. Samples were visualised on a 2 % agarose gel. For wild-type mice, the band was not cut, heterozygous mice had one cut and one uncut band and both bands were cut in homozygous mice.

2.4.3 *Gimap5$^{sph/sph}$* genotyping

Genomic DNA was obtained by digesting tail or ear biopsies in proteinase K and then isolating DNA by isopropanol precipitation. A section of genomic DNA encompassing the *sphinx* mutation was amplified using the following primer sequences: (forward) 5'-AAGCCCACTGTGTACAAGAATCTAGC-3' and (reverse) 5'-GGGTGAAGAGGACGATCATGTG-3'. Standard PCR conditions were used and a single 556 bp band was amplified with the following conditions: 94º C 10 min, (94º C 30 sec, 55º C 60 sec, 72º C 60 sec) x 33, 72º C 10 min, 4º C ∞. To distinguish between wild-type, heterozygous and homozygous mice, a restriction digest was carried out by incubating 10 μL

PCR product, 17 μL H_2O, 3 μL NEB buffer 3 and 0.3 μL AciI restriction enzyme at 37º C overnight. Samples were visualised on a 3% agarose gel. For wild-type mice, both bands were cut, heterozygous mice had one cut and one uncut band and homozygous mice had no cut bands.

2.4.4 Immunoblotting

Immunoblotting was performed using total spleen, thymus or bone marrow lysates, or splenic B cells that were isolated using MACS purification with anti-CD19 microbeads (Miltenyi Biotech) or splenic $CD4^+$ T cells isolated by MACS purification or CD4 and CD8 SP thymocytes that were sorted on a FACS Aria sorter. In some cases, cells were treated with 50 ng/mL PMA (SIGMA) and 500 ng/mL Ionomycin (SIGMA) in complete IMDM media with 5% FCS for the indicated number of minutes. Cells were pelleted and lysed by resuspending in RIPA lysis buffer containing phosphatase and protease inhibitor cocktails (Roche). Lysates were heated with Lamelli sample buffer at a 1:1 ratio at 85º C for 10 minutes before loading and resolving proteins in 4-20% pre-cast SDS-polyacrylamide gradient gels (BioRad). Gels were transferred onto nitrocellulose membranes (Amersham). Membranes were blocked with 5% powdered non-fat milk (Safeway/Vons) for 30 minutes, immunoblotted with primary antibodies diluted according to manufacturer recommendations, and then membranes were incubated with a horseradish peroxidase conjugated secondary antibody (Cell Signaling). Bands were visualised using an enhanced chemiluminescence detection kit (Amersham).

2.4.5 Cross-priming of $CD8^+$ T cells by DCs

To test the capacity of DCs to cross-prime $CD8^+$ T cells, lymphoid DCs were generated by culturing 1×10^7 bone marrow cells with 200 ng/ml human FMS-like tyrosine kinase 3 (Flt3)-ligand (Peprotech) in supplemented IMDM media for eight days. 1×10^5 DCs were then co-cultured with 2×10^5 γ-irradiated (1,500 rad) *Kb*$^{-/-}$*act-mOVA* splenocytes and 1×10^5 MACS

purified CFSE labelled $CD8^+$ OT-I T cells. CFSE dilution was assessed three days later by flow cytometry as an indication of cross-priming.

2.4.6 *In vitro* T cell activation

MACS purified splenic $CD4^+$ T cells were used to test T cell activation. Cells were plated in 48-well flat bottom plates that had been previously coated with antibody overnight at 4º C. To measure up-regulation of activation markers by flow cytometry, 1 x 10^5 cells were activated for 24 hours, and then stained for intra-cellular CD25 and CD69. IL-2 production was measured by culturing 2×10^5 cells/ml under activating conditions in complete IMDM media with 10% FCS. Supernatant was harvested at 18 hours and IL-2 was measured by ELISA (eBioscience). T cell proliferation assays entailed activating CFSE-labelled $CD4^+$ T cells using indicated concentrations of CD3ε and CD28 plate-bound antibodies (eBioscience) with or without 100 U/ml IL-2 (R&D systems). CFSE dilution was measured after four days by flow cytometry.

2.4.7 *In vitro* T_{reg} cell induction

Peripheral T_{reg} cells were generated in 24-well flat bottom plates by culturing 3×10^5 CFSE-labelled $CD4^+$ T cells in plates coated with CD3ε and CD28 antibodies with or without IL-2 (R&D Systems) and/or TGF-β (R&D Systems) in complete IMDM media with 10% FCS. CFSE dilution and intracellular expression of Foxp3 was measured after four days of culture. To examine the effect of other T cell produced cytokines, 2×10^5 CFSE-labelled C57BL/6-$CD45.1^+$ and *tut*-$CD45.2^+$ $CD4^+$ T cells were co-cultured in plates coated with 2 μg/ml CD3 and CD28 antibodies in complete IMDM media with 10% FCS and 5 ng/ml TGF-β, with or without 10 μg/ml neutralizing IL-2 antibody (clone JES6–1) (eBioscience). Additionally, CFSE dilution and Foxp3 induction was assessed in CFSE-labelled $CD4^+$ T cells activated by 2 μg/mL CD3 and 2 μg/mL CD28 plate-bound antibodies in complete IMDM media with 10% FCS and 5 ng/mL TGF-β1 and: 100 nM CpG oligonucleotides (SIGMA), 10 ng/mL IL-4, 10 ng/mL TNF-α, 100 U/mL IFN-α or 100 U/mL IFN-γ (all cytokines, R&D systems).

2.4.8 *In vitro* T_{reg} cell suppressor assays

The T_{reg} cell-mediated suppressor assay was performed under conditions previously described (Davidson et al., 2007; Thornton and Shevach, 1998). Briefly, MACS purified $CD4^+$ T cells were cultured in plates coated with 2 μg/mL anti-mouse CD3ε and 2 μg/mL anti-mouse CD28 antibodies, in 100 U/mL IL-2 (R&D Systems) and 5 ng/mL TGF-β1 (R&D Systems) in complete IMDM media with 10% FCS. After four days, $CD4^+$ T cells were again MACS purified. Foxp3 induction was assessed by flow cytometry, and in all experiments at least 90% of $CD4^+$ T cells expressed Foxp3. Induced T_{reg} cells were co-cultured at indicated ratios with 5×10^4 MACS purified CFSE-labelled $CD8^+$ T cells. Also included were 1×10^5 T cell-depleted, γ-irradiated (3,000 rad) splenocytes as bystander cells and 0.5 μg/mL soluble CD3ε antibody. CFSE dilution was assessed by flow cytometry after three days of co-culture.

2.4.9 B cell proliferation in response to various mitogens

To induce B cell proliferation *in vitro*, whole splenocytes were labelled with 5 μM CFSE and 4×10^5 cells were plated in 24-well flat bottom plates. Cells were cultured in complete IMDM media with 10% FCS and soluble anti-IgM (Jackson ImmunoResearch), anti-CD40 (eBioscience), IL-4 (R&D systems), LPS (Axxora) or PMA and Ionomycin (SIGMA). Four days later, CFSE dilution was assessed by flow cytometry for $B220^+CD19^+$ B cells.

2.4.10 MitoTracker green staining for mitochondrial content

Thymocytes or splenocytes were cultured in complete RPMI with 10% FCS for 30 minutes with 40 nM MitoTracker Green (Molecular Probes, Invitrogen). Cells were washed with PBS 2% FCS, then stained for cell surface markers with monoclonal antibodies (CD4-PerCp-Cy5.5 and TCRβ-APC). Mitochondrial content was measured by the mean fluorescence intensity of the FL1 channel on a FACS Sort flow cytometer.

2.4.11 Activation of innate lymphoid cells by IL-23

Liver leukocytes were isolated and plated in 48-well flat bottom plates with complete RPMI media with 10% FCS and 10 ng/mL recombinant IL-23 (R&D systems) overnight. The next day, brefeldin A was added for 2 hours, before cytokine expression was determined by intracellular cytokine staining.

2.5 Statistical analysis

Data were analyzed using the GraphPad Prism5 software (GraphPad Software, San Diego, CA). Generally a Mann Whitney test was used to determine statistically significant differences between groups. When sample sizes were n = 3 or n = 4, a Student's *t*-test was used instead. Multiple sequence alignments of DNA or protein sequences were created using publicly available web-based software (Ensembl).

Chapter III. Commitment to the Regulatory T Cell Lineage Requires Carma1 in the Thymus but not in the Periphery

3.1 Introduction

Two major mechanisms enforce self-tolerance in T cells: negative selection in the thymus and dominant tolerance in the periphery. The importance of both mechanisms is underscored by the phenotypes of *autoimmune regulator* (*Aire*) knockout (Anderson et al., 2002) and *scurfy* mutant mice (Brunkow et al., 2001), which have defects in negative selection or dominant tolerance, respectively. Humans with orthologous mutations develop autoimmune polyendocrinopathy-candidiasis-ectoderma dystrophy (APECED) (Consortium, 1997; Nagamine et al., 1997) or IPEX syndrome (Bennett et al., 2001), respectively. These mutations all result in systemic autoimmunity, though defects in dominant tolerance cause a more severe and fatal disease. The study of dominant tolerance accelerated after cloning of the *scurfy* locus, which identified Foxp3 as an essential molecule (Khattri et al., 2003). Foxp3 is a transcription factor expressed predominantly in $CD4^+$ T cells committed to the T_{reg} cell lineage (Fontenot et al., 2005b). Expression of Foxp3 programs T cells with suppressor function, allowing T_{reg} cells to effect dominant tolerance (Hori et al., 2003).

The majority of T_{reg} cells are derived from the thymus, although an unknown percentage of these cells may also develop in the peripheral lymphoid organs. Thymic T_{reg} cell lineage commitment occurs in CD4 single-positive (SP) thymocytes and requires intermediate affinity binding of the T cell receptor (TCR) (Hsieh et al., 2004), co-stimulation through CD80 and CD86 interactions with CD28 (Salomon et al., 2000; Tai et al., 2005) and the cytokines Interleukin-2 (IL-2) or IL-15 signalling through the shared IL-2Rβ chain (Burchill et al., 2007; Fontenot et al., 2005a; Soper et al., 2007; Yao et al., 2007). Peripheral commitment of naïve $CD4^+$ T cells to the T_{reg} cell lineage, modelled *in vitro*, requires exogenous TGF-β, in addition to TCR stimulation and concomitant IL-2 production, to induce Foxp3 expression

and T_{reg} cell function (Chen et al., 2003). Foxp3 induction can be enhanced *in vitro* by inhibition of Akt-mediated signalling or transient TCR stimulation (Haxhinasto et al., 2008; Sauer et al., 2008), and may be preferentially driven *in vivo* by retinoic acid made by macrophages and DCs residing in mucosal tissues (Coombes and Powrie, 2008). The difference in signalling pathways used in the development of thymic versus peripherally induced T_{reg} cells is currently a topic of intense investigation.

In this chapter, the characterization and positional cloning of a point mutation in the gene *Carma1* that blocked the development of T_{reg} cells in the thymus is described. The mutation was identified in a screen of G_3-ENU mice, homozygous for germline mutations induced by ENU, designed to detect defects in T cell effector function (see Chapter 2.1.1). Previously, Carma1 was discovered as a Bcl10 interacting protein that could activate NF-κB when overexpressed in cell lines (Gaide et al., 2001). *Carma1* expression is abundant in T, B and NK lymphocytes, mast cells and DCs (www.biogps.org). The Carma1 protein contains several functional domains, including a CARD domain that binds Bcl10, a coiled-coil domain important for oligomerisation, as well as PDZ, SH3 and guanylate kinase domains (Thome et al., 2010). A linker region controls the ability of Carma1 to assemble a signalling complex (the 'CBM-signalosome'). This can occur in T and B cells upon phosphorylation of the linker region by Pkc-θ and HPK1 (Brenner et al., 2009; Matsumoto et al., 2005; Sommer et al., 2005). Subsequently, Carma1 acts as a scaffolding protein that recruits Bcl10, Malt1, Tak1 and the Ikk complex, which drive the activation of the Jun and NF-κB transcription factors.

Previously, three separate strains of *Carma1*$^{-/-}$ mice (Egawa et al., 2003; Hara et al., 2003; Newton and Dixit, 2003) and one hypomorphic *Carma1* mutant mouse (Jun et al., 2003) had been reported. Less mature T and B cells and greatly diminished serum Igs are found in *Carma1*$^{-/-}$ mice. TCR- and BCR-induced cytokine production, activation of NF-κB and proliferation is impaired in T and B cells, respectively. However, the addition of exogenous

γ-chain cytokines can rescue the proliferative defects *in vitro*. NK cells require Carma1 for cytokine production, but not cytotoxicity, in response to activation by the NK1.1, Ly49D, Ly49H, or NKG2D receptors (Hara et al., 2008; Gross et al., 2008). Again, addition of exogenous cytokines, IL-12 or IL-18, can restore normal cytokine production by NK cells *in vitro*. The effect of Carma1-deficiency in mast cells and DCs remains poorly characterized. Interestingly, the hypomorphic *unmodulated* point mutation caused spontaneous atopic disease and markedly increased serum IgE concentrations in *Carma1*$^{unm/unm}$ mice (Jun et al, 2003). This finding raised the possibility that a tolerance mechanism required intact Carma1-signaling to operate and that, in the absence of a complete Carma1-signal, enough of a signal remained to sustain a pathogenic, atopic response. Spontaneous immunopathology was not observed in *Carma1*$^{-/-}$ mice, suggesting that some cell types with pathogenic potential, perhaps mast cells, retain functionality despite the *unmodulated* mutation.

In the homozygous mutant mice described here, no thymic T_{reg} cells were detected, but Foxp3 expression could be induced among peripheral $CD4^+$ T cells in response to the cytokines IL-2 and TGF-β. Prior to this study, no link between Carma1 and T_{reg} cell development had been reported. It was known that *CD4*^{cre}*Ikkβ*$^{flox/flox}$ and *Pkcθ*$^{-/-}$ mice had fewer peirpheral $CD4^+CD25^+$ T cells (Schmidt –Supprian et al., 2003; Schmidt –Supprian et al., 2004), however it remained unclear whether this simply reflected a role for IKKβ and Pkc-θ in promoting CD25 expression, or was indicative of a critical cell-intrinsic or –extrinsic role for these proteins $CD4^+Foxp3^+$ T_{reg} cell development (Liston and Rudensky, 2007). Thus, this mutant mouse allowed the role of the Carma1-dependent signalling pathway to be characterised in thymic and peripheral T_{reg} cell development *in vivo*.

3.2 Results

3.2.1 Identification of the *tut* mutation

To identify genes with non-redundant roles in T cell development, priming or effector function, a screen was designed to detect defective cytotoxic $CD8^+$ T cell (CTL) responses in mice immunized with γ-irradiated cells expressing ovalbumin (*act-mOVA*) (Ehst et al., 2003; Janssen et al., 2006). Among 2,500 ENU-mutagenised G_3 mice screened, two non-responsive mutations were bred to homozygosity. One of these mutants was termed *tut* (for T_{reg} cells undetectable in the thymus). While the primary screen used was an *in vivo* cytotoxicity assay (Barber et al., 2003), the mutation could also be scored using an *in vitro* assay. To do so, T cells were isolated seven days after immunization, at the peak of the $CD8^+$ T cell response, and antigen-specific $CD8^+$ T cells were expanded in culture with SIINFEKL peptide. $CD8^+$ T cells from *tut* mice did not undergo secondary expansion or produce interferon-γ (IFN-γ) after restimulation with peptide (Figure 3.1A). It was hypothesized that a mutation affecting DC cross-priming of $CD8^+$ T cells, T cell activation or T cell proliferation could cause such a phenotype. To test DC function, FMS-like tyrosine kinase 3 (Flt3) ligand was used to generate bone marrow-derived lymphoid DCs, a type of DC that efficiently cross-primes $CD8^+$ T cells (Janssen et al., 2006). When Flt3 ligand-derived DCs were exposed to ovalbumin-expressing apoptotic cells, *tut* DCs cross-primed ovalbumin-specific OT-I T cells as efficiently as wild-type DCs (Figure 3.1B). In addition, *tut* DCs showed normal up-regulation of co-stimulatory molecules CD40, CD80, CD86 and major histocompatibility complex (MHC) class I and II after activation by Toll-like receptor ligands (Hoebe et al., 2003) or apoptotic cells (Janssen et al., 2006) (data not shown), suggesting that the mutation did not affect co-stimulation. These results indicated that the *tut* mutation did not impair DC-mediated cross-priming of $CD8^+$ T cells.

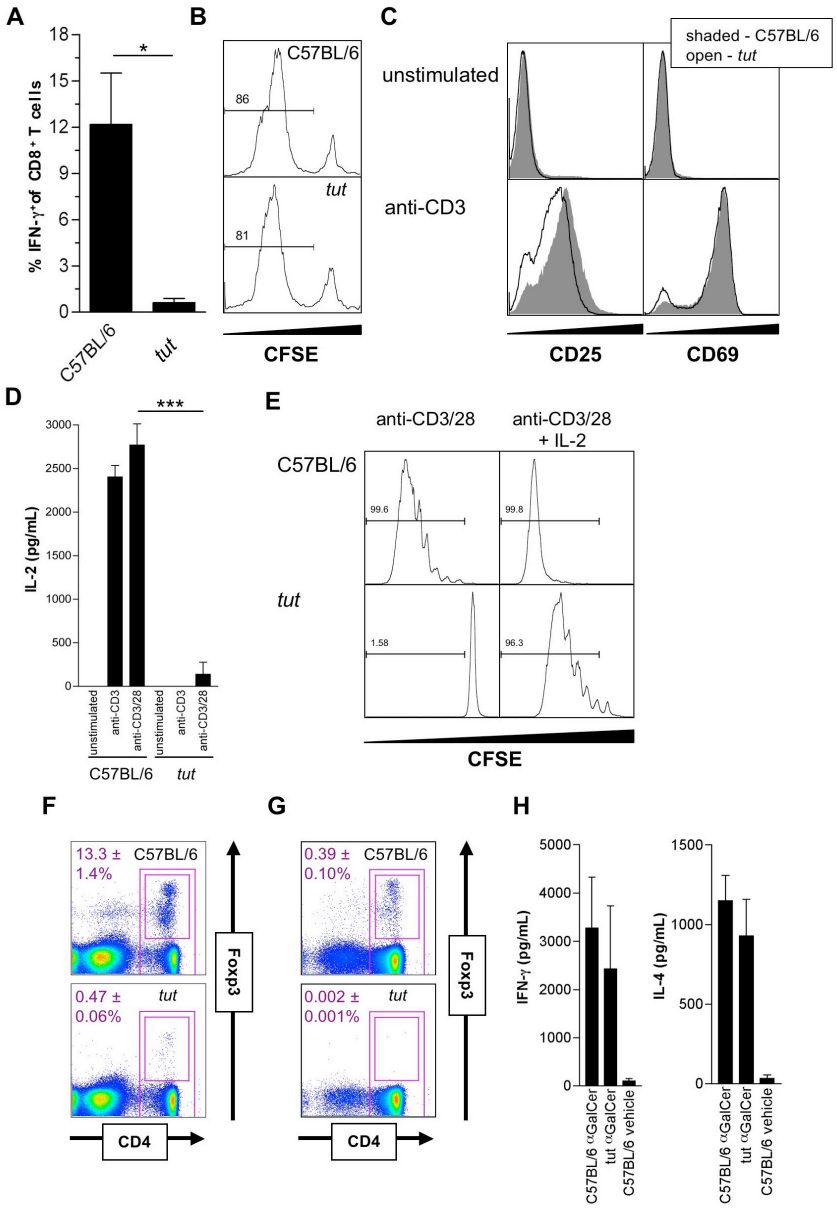

Figure 3.1. The *tut* mutation impairs T cell activation and T_{reg} cell development.
(A) To test the ability of mice to generate CTL responses, mice were immunized with 1 x 10^8 γ-irradiated *act-mOVA* splenocytes (1,500 rad) as an immunogen. Seven days later, splenocytes from immunized mice were cultured with the immunodominant OVA MHC class I epitope, SIINFEKL peptide, for three days to expand OVA-reactive cells. IFN-γ production among peptide-restimulated $CD8^+$ T cells was then assessed by intracellular cytokine staining. Error bars represent standard deviation (n = 5; * = $p < 0.05$).
(B) The cross-priming capacity of Flt3L-matured bone marrow-derived dendritic cells (BMDCs) was examined by culturing 2 x 10^4 BMDCs with 2 x 10^4 γ-irradiated (1,500 rad) *Kb*$^{-/-}$*act-mOVA* splenocytes (that were unable to prime $CD8^+$ T cells directly) and 1 x 10^5 CFSE labelled OT-I cells. After three days of culture, cross-priming was assessed by CFSE dilution.
(C) T cell activation was examined by assessing expression of the activation markers CD25 and CD69 (intracellular) on purified $CD4^+$ T cells cultured with plate-bound anti-CD3ε antibodies (1 μg/mL) for 24 hours.
(D) IL-2 secretion by $CD4^+$ T cells was measured in supernatants 18 hours after activation by plate-bound anti-CD3ε (1 μg/mL) + CD28 (1 μg/mL) antibodies. Error bars represent standard deviation (n = 3; *** = $p < 0.001$).
(E) $CD4^+$ T cell proliferative capacity was assessed by culturing CFSE-labelled cells for 96 hours with plate-bound anti-CD3ε (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies, with or without exogenous IL-2 (100 U/mL).
(F-G) Foxp3 expression in $CD4^+$ (F) splenocytes and (G) thymocytes was measured by intracellular cytokine staining. The percentage of $CD4^+$ cells that express Foxp3 is indicated, along with standard deviation.
(H) Cytokine production by *i*NKT cells was induced by injecting mice with 2 μg αGalCer. Serum was collected 90 minutes later and cytokine concentrations of IFN-γ and IL-4 were measured by ELISA. Error bars represent standard deviation (n = 5).
All experiments were repeated at least three times and representative data are shown.

T cell activation was investigated next. $CD4^+$ T cells and $CD8^+$ T cells from *tut* mice normally up-regulated CD69, but not CD25 (IL-2Rα) after TCR ligation *in vitro* (Figure 3.1C). As IL-2 can positively regulate CD25 expression, IL-2 production by TCR-stimulated *tut* $CD4^+$ T cells was measured and found to be undetectable (Figure 3.1D). These data implied a partial defect in T cell activation. T cell proliferative capacity was assessed next. T cells from *tut* mice failed to proliferate in response to TCR stimulation, although proliferation could be partially rescued by exogenous IL-2 (Figure 3.1E). As IL-2 is required to maintain $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the periphery (Fontenot et al., 2005a), the development of these cells was assessed in *tut* mice. $Foxp3^+$ T_{reg} cells were reduced by an order of magnitude in the periphery (Figure 3.1F), but were absent in the thymus (Figure 3.1G), indicating that the *tut* mutation blocked commitment of developing thymocytes to the T_{reg} cell lineage. The function of another population of self-reactive T cells that develop in the thymus, invariant natural killer T (*i*NKT) cells, was also investigated. 90 minutes after injection of the *i*NKT cell-specific agonist alpha-galactosylceramide (αGalCer), elevated concentrations of IL-4 and IFN-γ were measured in the serum of *tut* mice indicating that the mutation did not impair pan-T cell function (Figure 3.1H).

Unlike most other mutations that impair T_{reg} cell development, *tut* mice did not exhibit gross signs of autoimmunity. Even in a cohort of *tut* mice monitored for over nine months, no detectable anti-chromatin auto-antibodies (Figure 3.2) were found in the serum, nor did the mice develop splenomegaly, lymphoproliferative disease or signs of chronic inflammation—all aspects of autoimmunity normally controlled by T_{reg} cells.

3.2.2 *tut*, a mutation in *Carma1*

To find the causative mutation, the *tut* phenotype was mapped by outcrossing the *tut* stock (C57BL/6 background) to C3H/HeN mice, backcrossing F_1 offspring to the *tut* stock and measuring the percentage of circulating $CD4^+$ T cells expressing Foxp3 in the blood of F_2 mice (Figure 3.3A). By analyzing 134 microsatellite markers that differed between C57BL/6

and C3H/HeN mice and were dispersed throughout the genome on 39 meioses, the *tut* mutation was localized to the distal region of Chromosome 5 with a peak logarithm of odds (Lod) score of 11.74 (Figure 3.3B). Further analysis of 268 meioses confined the mutation to a 1.03-megabase critical region, bounded by the markers D5Mit292 and D5Mit101. This region contained only six annotated genes (http://www.informatics.jax.org), and among these was *caspase recruitment domain 11* [*Card11*, more commonly known as *Carma1* (from CARD-MAGUK1)]. Either the genomic DNA or cDNA of all coding base pairs within the critical region was sequenced and a single point mutation in *Carma1* (Figure 3.3C) was identified, which resulted in an L525Q substitution. The mutation occurred in α-helix2 of the NORS (no regular secondary structure) domain in the Carma1 linker region (Figure 3.3D). In naïve T cells, the Carma1 protein adopts a conformation in which the linker domain associates with the CARD domain. Upon T cell activation, protein kinase C theta (Pkcθ) phosphorylation of residues in the linker domain reduces intermolecular affinity for the CARD domain. This liberates both the CARD and coiled-coil domains, allowing Carma1 oligomerisation and recruitment of B cell CLL/lymphoma 10 (Bcl10) and mucosa associated lymphoid tissue lymphoma translocation gene 1 (Malt1) to the Carma1 signalling module (Matsumoto et al., 2005; Sommer et al., 2005). Following activation, degradation of both Bcl10 and Carma1 terminates Carma1-dependent signalling (Moreno-Garcia et al., 2010; Scharschmidt et al., 2004; Zeng et al., 2007). Carma1 has a similar function in B cells, downstream of Pkcβ. No Carma1 expression was detected by immunoblotting in the thymus, spleen or lymph nodes of *tut* homozygotes ($Carma1^{tut/tut}$) (Figure 3.3E). Furthermore, Carma1 was not detectable in CD4 SP or CD8 SP thymocyte lysates (Figure 3.3F). The L525Q mutation may have the effect of destabilizing the $Carma1^{tut}$ protein or marking it for degradation in mature T cells and B cells.

Several other groups have generated targeted knockouts or hypomorphs of *Carma1* (Egawa et al., 2003; Hara et al., 2003; Jun et al., 2003; Newton and Dixit, 2003). Like these other mutant mice, $Carma1^{tut/tut}$ mice have reduced basal serum immunoglobulin levels (Figure

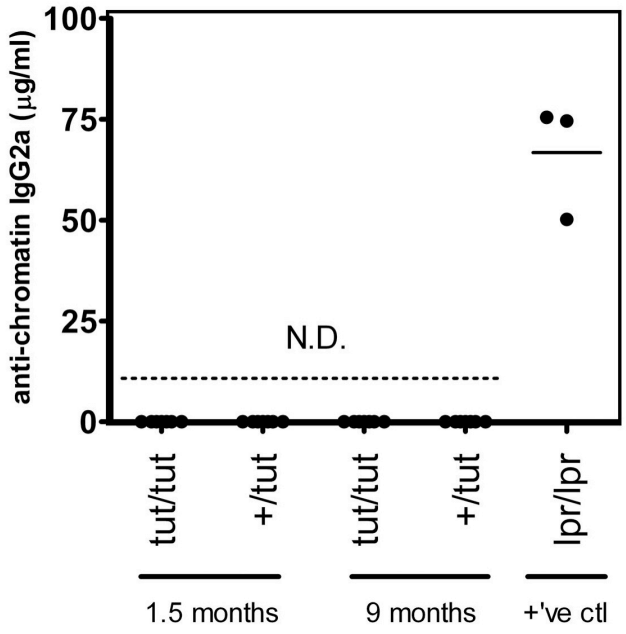

Figure 3.2. Auto-antibodies are absent in aged *tut* mice.
Serum concentrations of anti-chromatin IgG2a auto-antibodies were measured in cohorts of 6-week-old and 9-month-old *tut* homozygote and heterozygote mice (n = 5).

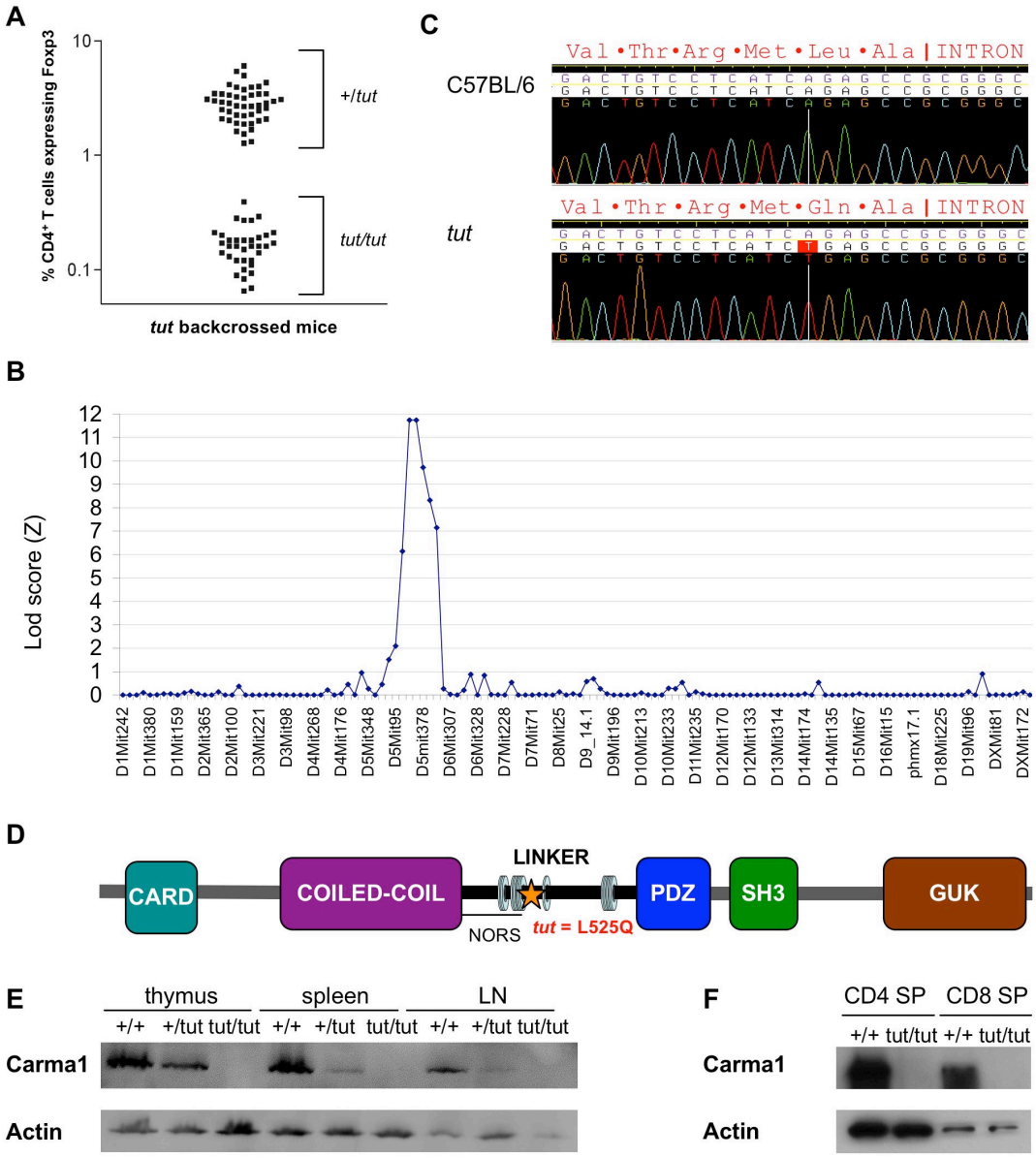

Figure 3.3. Positional cloning of the *tut* mutation.
(A) The percentage of circulating $CD4^+$ T cells expressing Foxp3 was measured in the blood of *tut* mice (C57BL/6 background) that were backcrossed with wild-type C3H/HeN mice in order to distinguish *tut* homozygotes from heterozygotes in F_2 mice.
(B) Using 134 informative microsatellite markers dispersed across the genome, backcrossed *tut* mice were genotyped and the T_{reg} cell deficiency was linked to a critical region on Chromosome 5.
(C) The *tut* critical region on Chromosome 5 contained six genes. Sequencing all predicted coding regions uncovered one nucleotide that differed between the C57BL/6 and *tut* genomes, which caused a predicted leucine to glutamine amino acid substitution in Carma1.
(D) Carma1 is a large protein with several distinct domains. The *tut* mutation occurred within the linker region (★, *tut* mutation; α-helices, indicated by rings; NORS, no regular secondary structure; SH3, Src homology 3; GUK, guanylate kinase).
(E-F) Immunoblotting was used to measure Carma1 expression in (E) total thymus, spleen and lymph node lysates and (F) in $CD4^+CD8^-CD3\varepsilon^+$ (CD4 SP) and $CD4^-CD8^+CD3\varepsilon^+$ (CD8 SP) sorted thymocytes. Representative blots from five independent experiments are shown.

3.4A), fail to mount antigen-specific immunoglobulin M (IgM) and IgG responses after immunization with ovalbumin in CFA (Figure 3.4B) and exhibit impaired B cell proliferation in response to anti-IgM, but not LPS or anti-CD40 with exogenous IL-4 stimulation (Figure 3.4C). Lymphocyte development was abnormal in *Carma1*$^{tut/tut}$ mice, as in other *Carma1* mutants. In the peritoneum, this included a deficiency in peritoneal B1 cells (Figure 3.5A). In the thymus, the cellularity and percentage of CD4 SP, CD8 SP and DP thymocyte populations were normal, but DN thymocyte populations were skewed with an increased percentage of TCRβ^+ cells and fewer NK1.1$^+$ and TCR$\gamma\delta^+$ cells (Figure 3.5B–F). Bone marrow cellularity and pro B cell development appeared to be grossly normal (Figure 3.5G). In the periphery, splenic natural killer (NK) cells, *i*NKT cells, γδ T cells, memory CD4$^+$ T cells and mature B cells were reduced in both percentage (Figure 3.5H–L) and cell number (data not shown). In addition, some *Carma1*$^{tut/tut}$ mice developed dermatitis that resolved after topical antibiotic treatment, as reported in homozygotes for the *unmodulated* allele of *Carma1* (Jun et al., 2003). Collectively, these data suggest that the L525Q tut mutation abolishes Carma1 activity and uncover an essential requirement for Carma1 in thymic T_{reg} cell development.

3.2.3 A cell-intrinsic role for Carma1 in T_{reg} cell lineage commitment

Prior to expression of Foxp3, T_{reg} cell precursors also express CD25, glucocorticoid-induced tumour necrosis factor receptor (Gitr) and cytotoxic T-lymphocyte antigen-4 (Ctla-4) (Gavin et al., 2007; Lin et al., 2007). To determine whether *Carma1*$^{tut/tut}$ thymocytes begin differentiation into the T_{reg} cell lineage but fail to express Foxp3, a distal marker of T_{reg} cell differentiation, expression of these additional markers was examined (Figure 3.6A). The lack of expression of these markers suggests that Carma1 acts early in T_{reg} cell lineage commitment. The absence of thymic T_{reg} cells in *Carma1*$^{tut/tut}$ mice could result from altered selection by thymic epithelial cells, a defect in the TCR and co-stimulatory signalling pathways or a lack of signalling through the IL-2Rβ chain

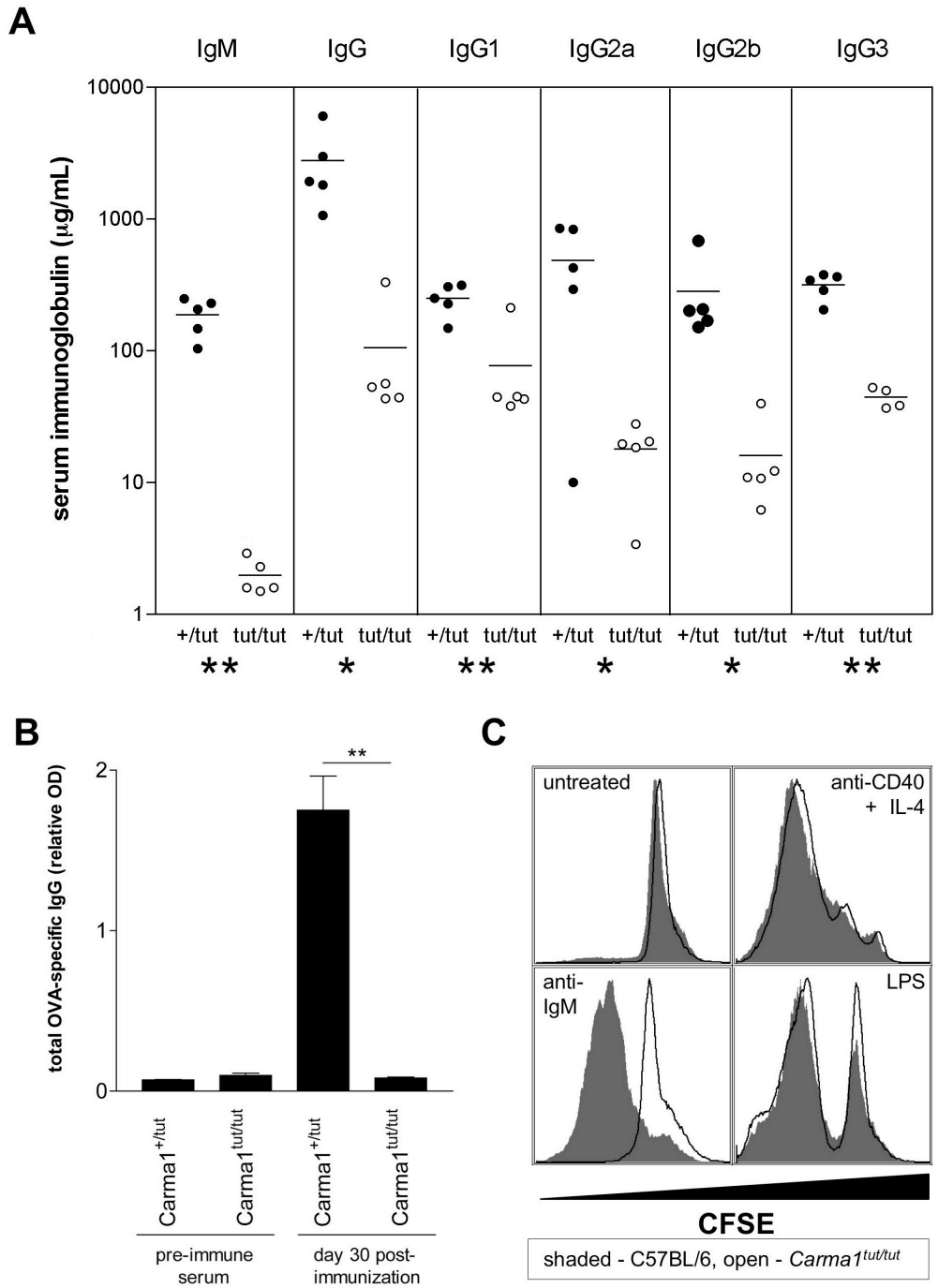

Figure 3.4. B cell function is impaired in *Carma1*$^{tut/tut}$ mice.
(A) Basal serum immunoglobulin concentrations were measured in heterozygote (+/tut) and *Carma1*$^{tut/tut}$ (tut/tut) littermates. A horizontal bar indicates the mean of each column a Mann-Whitney test was used to determine statistically significant differences between groups (* = $p < 0.05$; ** = $p < 0.01$).
(B) To assess T-dependent B cell responses, mice were immunized with OVA and CFA. 30 days later, OVA-specific IgG was measured in the serum by ELISA (n = 5; ** = $p < 0.01$).
(C) B cell proliferative capacity was examined by culturing CFSE-labelled splenocytes with the mitogens anti-IgM (10 μg/ml), anti-CD40 (10 μg/ml) + IL-4 (10 ng/ml) or LPS (2 μg/ml) for 72 hours, then measuring CFSE dilution in CD19^{+} cells. Representative histograms from three independent experiments are shown.

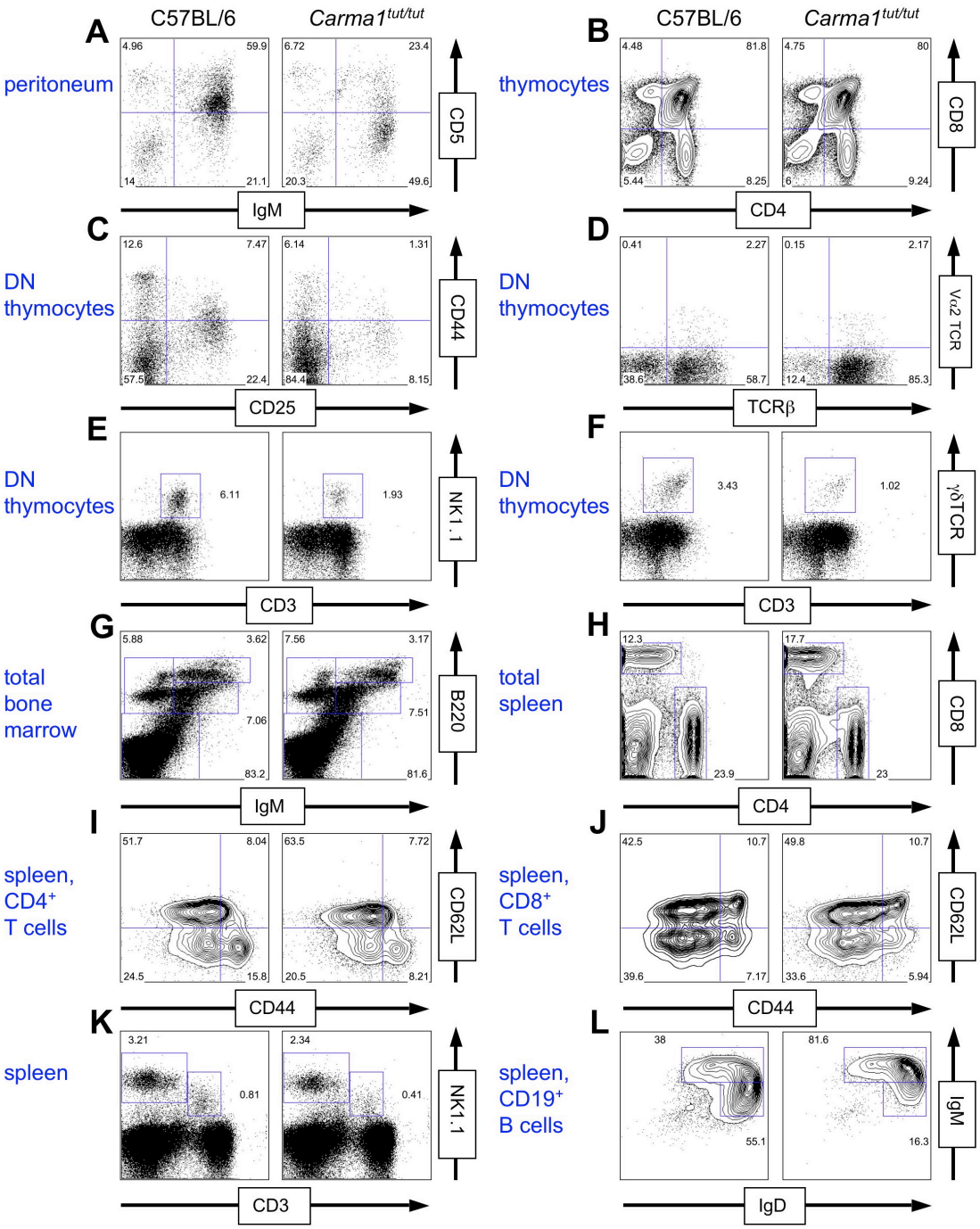

Figure 3.5. Abnormal lymphocyte development occurs in *Carma1*$^{tut/tut}$ mice. Cells from the (A) peritoneal cavity, (B-F) thymus, (G) bone marrow and (H-L) spleen of eight-week-old mice were stained with cell-surface markers for various lymphocyte populations. Representative plots show all cells in the live lymphocyte gate, except where only (C-F) CD4$^-$CD8$^-$ DN thymocytes, (I) CD4$^+$ splenocytes, (J) CD8$^+$ splenocytes or (L) CD19$^+$ splenocytes are shown. Because these results mirrored published data for *Carma1*$^{-/-}$ mice, larger cohorts of mice were not analysed multiple times.

(Burchill et al., 2007; Liston and Rudensky, 2007; Soper et al., 2007; Yao et al., 2007). To understand the role of Carma1 in thymic T_{reg} cell development, reciprocal and mixed bone-marrow chimaeric mice were generated. Foxp3 expression was absent in *Carma1*$^{tut/tut}$ thymocytes that developed in a wild-type thymus (Figure 3.6B), but normal in wild-type thymocytes that developed in a *Carma1*$^{tut/tut}$ thymus (Figure 3.6C). Therefore, the T_{reg} cell deficiency in *Carma1*$^{tut/tut}$ mice results from an intrinsic defect in haematopoietically-derived precursors and not from an altered thymic stromal cell environment. When wild-type mice were reconstituted with *Carma1*$^{tut/tut}$ and wild-type bone marrow at a 4:1 ratio, 1:1 chimaerism was achieved among lymphocytes. While wild-type thymocytes differentiated into the T_{reg} cell lineage at normal frequencies, *Carma1*$^{tut/tut}$ thymocytes failed to develop into thymic T_{reg} cells and expressed lower levels of CD25, Gitr and Ctla-4 among CD4 SP thymocytes (Figure 3.6D). As trans-acting common γ-chain cytokines produced by wild-type thymocytes did not rescue Foxp3 induction in *Carma1*$^{tut/tut}$ thymocytes, it is likely that impaired signalling downstream of the TCR or CD28 underlies the absence of the thymic T_{reg} cells.

Pathways distinct from those involved in thymic development regulate the development and homeostasis of peripheral T_{reg} cells (Fontenot et al., 2005a; Marie et al., 2006). In wild-type mice reconstituted with *Carma1*$^{tut/tut}$ bone marrow, peripheral T_{reg} cells were found at reduced frequencies, similar to those observed in *Carma1*$^{tut/tut}$ mice (Figure 3.6E). Conversely, in *Carma1*$^{tut/tut}$ mice reconstituted with wild-type bone marrow, peripheral Foxp3^{+} T_{reg} cells occurred at frequencies similar to those observed in wild-type mice (Figure 3.6F). Therefore, the *Carma1*$^{tut/tut}$ environment can support T_{reg} cell homeostasis and a cell-intrinsic defect in development causes the T_{reg} cell deficiency observed in these mice. It has been proposed that IL-2 regulates T_{reg} cell homeostasis and that T_{reg} cells may function as an IL-2 "sink" (Fontenot et al., 2005a; Thornton and Shevach, 1998). However, in wild-type mice reconstituted with mixed wild-type and *Carma1*$^{tut/tut}$ bone marrow, the *Carma1*$^{tut/tut}$ T_{reg} cell population did not expand in the periphery (Figure 3.6G). Interestingly, the wild-type T_{reg}

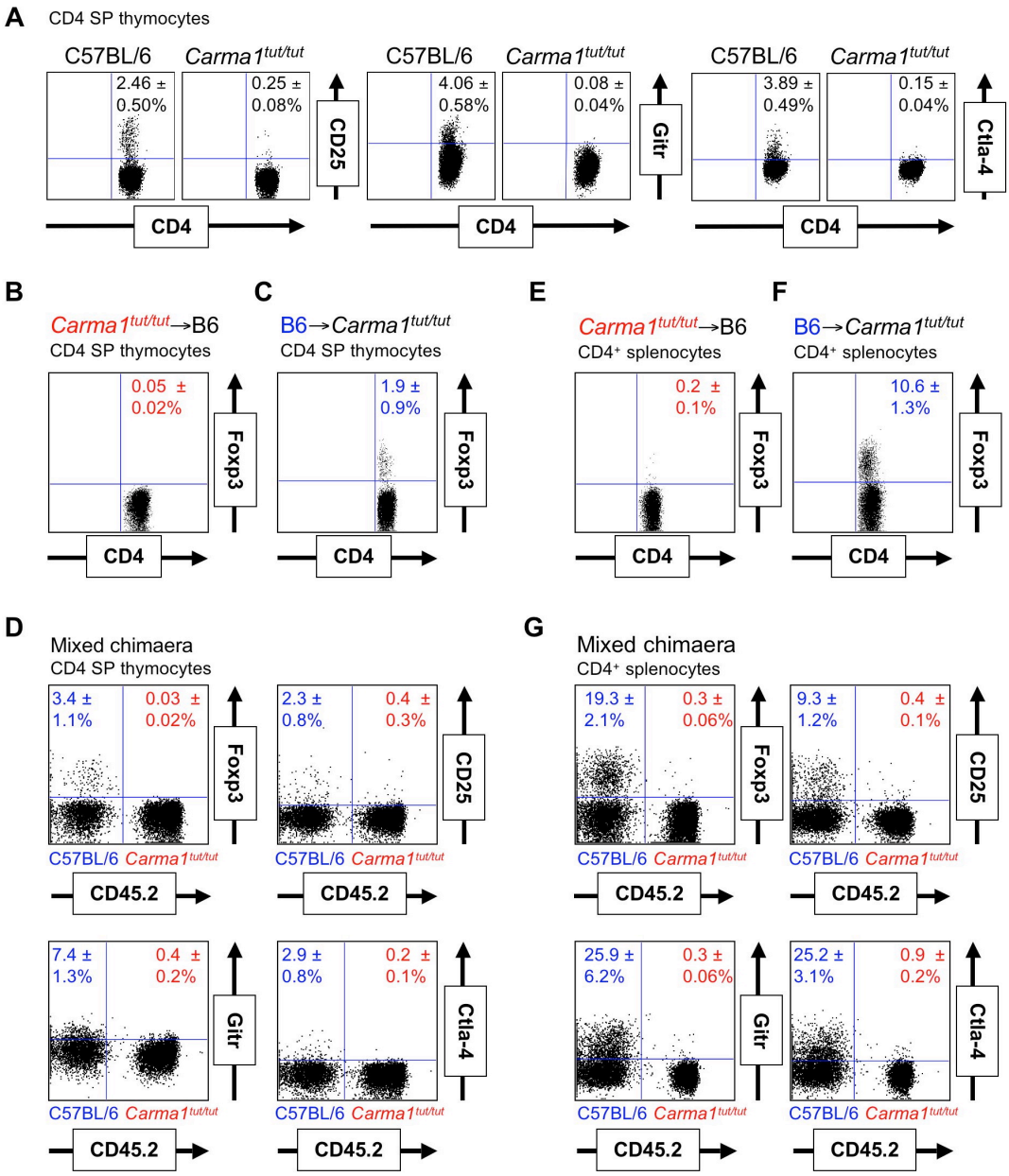

Figure 3.6. T_{reg} cell development requires cell-intrinsic expression of Carma1.
(A) To assess the early stages of T_{reg} cell development, cell-surface expression of CD25, Gitr and Ctla-4 was measured in CD4 SP thymocytes from eight-week-old mice.
(B-C) Reciprocal bone marrow chimaeric mice were generated to determine whether the T_{reg} cell deficit had a haematopoietic cell origin. The percentage of CD4 SP thymocytes expressing Foxp3 was assessed in (B) irradiated congenically marked C57BL/6.SJL mice ($CD45.2^-$) reconstituted with *Carma1*$^{tut/tut}$ bone marrow cells ($CD45.2^+$), and in (C) *Carma1*$^{tut/tut}$ mice reconstituted with C57BL/6.SJL bone marrow cells.
(D) Mixed bone marrow chimaeric mice were generated by reconstituting irradiated congenic C57BL/6.SJL ($CD45.2^-$) mice with *Carma1*$^{tut/tut}$ ($CD45.2^+$) and C57BL/6.SJL ($CD45.2^-$) bone marrow cells at a 1:4 ratio (resulting in 1:1 chimaerism among thymocytes and splenocytes). The percentage of CD4 SP thymocytes of C57BL/6.SJL or *Carma1*$^{tut/tut}$ origin expressing each T_{reg} cell marker is shown in blue or red, respectively.
(E-G) Using the same mice as in (B-D), the percentage of splenic $CD4^+$ T cells expressing Foxp3 in reciprocal (E, F) and mixed (G) bone marrow chimaeric mice was determined.
Five mice were analysed in each experiment and standard deviation is shown (n = 5).

cell population expanded to comprise ~ 20 % of the wild-type $CD4^+$ T cells, and ~ 10 % of the total $CD4^+$ T cell pool. These data are consistent with the idea that a cell-extrinsic homeostatic mechanism regulates the size of the T_{reg} cell compartment in the periphery of naïve wild-type mice (Li et al., 2006).

Our data suggested that the *tut* mutation blocks thymic commitment to the T_{reg} cell lineage by disrupting signaling downstream of the TCR or CD28. Other groups have reported that *Carma1*-deficient T cells exhibit a lack of NF-κB nuclear translocation (Bertin et al., 2001; Gaide et al., 2002; Wang et al., 2002) and Jnk2 phosphorylation (Blonska et al., 2007) after TCR stimulation. Therefore, it remained unclear whether the T_{reg} cell deficiency in *Carma1*$^{tut/tut}$ mice was caused by a failure to activate NF-κB or Jnk2, or both. To address this issue, thymic and peripheral T_{reg} cells in *Pkcθ*$^{-/-}$ and *Jnk2*$^{-/-}$ mice were analyzed (Figure 3.7A). Pkcθ acts upstream of Carma1 and *Pkcθ*$^{-/-}$ mice fail to activate NF-κB, but normally phosphorylate Jnk after TCR stimulation (Sun et al., 2000). It has also been reported that *Pkcθ*$^{-/-}$ mice have a reduced $CD4^+CD25^+$ T cell population in the thymus and periphery (Schmidt-Supprian et al., 2004). The numbers of thymic $CD4^+Foxp3^+$ T_{reg} cells were reduced by an order of magnitude, but were not absent (as in *Carma1*$^{tut/tut}$ mice) in *Pkcθ*$^{-/-}$ mice, whereas peripheral T_{reg} cells showed only a modest reduction in frequency. Frequencies of both thymic and peripheral T_{reg} cells were unaffected in *Jnk2*$^{-/-}$ mice. These results suggest that *Carma1* deficiency likely blocks thymic T_{reg} cell development due to a failure to activate of NF-κB rather than Jnk2. It also appears that Carma1 has Pkcθ-independent roles in T_{reg} cell development.

3.2.4 TGF-β and IL-2 can drive Carma1-independent Foxp3 induction

While *Carma1*$^{tut/tut}$ mice lack thymic T_{reg} cells, they do have peripheral T_{reg} cells, although this cellular compartment is substantially smaller than in wild-type mice. Peripheral expansion and conversion of $CD4^+Foxp3^+$ T cells can be modelled *in vitro* by culturing

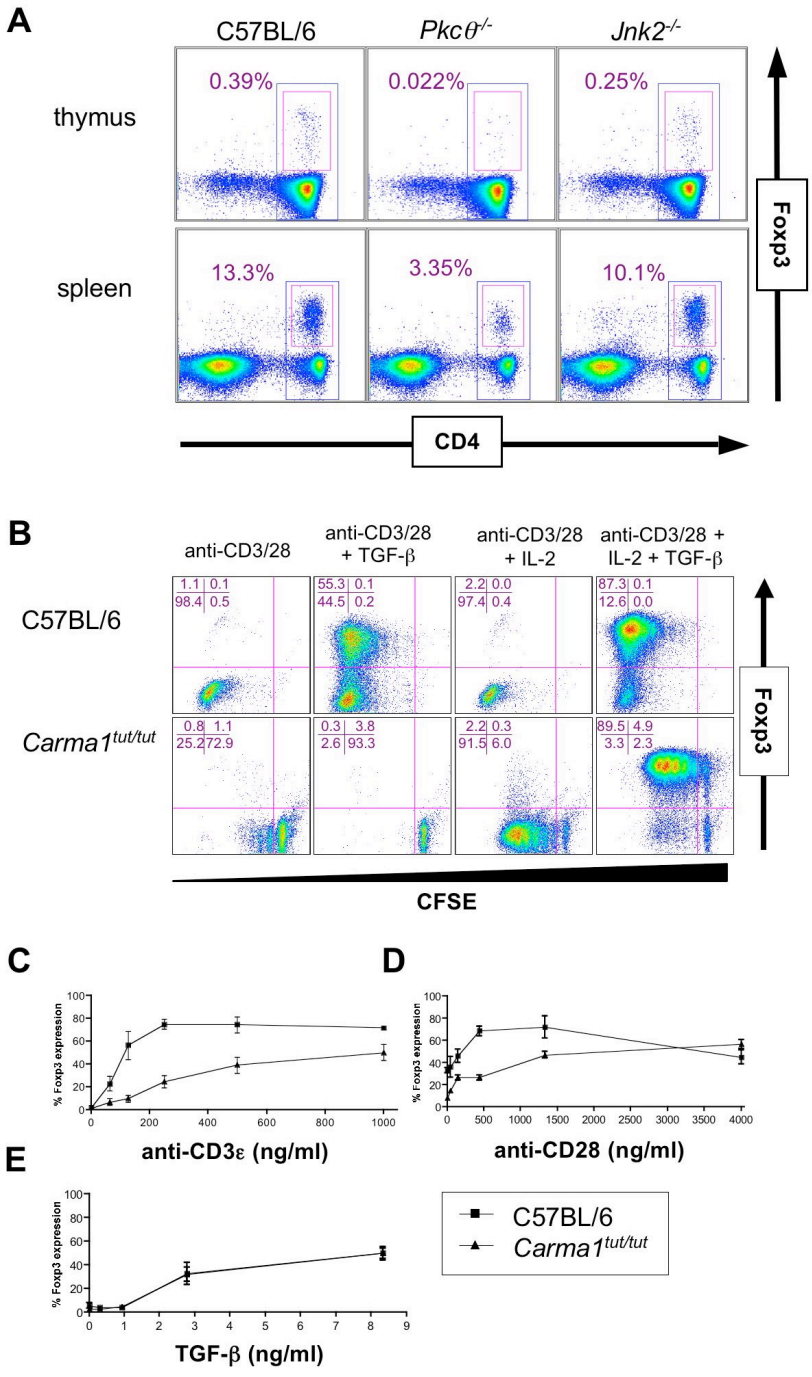

Figure 3.7. Cytokine driven induction of Foxp3 expression does not require Carma1.
(A) Foxp3 expression in $CD4^+$ thymocytes and splenocytes was measured by intracellular cytokine staining. Representative plots show the percentage of $CD4^+$ cells expressing Foxp3.
(B) To induce proliferation and Foxp3 expression in CFSE-labelled $CD4^+$ T cells, cells were cultured with plate-bound anti-CD3ε (2 μg/ml) and anti-CD28 (2 μg/ml) antibody for 96 hours in the presence of IL-2 (100 U/ml) and/or TGF-β (5 ng/ml).
(C-E) To identify Foxp3 induction pathways affected by Carma1 deficiency, dose response analyses were performed. $CD4^+$ T cells were cultured with IL-2 (100 U/mL) and: (C) indicated concentrations of anti-CD3ε antibody with anti-CD28 (1 μg/ml) and TGF-β (5 ng/mL), (D) indicated concentrations of anti-CD28 antibody with anti-CD3ε (1 μg/ml) and TGF-β (5 ng/ml) or (E) indicated concentrations of TGF-β with anti-CD3ε (1 μg/ml) and anti-CD28 (1 μg/ml). Error bars represent standard deviation. Lines representing C57BL/6 and *Carma1*$^{tut/tut}$ cells overlap in panel (E).

activated $CD4^+$ T cells in the presence of the cytokine TGF-β (Chen et al., 2003). When *Carma1*$^{tut/tut}$ $CD4^+$ T cells were activated with plate-bound anti-CD3ε and anti-CD28 in the presence of exogenous TGF-β, they did not proliferate or express Foxp3. However, the combination of exogenous IL-2 and TGF-β was sufficient to rescue both proliferation and Foxp3 induction (Figure 3.7B). Importantly, the percentage and number of *Carma1*$^{tut/tut}$ $CD4^+Foxp3^+$ T cells that did not divide increased, indicating that Foxp3 expression was induced from $CD4^+Foxp3^-$ T cells rather than just expanding pre-existing $Foxp3^+$ T_{reg} cells.

To determine whether *Carma1*$^{tut/tut}$ $CD4^+$ T cells were more or less prone than wild-type cells to express Foxp3, a dose-response analysis of Foxp3 induction was preformed by titrating anti-CD3ε, anti-CD28 and TGF-β concentrations. In the absence of Carma1, the TCR-signalling threshold for Foxp3 induction was increased (Figure 3.7C). Co-stimulatory signals were also required for efficient Foxp3 induction in wild-type and *Carma1*$^{tut/tut}$ $CD4^+$ T cells (Figure 3.7D). Carma1-deficiency did not alter the ability of $CD4^+$ T cells to respond to limiting concentrations of TGF-β (Figure 3.7E). These data suggest that Foxp3 induction can occur without Carma1, and reveal a partial, but dispensable role for Carma1 in transmitting TCR-mediated signals for peripheral Foxp3 induction.

The absence of detectable thymic T_{reg} cells and presence of a small peripheral T_{reg} cell population in *Carma1*$^{tut/tut}$ mice suggests that induction of T_{reg} cells can occur in the periphery of naïve mice without activation of the Carma1-dependent signalling pathway. Also consistent with this conclusion, *Carma1*$^{tut/tut}$ mice had increased frequencies of $CD4^+Foxp3^+$ T_{reg} cells in the colon LP and mLN compared to other secondary lymphoid organs (Table 3.1). The LP of the intestine is a site where peripheral conversion of T_{reg} cells may preferentially occur, and the mLN is one site where LP lymphocytes can drain (Coombes and Powrie, 2008; Maynard et al., 2007). Additionally, peripheral T_{reg} cells in *Carma1*$^{tut/tut}$ mice showed normal expression of the T_{reg} cell surface markers CD25, Ctla-4 and Gitr (data not shown). Together,

Table 3.1. Distribution of T_{reg} cells in *Carma1*$^{tut/tut}$ mice[1]

organ	C57BL/6	*Carma1*$^{tut/tut}$
thymus	0.39 ± 0.10 %	0.002 ± 0.001 %
spleen	13.3 ± 1.4 %	0.47 ± 0.06 %
mLN[2]	12.6 ± 0.8 %	0.96 ± 0.06 %
smLN[3]	14.1 ± 1.5 %	0.61 ± 0.16 %
siLN[4]	12.4 ± 1.7 %	0.32 ± 0.07 %
colon LP[5, 6]	22.5 %	8.4 %

[1]The percentage of $CD4^+$ T cells expressing Foxp3 is indicated along with standard deviation. Eight-week-old C57BL/6 and *Carma1*$^{tut/tut}$ mice were analysed (n = 5).

[2]mLN—mesenteric lymph node.

[3]smLN—submandibular lymph node.

[4]siLN—superficial inguinal lymph node.

[5]LP—lamina propria.

[6]Tissues from three mice were pooled, data reflects the average of two groups of pooled tissue.

these data suggest that the peripheral induction of $CD4^+Foxp3^+$ T_{reg} cells can occur in the absence of Carma1.

3.2.5 Characterisation of Carma1-independent Foxp3 induction

As IL-2 and TGF-β are sufficient to induce Foxp3 in *Carma1*$^{tut/tut}$ $CD4^+$ T cells, the possibility that these cytokines activated signalling pathways downstream of Carma1, or induced Foxp3 expression via an alternative pathway was explored. First, wild-type and *Carma1*$^{tut/tut}$ $CD4^+$ T cells were activated with the Pkc activator phorbol myristate acetate (PMA) and ionomycin, without exogenous cytokines (Figure 3.8A). Upon activation, Bcl10 phosphorylation, NF-κB inhibitor alpha (IκBα) degradation and phosphorylation of both Jun N-terminal kinase (Jnk) isoforms occurred in wild-type T cells. In contrast, Bcl10 was constitutively phosphorylated, amounts of Iκbα were constantly elevated, and the Jnk-p54 isoform remained unphosphorylated in *Carma1*$^{tut/tut}$ T cells. Interestingly, decreased abundance and constitutive phosphorylation of Bcl10 was observed in total *Carma1*$^{tut/tut}$ lymph node T cells, but not thymocytes (Figure 3.8B). Normally, TCR-triggered Carma1 phosphorylation by Pkcθ and Hpk1 allows it to interact directly with Bcl10 (Brenner et al., 2009; Matsumoto et al., 2005; Sommer et al., 2005). After assembly of the Carma1 signalling complex, Carma1 and Bcl10 undergo K48 ubiquitination and subsequent degradation thereby terminating Carma1-dependent signalling (Moreno-Garcia et al., 2010; Scharschmidt et al., 2004; Zeng et al., 2007). The lack of Carma1 protein (Figure 3.3E) and constitutive degradation of Bcl10 in *Carma1*$^{tut/tut}$ T cells indicates that a stable Carma1 signalling complex cannot be assembled; this is also reflected by the elevated amounts of TGF-β-activated kinase 1 (Tak1) expressed by resting and activated *Carma1*$^{tut/tut}$ T cells (Figure 3.8A), likely due to reduced protein turnover.

Although the Carma1 signalling complex was inoperative in *Carma1*$^{tut/tut}$ mice, it was possible that exogenous IL-2 and TGF-β retained the ability to activate NF-κB or Jnk.

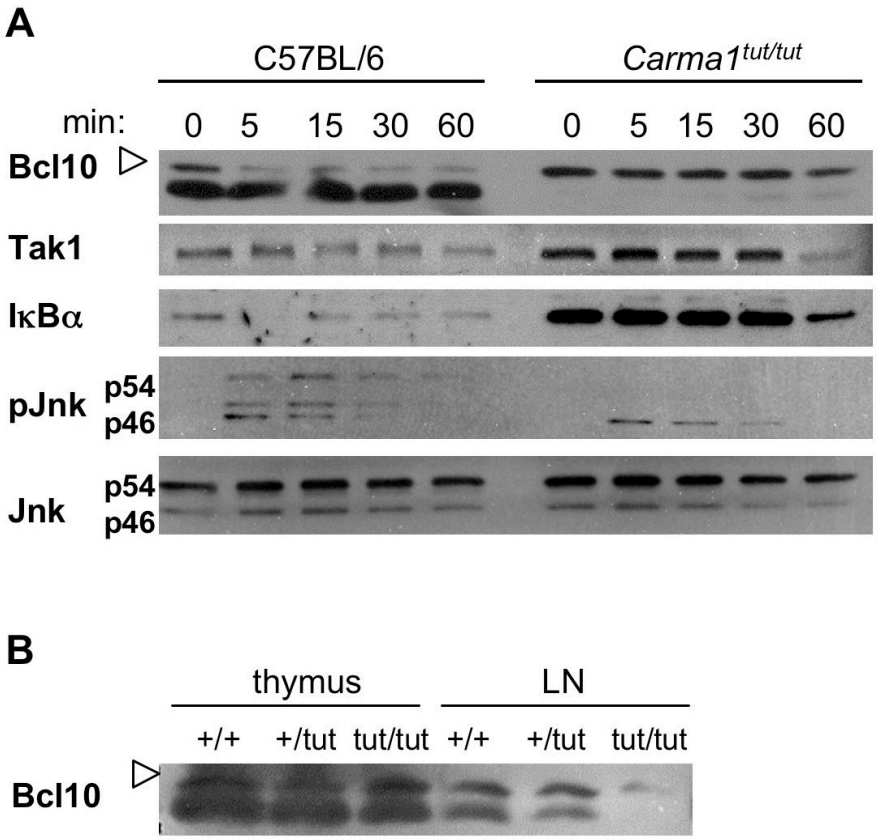

Figure 3.8. Impaired IκB degradation, Jnk phosphorylation and Bcl10 stability in *Carma1*$^{tut/tut}$ CD4^{+} T cells.
(A) To induce Pkc-driven activation of Carma1, purified CD4^{+} T cells were stimulated with PMA and ionomycin for the indicated number of minutes. Expression of indicated proteins and phosphorylated proteins was determined by immunoblotting. An open arrow indicates phosphorylated Bcl10.
(B) Expression of Bcl10 in untreated thymus and lymph node lysates was measured by immunoblotting. An open arrow indicates phosphorylated Bcl10.
These experiments were each repeated three times with consistent results and representative data are presented.

However, when *Carma1*$^{tut/tut}$ T cells were activated with PMA and ionomycin in the presence of these cytokines, neither Iκbα degradation or Jnk-p54 phosphorylation occurred up to 60 minutes after activation (data not shown). Similar results were obtained when T cells were activated by anti-CD3ε and anti-CD28 antibodies.

The ability of other cytokines to drive Foxp3 expression was investigated next. To determine whether trans-acting cytokines produced by wild-type CD4$^+$ T cells could drive Foxp3 induction, co-cultured wild-type and *Carma1*$^{tut/tut}$ CD4$^+$ T cells were activated in the presence of TGF-β. The presence of activated wild-type cells allowed proliferation and Foxp3 induction in *Carma1*$^{tut/tut}$ CD4$^+$ T cells. Co-culture in the presence of IL-2 neutralizing antibody abolished proliferation of *Carma1*$^{tut/tut}$ CD4$^+$ T cells and Foxp3 induction in both wild-type and *Carma1*$^{tut/tut}$ CD4$^+$ T cells (Figure 3.9A). However, other common γ-chain cytokines can also substitute for exogenous IL-2 in Foxp3 induction (Davidson et al., 2007). To test whether this could occur in the absence of Carma1, *Carma1*$^{tut/tut}$ CD4$^+$ T cells were activated in the presence of TGF-β and IL-4 (a common γ-chain cytokine). Exogenous IL-4 induced proliferation and Foxp3 induction, although, as expected, it was less potent than exogenous IL-2 (Wei et al., 2007). However, neutralizing IL-2 antibody abrogated this effect (Figure 3.9B), suggesting that other common γ-chain cytokines can drive IL-2 production and T cell proliferation independently of TCR-mediated Carma1 activation. It has also been reported that CpG oligonucleotides or associated proinflammatory molecules can act, *in vitro*, directly on T cells to restore TCR-mediated proliferation and induce CD4$^+$ T cell polarization in cells from *Pkcθ*$^{-/-}$ mice (Marsland et al., 2007). However, neither proliferation nor Foxp3 induction occurred in *Carma1*$^{tut/tut}$ T cells cultured with TGF-β and CpG, TNF-α, IFN-α or IFN-γ (Figure 3.9B).

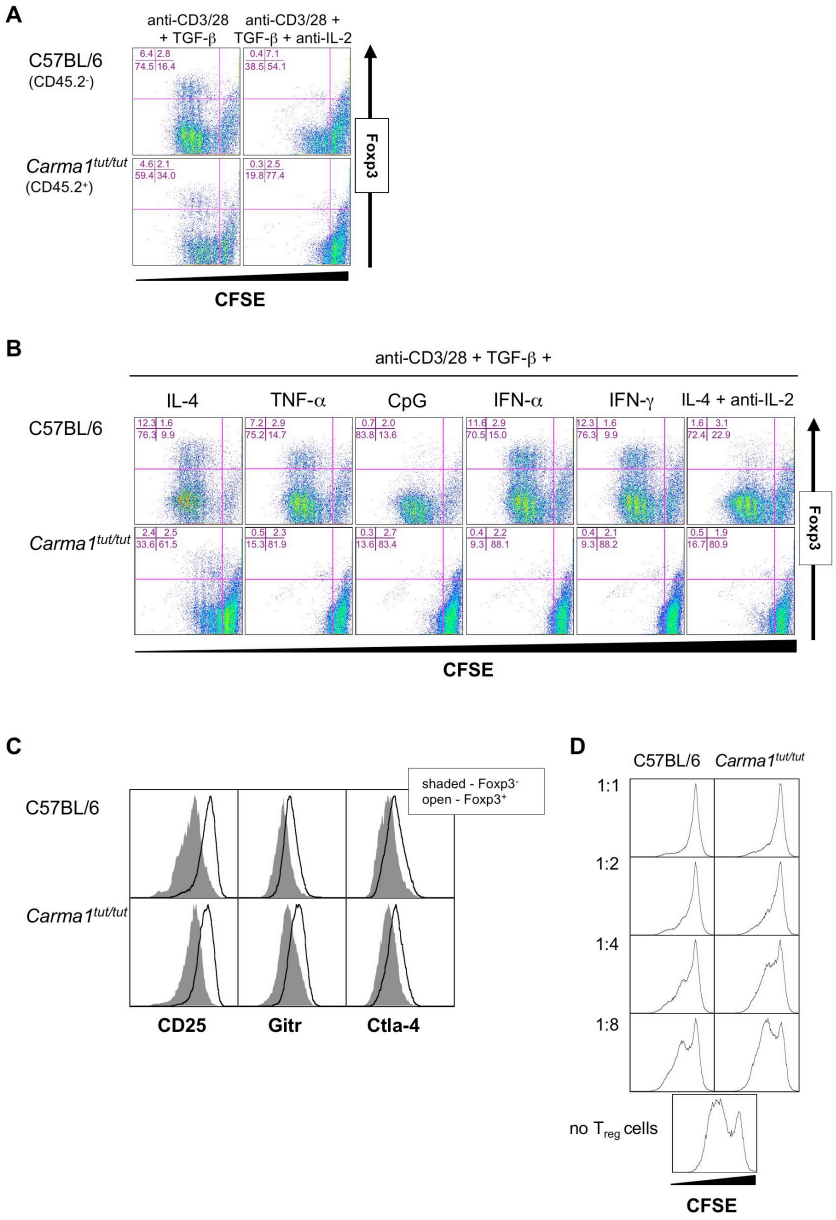

Figure 3.9. Induced *Carma1*$^{tut/tut}$ T_{reg} cells generated with TGF-β and IL-2 are functional.
(A) To examine the requirement for IL-2 in Foxp3 induction *in vitro*, purified CD4$^+$ T cells from congenic C57BL/6 (CD45.2$^-$) and *Carma1*$^{tut/tut}$ (CD45.2$^+$) mice were co-cultured with plate-bound anti-CD3ε (2 μg/mL) and anti-CD28 (2 μg/mL) in the presence of TGF-β (5 ng/mL), with or without anti-IL-2 (10 μg/mL) neutralising antibodies for 72 hours.
(B) Other cytokines or inflammatory molecules have been reported to drive T cell proliferation independently of Pkcθ, the activating kinase 'upstream' of Carma1. To assess the effect of these molecules on proliferation and Foxp3 induction *in vitro*, purified CD4$^+$ T cells were cultured with plate-bound anti-CD3ε (2 μg/mL) and anti-CD28 (2 μg/mL) in the presence of TGF-β (5 ng/mL) and either IL-4 (10 ng/mL), TNF-α (10 ng/mL), CpG oligonucleotides (100 nM), IFN-α (100 U/mL), IFN-γ (100 U/mL) or IL-4 (10 ng/mL) and anti-IL-2 (10 μg/mL) neutralising antibodies for 72 hours.
(C) Expression of the T_{reg} cell surface markers CD25, Gitr and Ctla-4 (intracellular) were measured in induced T_{reg} cells (generated as in Figure 3.7B) stained for intracellular Foxp3 expression.
(D) The functional activity of induced T_{reg} cells (generated as in Figure 3.7B) was investigated using an *in vitro* co-culture suppression assay. Naïve C57BL/6 CD8$^+$ T cells were purified from splenocytes, labelled with CFSE and cultured with γ-irradiated (3,000 rad) Thy-1-depleted splenocytes with soluble anti-CD3ε (0.5 μg/mL) for 72 hours. Indicated ratios of iT_{reg}: CD8$^+$ T cells were added to each well (iT_{reg} cells were generated as in Figure 3.7B) and CD8$^+$ T cell CFSE dilution was measured to assess suppression.
These experiments were each repeated at least two times with consistent results and representative data are presented.

3.2.6 T_{reg} cell suppressor function does not require Carma1

It remained unclear whether Carma1 was required for T_{reg} cell-mediated suppressor function. In addition to Foxp3, *in vitro* generated T_{reg} cells express higher amounts of CD25, Ctla-4 and Gitr on the cell surface, similar to T_{reg} cells found *in vivo*. *Carma1*$^{tut/tut}$ T_{reg} cells generated *in vitro* express normal amounts of these markers (Figure 3.9C). T_{reg} cells have the ability to suppress T cell proliferation by a cell-contact dependent mechanism *in vitro* (Thornton and Shevach, 1998). Both wild-type and *Carma1*$^{tut/tut}$ induced T_{reg} cells, generated *in vitro* with IL-2 and TGF-β, suppressed the proliferation of wild-type $CD4^+$ T cells in a co-culture assay (Figure 3.9D). The difference in observed suppression at 1:4 and 1:8 dilutions likely reflects the proliferation defect in *Carma1*$^{tut/tut}$ T_{reg} cells. These results suggest Carma1 is neither required for TGF-β-mediated induction of the T_{reg} cell phenotype, nor for suppressor function.

3.2.7 *Carma1*$^{tut/tut}$ T_{reg} cells expand after mouse cytomegalovirus infection

Peripheral T_{reg} cells comprise a small percentage of *Carma1*$^{tut/tut}$ $CD4^+$ T cells in the steady-state. It has been suggested that peripheral T_{reg} cells may expand during conditions of lymphopaenia (Hsieh et al., 2004), at the site of tumours (Ghiringhelli et al., 2005) or in response to pathogens (Belkaid, 2007). The absence of thymic-derived T_{reg} cells in *Carma1*$^{tut/tut}$ mice allows the dynamics of peripheral T_{reg} cells to be tracked during infection. To do this, *Carma1*$^{tut/tut}$ mice were infected with a pathogen that establishes persistent infection in mice—mouse cytomegalovirus (MCMV). *Carma1*$^{tut/tut}$ mice mounted T-dependent B cell responses that were reduced compared to the wild-type response (Figure 3.10A), but sufficient to allow survival without any signs of virus-induced immunopathology or detectable virus in the spleen fourteen days after infection. Yet, at the peak of the effector $CD4^+$ T cell response eight days after infection, no T_{reg} cell accumulation was observed in the spleen (Figure 3.10B). However, splenic $CD4^+Foxp3^+$ T_{reg} cell numbers increased by an order of magnitude in *Carma1*$^{tut/tut}$ mice fourteen days after MCMV infection (Figure 3.10B).

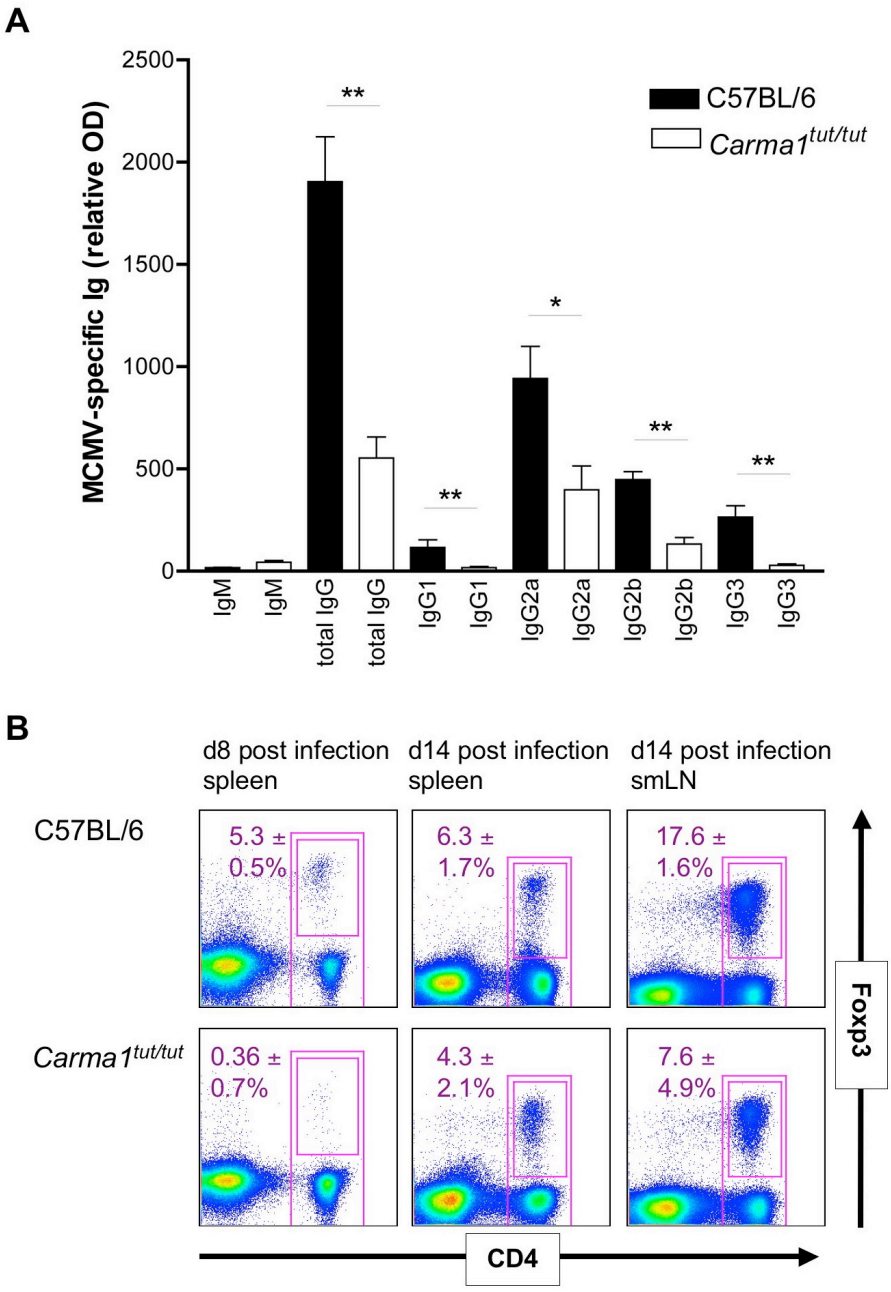

Figure 3.10. Peripheral T_{reg} cells accumulate in *Carma1*$^{tut/tut}$ mice after MCMV infection. (A) To assess the magnitude of the immune response to MCMV, the T-dependent and T-independent antibody responses were measured. MCMV-specific Ig concentrations for indicated isotypes were determined in serum collected fourteen days after infection (n = 5; * = $p < 0.05$, ** = $p < 0.01$).
(B) Foxp3 expression in splenic $CD4^+$ T cells was measured eight or fourteen days after infection, and in submandibular LN $CD4^+$ T cells fourteen days after infection. The percentage of $CD4^+$ T cells expressing Foxp3 is indicated with standard deviation (n = 5).

MCMV establishes persistent infection in the salivary glands and T cells drain from the salivary glands to the submandibular LNs. Here, T_{reg} cell expansion was also observed in *Carma1*$^{tut/tut}$ mice (Figure 3.10B). No Foxp3 expression was detected in the thymus eight or fourteen days after infection (data not shown), suggesting that T_{reg} cell accumulation resulted from either *de novo* induction of Foxp3 in naïve T cells or expansion of the pre-existing peripheral T_{reg} cell pool. However, thymectomy would be needed to make definitive conclusions about the role of the thymus in this system. Additionally, MCMV infected *Carma1*$^{tut/tut}$ mice did not develop signs of autoimmune or lymphoproliferative disease when monitored for 80 days after infection.

3.3 Discussion

Previously, it was known that mice lacking components of the Carma1-dependent signalling pathway – Pkcθ (Gupta et al., 2008; Schmidt-Supprian et al., 2004), Bcl10 (Deenick et al., 2010; Schmidt-Supprian et al., 2004), Tak1 (Sato et al., 2006; Wan et al., 2006), Ikkβ (Schmidt-Supprian et al., 2003) or c-Rel and NF-κB1 (Zheng et al., 2003) – had reduced $CD4^+CD25^+$ or $CD4^+Foxp3^+$ T_{reg} cell populations. Here, cell-intrinsic Carma1-dependent signalling is shown to be required for the development of $CD4^+Foxp3^+$ T_{reg} cells in the thymus, but dispensable for the peripheral induction of $Foxp3^+$ T_{reg} cells. Recently, several other studies have also confirmed that *Carma1*$^{-/-}$ mice lack T_{reg} cells in the thymus (Medoff et al., 2009; Molinero et al., 2009) due to a cell-intrinsic signalling defect (Molinero et al., 2009).

A two-step model for thymic T_{reg} cell development proposes that instructive TCR signals promote the development of $CD4^+CD25^+Foxp3^-$ SP thymocytes before Foxp3 expression can be induced upon encounter with IL-2, independently of TCR stimulation (Lio and Hsieh, 2008). In the context of Carma1-deficiency, $CD4^+CD25^+Foxp3^-$ T_{reg} cell precursors do not develop. Because Carma1-mediated TCR signalling to NF-κB can promote cell survival, one possibility is that Carma1-deficient T_{reg} cell precursors undergo apoptosis early in their development. Others have reported that transgenic expression of the pro-survival gene *Bcl-2* in *Carma1*$^{-/-}$ mice did not affect the thymic T_{reg} cell deficiency (Molinero et al., 2009). However, early in thymocyte development, between the DN and DP stages, a TCR-independent, p53-dependent checkpoint controls thymocyte survival (Jiang et al., 1996), whereas Bcl-2 promotes survival after thymocytes reach the DP stage (Linette et al., 1994). It is interesting to note that both *Carma1*$^{-/-}$ and *Carma1*$^{tut/tut}$ mice have skewed DN thymocyte populations, with an increased percentage of DN cells undergoing apoptosis (Egawa et al., 2003). Thus, it remains possible that an early step in the development of T_{reg} cell precursors requires Carma1. It would be interesting to generate and examine whether T_{reg} cells or their

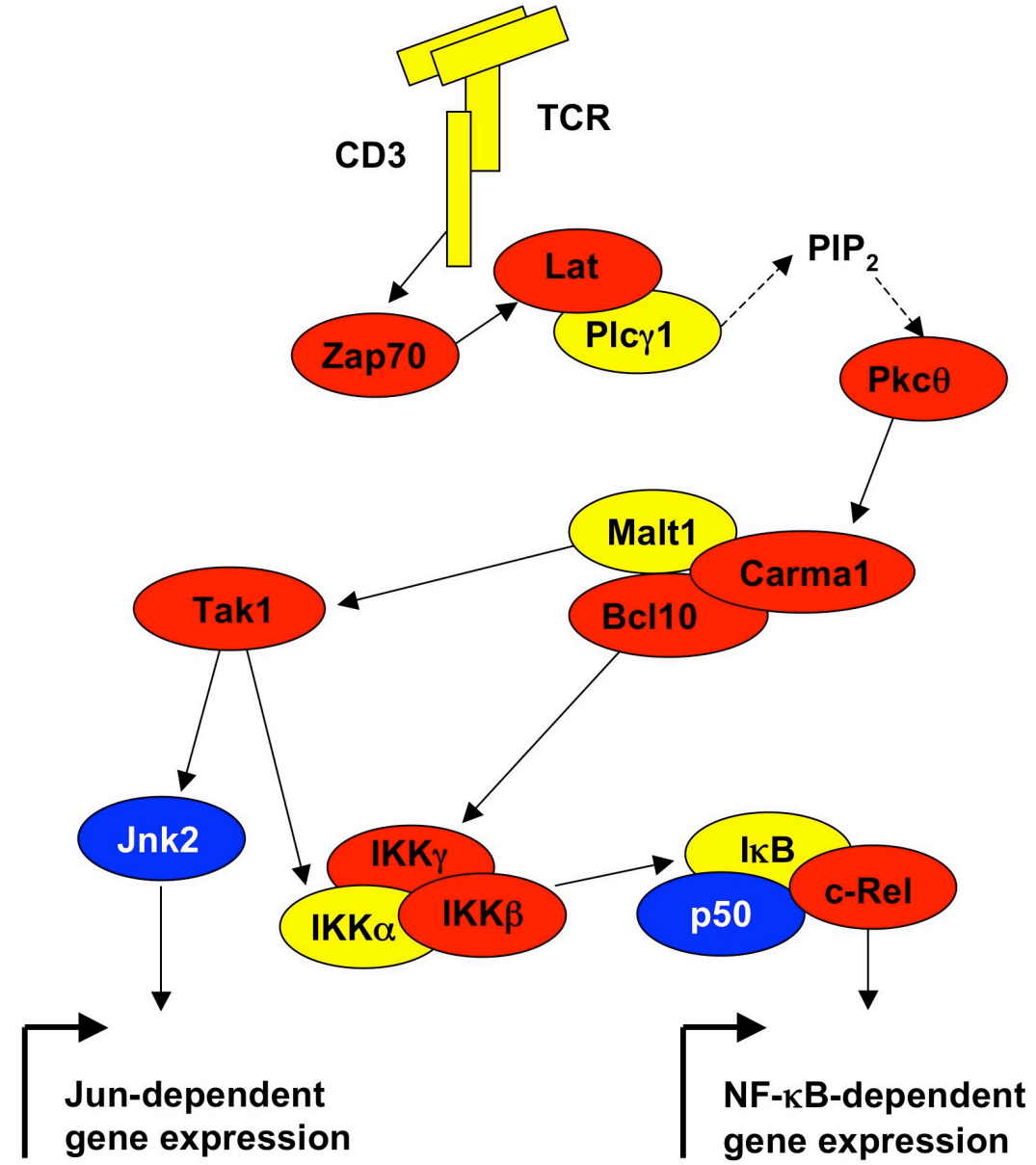

Figure 3.11. The role of TCR-mediated NF-κB activation in T_{reg} cell development. Red ovals highlight genes for which mutant or knockout mice display reduced numbers of $CD4^+CD25^+$ or $CD4^+Foxp3^+$ T_{reg} cells in the steady state. Blue ovals represent genes for which knockout mice have normal numbers of $CD4^+Foxp3^+$ T_{reg} cells. The phenotype of mice deficient in genes represented by yellow circles has not been reported.

precursors develop in the thymus of *Carma1*$^{-/-}$*p53*$^{-/-}$ mice or in *CD4*^{cre}*Carma1*$^{flox/-}$ mice that would retain Carma1 expression in the DN developmental stages, but lack it in DP and SP thymocytes. In such mice, T_{reg} cell precursors might survive to reach the $CD4^+CD25^+Foxp3^-$ stage of T_{reg} cell development described previously (Lio and Hsieh, 2008).

A second possible reason for the T_{reg} cell deficiency is that Carma1-dependent signalling directly promotes expression of Foxp3. Recently, several groups reported that mice lacking the transcription c-Rel have a severe reduction in thymic T_{reg} cells due to a cell-intrinsic defect (Deenick et al., 2010; Isomura et al., 2009; Long et al., 2009; Ruan et al., 2009; Visekruna et al., 2010; Zheng et al., 2010). c-Rel can bind directly to the Foxp3 promoter at a conserved nucleotide sequence and act to directly promote Foxp3 transcription (Long et al., 2009; Zheng et al., 2010). Additionally, IL-2-mediated signals promote Foxp3 expression in thymocytes and others have shown that IL-2 induced phosphorylation of Stat5 is defective in rare *Carma1*$^{-/-}$ $CD4^+CD25^+$ thymocytes (Lee et al., 2010a). Since the 'second-step' in thymic T_{reg} cell development requires intact IL-2 (or IL-15) signalling to induce the transcription of *Foxp3* (Lio and Hsieh, 2008), this stage of T_{reg} cell development is also predicted to require Carma1.

In the peripheral tissues, or in response to TGF-β and IL-2 *in vitro*, Foxp3 induction could still occur in the absence of Carma1, but only at higher doses of TCR stimulation. This suggests that either a different signalling-strength threshold or signalling pathway allows for T_{reg} cell development in the thymus versus the peripheral tissues. Available data support aspects of both possibilities. The peripheral T_{reg} cell population is considerably reduced in the absence of Carma1. The finding reported here that titration of TCR stimulation affects Foxp3 induction in Carma1-deficient $CD4^+$ T cells more than in wild-type cells suggests that only a fraction of TCR triggering events that would normally trigger Foxp3 induction are productive in the absence of Carma1. This might mean that Carma1-mediated activation of c-Rel allows for efficient transcription of *Foxp3* in the peripheral tissues. In its absence, TGF-β signals

and other TCR-triggered signals can allow for *Foxp3* transcription by mobilising transcription factors that bind other promoter elements at the *Foxp3* locus. Indeed, a conserved nucleotide sequence in the *Foxp3* promoter has been recently described that does not bind c-Rel, but does bind TGF-β mobilized transcription factors (Zheng et al., 2010). Also, proliferation of $CD4^+$ T cells is reduced *in vivo* (Isomura et al., 2009) and *in vitro* in the absence of Carma1 or c-Rel, as is the production of IL-2 by T cells. The small T_{reg} cell population observed in Carma1-deficient mice might be supported by myeloid cell-derived production of IL-2, which does not require Carma1 (Hara et al., 2007). Together these factors can account for the reduced peripheral T_{reg} cell population in Carma1-deficient mice.

A second pathway, through the PI3K→mTor signalling axis, has been recently shown to affect Foxp3 induction. In the thymus, $S1P_1$-mediated signalling through mTor inhibits Foxp3 expression (Liu et al., 2009). In peripheral $CD4^+$ T cells, treatment with the chemical inhibitor of mTor, rapamycin, or genetically-induced mTor deficiency (in cells from $CD4^{cre}Frap1^{flox/-}$ mice) caused enhanced Foxp3 expression when cells were activated in the presence of TGF-β and IL-2 (Delgoffe et al., 2009; Sauer et al., 2008). Additionally, $CD4^+$ T cells that were transfected with a constitutively active form of Akt, an upstream activator of mTor, showed diminished Foxp3 induction in similar conditions (Haxhinasto et al., 2008). By contrast, deficiencies in components of the TCR→Carma1→c-Rel signalling axis cause a loss of thymic T_{reg} cells and somewhat less efficient Foxp3 induction of peripheral T_{reg} cells. These two signalling pathways could act as opposing forces in the differentiation of $CD4^+$ T_{reg} cells. Conditions that activate the PI3K→mTor signalling axis through $S1P_1$ or other receptors could inhibit Foxp3 expression and favour inflammatory responses, whereas TCR→Carma1→c-Rel signals, or other TCR-independent signals that activate c-Rel such as TNF-α-signalling (Hara et al., 2003), might favour Foxp3 induction and the mitigation of inflammatory responses. It will be interesting to examine the interplay between these two pathways in various infectious or disease models.

Perhaps the most surprising finding in this study is that Carma1-deficient mice do not develop autoimmune pathology, either in the steady-state or upon infection. Peripherally induced T_{reg} cells accumulate in the colon LP of Carma1-deficient mice, in agreement with many previous studies that show intestine LP and mLN cells can promote the generation of induced T_{reg} cells and expand pre-existing T_{reg} cells (Coombes et al., 2007; Denning et al., 2007; Izcue et al., 2008; Mucida et al., 2005; Sun et al., 2007). It is likely that the intestinal microbial flora (or chronic infection) is required to drive peripheral T_{reg} cell expansion *in vivo* and it would be interesting to rederive Carma1-deficient mice under germ free conditions. Such mice would be predicted to completely lack Foxp3$^+$ T_{reg} cells in the thymus and peripheral tissues.

This study shows that Carma1-deficient T_{reg} cells are suppressive *in vitro*, and others have shown that or *c-Rel*$^{-/-}$ T_{reg} cells have normal protective capacity *in vivo* in the T-cell-transfer colitis model (Isomura et al., 2009) and T_{reg} cells treated with *Pkcθ*$^{-/-}$ inhibitors have even been reported to have enhanced activity in this model (Zanin-Zhorov et al., 2010). It is therefore a safe assumption that the small Foxp3$^+$ T_{reg} cell population in Carma1-deficient mice retains functionality. If Carma1-deficient mice were depleted of Foxp3$^+$ T_{reg} cells or crossed with T_{reg} cell-deficient *Foxp3*^{scurfy} or *Foxp3*$^{-/Y}$ mice, it would be interesting to observe whether spontaneous colitis occurred due to the absence of T_{reg} cells in the GI tract, and the presence of microbes that might be able to promote inflammatory responses. The lymphoproliferative aspect of the disease that occurs in *Foxp3*^{scurfy} or *Foxp3*$^{-/Y}$ mice would be predicted to be absent from *Carma1*$^{tut/tut}$*Foxp3*^{scurfy} mice for two reasons. First, IL-2 is not produced by Carma1-deficient T cells and is likely to be required to sustain a lymphoproliferative response in the absence of Carma1 because *Carma1*$^{tut/tut}$ CD4$^+$ T cells require IL-2 to proliferate (at least *in vitro*). Second, Carma1- or c-Rel-deficiency does not impede the peripheral differentiation of inflammatory CD4$^+$ T cell subsets (Visekruna et al., 2010). Because LCMV or *Listeria monocytogenes* infection induced a T-cell dependent host-protective immune response in *Carma1*$^{tut/tut}$ mice (data not shown) without causing Foxp3$^+$

T_{reg} cell expansion or autoimmunity, the TCR repertoire in Carma1-deficient mice might include fewer autoreactive clones. Alternatively, LCMV and *L. monocytogenes* might provide a weaker TCR stimulus than MCMV at the doses used in this study. This point merits further investigation.

It is interesting that MCMV can cause the accumulation of T_{reg} cells after the acute phase of infection ends around day eight and as the persistent phase of infection begins around day fourteen post-infection. Notably, DCs increase cell-surface expression of PD-L1 after MCMV infection (Benedict et al., 2008), and PD-L1 engagement by $CD4^+$ T cells has been shown to promote Foxp3 induction (Wang et al., 2008). Exhausted T cells increase PD-1 expression (the PD-L1 ligand) (Fife and Bluestone, 2008), so Foxp3 induction might occur when exhausted effector $CD4^+PD\text{-}1^{high}$ T cells encounter MCMV-infected DCs. To prove that MCMV-induced T_{reg} cell expansion is not an artefact of the Carma1-deficient environment, it will be important to examine whether MCMV induces T_{reg} cells in wild-type mice, using adoptively transferred $Foxp3^{gfp+}$ reporter $CD4^+$ T_{reg} cells and conventional $CD4^+Foxp3^{gfp-}$ T cells (Fontenot et al., 2005b). If this is also found to occur in wild-type mice, local T_{reg} cell induction could be one way that MCMV facilitates persistent infection in mice.

Finally, the nature of the *tut* mutation warrants some discussion. The defects in the development of the haematopoietic system and in lymphocyte activation were similar in $Carma1^{tut/tut}$ mice and $Carma1^{-/-}$ mice (Egawa et al., 2003; Hara et al., 2003; Jun et al., 2003; Medoff et al., 2009; Newton and Dixit, 2003). Therefore, it was surprising to find reduced protein concentration and constitutive phosphorylation of Bcl10 in $Carma1^{tut/tut}$ $CD4^+$ T cells. In CARMA1-deficient human JPM50.6 cells, BCL10 expression is normal (Wang et al., 2002). The normal expression of Bcl10 in $Carma1^{tut/tut}$ thymocytes, but not lymph node cells suggests that Bcl10 degradation may be confined to mature T cells and B cells. As the *tut* mutation occurred in a region of Carma1 predicted to regulate accessibility of the CARD

domain (Matsumoto et al., 2005; Sommer et al., 2005), a model is proposed in which Bcl10 is constitutively recruited to the Carma1^{tut} protein in mature T and B cells. This interaction would not be sufficient to assemble the full Carma1-Bcl10-Malt1 signalling complex or to activate NF-κB, but would allow for Bcl10 phosphorylation and the subsequent degradation of Bcl10 and the Carma1^{tut} proteins. As a result, the Carma1-Bcl10-Malt1 complex cannot be assembled upon TCR stimulation. The net result is that *Carma1$^{tut/tut}$* mice phenocopy *Carma1$^{-/-}$* mice and the conclusions made here about Carma1-dependent signalling are in accordance with other recent studies using Carma1-knockout mice. However, this model needs to be tested biochemically and it may be that the *tut* mutation could offer additional insights into the mechanisms of TCR signalling and protein turnover.

IV. Resident intestinal bacteria promote IL-10 production by $CD4^+$ T cells

4.1 Introduction

The cytokine interleukin 10 (IL-10; originally termed cytokine inhibitory factor) plays a critical role in limiting the magnitude of an immune response. Its importance is underscored by the lethal immunopathology that occurs when *Il10*$^{-/-}$ mice are infected with various pathogens (Gazzinelli et al., 1996; Grünig et al., 1997; Hunter et al., 1997) and by the multitude of cell types—B, $CD4^+$, $CD8^+$, DC, macrophage, mast, myeloid, NK and neutrophil cells—that can produce IL-10 when activated (Saraiva and O'Garra, 2010). IL-10 signalling is transmitted through the IL-10R (Spencer et al., 1998), which is coupled to activation of Jak1, Tyk2 and the Stat3 transcription factor (Finbloom and Winestock, 1995; O'Farrell et al., 1998; Rodig et al., 1998). Although lymphocytes and epithelial cells are targets of IL-10 (Murai et al., 2009; Shkoda et al., 2007), signalling through Stat3 into myeloid cells seems to be essential for limiting immune activation where it acts to shut down production of pro-inflammatory cytokines and prevent the upregulation of co-stimulatory molecules (Ding et al., 1993; Fiorentino et al., 1991; Takeda et al., 1999). Expression of IL-10 can be initiated by a wide variety of different innate immune stimuli, making it an especially difficult cytokine to manipulate *in vivo*. Yet, this remains a worthy goal because blocking IL-10 signalling can profoundly enhance protective immune responses in the contexts of infection and vaccination (Brooks et al., 2008; Couper et al., 2008; Stober et al., 2005), and, alternatively, enhancing production of IL-10 might help to control allergies, asthma, inflammatory bowel disease or other types of chronic immunopathologies (Campbell et al., 2009; Meiler et al., 2008; Steidler et al., 2000; Sundstedt et al., 2003).

Accessibility of the *Il10* promoter is regulated by chromatin modifications and the promoter contains binding sites for several transcription factors—C/EBPβ, Gata3, Irf1, c-Maf, NF-κBp50, Smad4, Sp1, Sp3 and Stat3 (Saraiva and O'Garra, 2010). Whether any of these

individual transcription factors are absolutely required for *Il10* transcription, or whether certain combinations of these transcription factors are sufficient to drive *Il10* transcription remains the subject of investigation. In effector $CD4^+$ T cells, activation of the Erk MAP kinases and at least one of the Stat transcription factors seems to be important for IL-10 production (Saraiva et al., 2009). Specifically, activation of Stat4 by IL-12 (Saraiva et al., 2009), activation of Stat6 by IL-4 (Chang et al., 2007) or activation of Stat3 by IL-6 (Stumhofer et al., 2007; Xu et al., 2009), in the presence of strong TCR stimulation can result in IL-10 production in T_H1, T_H2 or T_H17 cells, respectively. IL-27 has the capacity to induce IL-10 expression in all classes of effector $CD4^+$ T cells and it might act through Stat1 and Stat3 (Batten et al., 2008; Stumhofer et al., 2007; Xu et al., 2009). Apart from cytokines, ligands for the aryl hydrocarbon receptor (Ahr) have been reported to act as cofactors for IL-27-mediated induction of IL-10 (Apetoh et al., 2010; Gandhi et al., 2010), and Notch ligands can promote IL-10 expression via Stat4 (Rutz et al., 2008).

In addition to activated effector $CD4^+$ T cells, two types of regulatory T cells, $Foxp3^+$ T_{reg} and Tr1-like cells, also produce IL-10. Tr1-like cells that express IL-10, but not effector cytokines, can be differentiated *in vitro* by activating cells in the presence of TGF-β, exogenous IL-10 and IFN-α (Groux et al., 1997; Levings et al., 2001; Maynard et al., 2007) or the small molecules dexamethasone and Vitamin D_3 (Barrat et al., 2002) and *in vivo* by repetitive stimulation through the TCR (Nicolson et al., 2006). One subject of debate concerns whether $Foxp3^-$ Tr1-like cells represent 'exhausted' effector cells or a distinct subset of $CD4^+$ T cells. Combinations of cell-fate mapping, cytokine neutralisation and gene expression studies will be needed to resolve this debate. Even less is known about the mechanisms that regulate IL-10 production by $Foxp3^+$ T_{reg} cells. Although some studies suggest that IL-2 signalling into $CD4^+$ T cells can increase IL-10 expression (Barthlott et al., 2005; Brandenburg et al., 2008; de la Rosa et al., 2004), $Foxp3^+$ T_{reg} cells from *Il2*$^{-/-}$ mice have been reported to express normal amounts of *Il10* mRNA (Fontenot et al., 2005a). Also, IL-2 signalling activates Stat5 in $Foxp3^+$ T_{reg} cells, but these cells also require Stat5 to survive

(Josefowicz and Rudensky, 2009), making the functional role of Stat5 in promoting IL-10 expression *in vivo* difficult to assess.

Expression of IL-10 by regulatory T cell populations is essential to maintain intestinal homeostasis. This requirement first became apparent when *Il10*$^{-/-}$ mice were found to develop 'spontaneous' colitis (Kuhn et al., 1993), which was later shown to require the presence of certain type of resident intestinal bacteria that are present in most animal facilities (Madsen et al., 2000; Sellon et al., 1998). Studies using the T-cell-transfer colitis model showed that CD4+CD25+ regulatory T cells were an essential source of IL-10 in the intestine (Asseman et al., 1999). Subsequently, work with mice in which *Il10* was deleted from certain cell-types demonstrated that CD4$^+$ T cells were a non-redundant source of IL-10 in the intestine (Roers et al., 2004), and, among CD4$^+$ T cell subsets, Foxp3$^+$ T_{reg} cells were the most important (Rubtsov et al., 2008). Humans or mice lacking a functional IL-10R also develop intestinal inflammation (Glocker et al., 2009; Spencer et al., 1998). Imaging studies showed that CD4$^+$ T cells, especially Foxp3$^+$ T_{reg} cells, in the intestine were the major cell type in healthy mice or humans that produced detectable IL-10 (Uhlig et al., 2006). In the chapter, the cytokines and environmental stimuli that regulate IL-10 expression in the GI tract are investigated.

4.2 Results

4.2.1 Tracking IL-10 and Foxp3 expression *in vivo* with a dual-reporter mouse

One difficulty in identifying the cellular sources of IL-10 *in vivo* is the difficulty in detecting intracellular IL-10 expression in mouse cells using monoclonal antibodies. To circumvent this problem, other groups have generated reporter mice that express *Gfp* at a locus distal to the *Il10* coding region (Kamanaka et al., 2006) or a transgene encoding the *Thy1.1* cell-surface molecule under control of the *Il10* promoter (Maynard et al., 2007). Studies using these reporter mice showed that $CD4^+$ T cells are the major source of IL-10 in the GI tract of healthy mice, although subsequent studies indicated that some myeloid cells in the colon LP also express IL-10 and are functionally important in preventing intestinal inflammation [(Murai et al., 2009) and Mark Asquith, Kevin Maloy, Fiona Powrie, unpublished observations]. Additionally, within the $CD4^+$ T cell population, others have been able to track "IL-10-competent" Tr1-like and $Foxp3^+$ T_{reg} cells *in vivo* by crossing *Il10-Thy1.1* reporter mice (called 10BiT mice) with reporter mice that express a functional Foxp3-gfp fusion protein (Maynard et al., 2007; Maynard et al., 2009).

An analogous reporter mouse is described here that was generated by crossing $Il10^{gfp/gfp}$ with $Foxp3^{hCD2/hCD2}$ reporter mice. The latter expressed a non-signalling human *Cd2* allele 'downstream' of the Foxp3 locus, marking all $Foxp3^+$ T_{reg} cells with the expression of cell-surface hCD2 (Komatsu et al., 2009). Mice with an $Il10^{gfp/+}Foxp3^{hCD2/hCD2}$ genotype were used throughout this study in order to exclude the possibility that mice homozygous for the *Gfp* allele had aberrant expression of IL-10. Expression of IL-10 and Foxp3 throughout the GI tract and in the spleen and inguinal lymph node of healthy adult mice was examined (Figure 4.1A). Consistent with other reports (Coombes et al., 2005; Kamanaka et al., 2006; Maynard et al., 2007), nearly all $IL\text{-}10^{high}$ cells were $CD4^+$ T cells (data not shown) and the frequency of $Foxp3^+$ T_{reg} cells was increased in the large intestine LP and reduced in the liver (Figure 4.1A). Between 5 - 10 % of $Foxp3^+$ T_{reg} cells expressed IL-10 in the lymph nodes, and this frequency increased to 30 - 40 % in lymphocytes residing in either the IEL or LP of

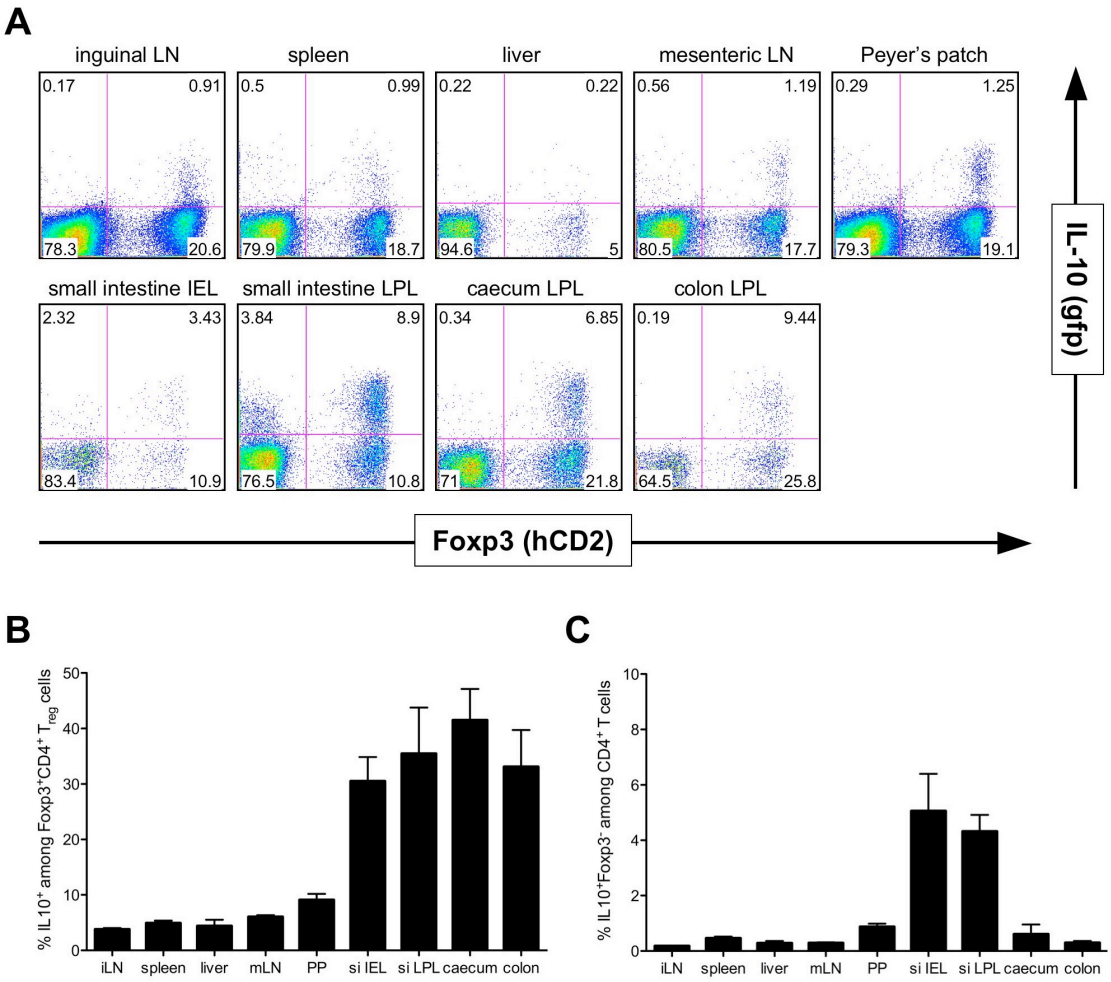

Figure 4.1. Characterisation of intestinal T_{reg} cells in *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice.

(A) To track the cellular sources of IL-10 *in vivo*, mice expressing an *Ires-Egfp* allele distal to the *Il10* locus were crossed with mice that expressed a non-signalling human *CD2* reporter allele knocked into the distal end of the *Foxp3* locus. Lymphocytes were harvested from indicated tissues of *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice and analysed for expression of Foxp3 and IL-10 *ex vivo*, without restimulation. The majority of IL-10^{gfp+} cells were CD4^{+} T cells. Representative flow cytometry plots show Foxp3 and IL-10 expression in CD4^{+}TCRβ^{+} lymphocytes.

(B-C) The percentages of (B) IL-10^{gfp+} cells among Foxp3^{hCD2+}CD4^{+}TCRβ^{+} T_{reg} cells and (C) IL-10^{gfp+}Foxp3^{hCD2-} Tr1-like cells among total CD4^{+}TCRβ^{+} T cells are shown. For each column, standard deviation is indicated (n = 5).

the large and small intestine (Figure 4.1B). Somewhat surprisingly, Foxp3$^-$ Tr1-like cells were largely confined to the small intestine and were less abundant than Foxp3$^+$IL-10$^+$ T_{reg} cells (Figure 4.1C). Other reports have found, using 10BiT mice, that small intestine Tr1-like cells were the most frequent IL-10 producing CD4$^+$ T cell population in the small intestine and that the colon LP also contained a population of Tr1-like cells (Maynard et al., 2007; Maynard et al., 2009). This discrepancy could reflect differences in the *Il10*-reporter constructs used to make IL-10 reporter mice or in the resident intestinal bacteria present in mouse colonies at different institutions.

4.2.2 IL-10 producing T_{reg} cells use a broad repertoire of TCR-V_β chains and have an activated phenotype

To begin to characterise intestinal IL-10-producing Foxp3$^+$ T_{reg} and Tr1-like cells, the TCR usage of these cells was investigated. In C57BL/6 mice, expression of the majority of TCR-V_β chains can be tracked using chain-specific monoclonal antibodies (Necker et al., 1991). Different combinations of V_β chains are formed early during T cell development by V(D)J recombination and examining the expression of different V_β isoforms offers a low-resolution way to assess the diversity of a T cell population (Goldrath and Bevan, 1999). For example, V_β-8$^+$ *i*NKT cells and V_β-6$^+$/V_β-8$^+$ MAIT CD8$^+$ T cells show developmentally restricted TCR-V_β chain usage (Park et al., 2001; Treiner et al., 2003), and during a pathogen-driven immune response, the expansion of immunodominant effector T cell clones can alter the distribution of TCR-V_β chains in CD4$^+$ T cell populations (Winslow et al., 2003).

One hypothesis was that IL-10-producing Foxp3$^+$ T_{reg} cells might recognise intestine-specific antigens, derived from dietary components or resident intestinal bacteria, which would promote their proliferation and secretion of IL-10. If this response was driven by a limited number of antigens, it was expected that one or several TCR-V_β chains would be over-represented in colon LP Foxp3$^+$ T_{reg} cells that produced IL-10. To first determine the baseline

'skewing' of colon LP Foxp3$^+$ T_{reg} cells, the differences in TCR-V_β chain usage were compared between Foxp3$^+$ T_{reg} cells in the mLN and colon LP of *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice. Foxp3$^+$ T_{reg} cells expressing the V_β-2, V_β-7 and V_β-10b chains were found to be over-represented in the colon LP, and those that expressed the V_β-6 and V_β-14 chains were under-represented compared to the mLN T_{reg} cell population (Figure 4.2A). Within the colon LP Foxp3$^+$ T_{reg} cell population, all groups expressed IL-10 and only cells that expressed the V_β-11 chain showed a modest, but statistically significant increase in the frequency of IL-10-producing cells compared to the total Foxp3$^+$ T_{reg} cell population (Figure 4.2B). V_β-11$^+$ Foxp3$^+$ T_{reg} cells were not over-represented in the colon LP compared to cells in the mLN (Figure 4.2A). Overall, it appears that IL-10$^+$Foxp3$^+$ T_{reg} cells are not an oligoclonal population that recognises a very limited number of immunodominant antigens; TCR-V_β chain usage by small intestinal Tr1-like cells remains to be determined.

An interesting, but unexpected finding was that V_β-14$^+$ T_{reg} cells were found at a reduced frequency in the colon LP compared to the mLN and contained fewer IL-10-producing cells compared to CD4$^+$ T cells that express other V_β chains (Figure 4.2A-B). The *Vb14* allele is unique compared to other *Vb* alleles as it can be expressed in a biallelic fashion, is more prone to undergo recombination and is not subject to allelic exclusion (Glusman et al., 2001; Ranganath et al., 2008; Senoo and Shinkai, 1998). Also, the genomic orientation of the *Vb14* allele is inverted compared to all other *Vb* alleles, which are clustered on mouse Chromosome 6. Previous studies showed that *Il2rg*$^{-/-}$ mice (common γ-chain knockout) that develop bacteria-dependent, 'spontaneous' colitis have an abundance of IL-6-producing V_β-14$^+$ CD4$^+$ T effector cells in the colon LP (Kai et al., 2005), and V_β-14$^+$ CD8$^+$ T cells proliferate extensively in wild-type mice treated with the colitis-inducing chemical *2*,*4*,*6*-trinitrobenzenesulfonic acid **(**TNBS) (Kanetake et al., 2003). Together, these data suggest that V_β-14$^+$ T cells might have properties that predispose them to become an effector, rather than regulatory cell type and merit further investigation.

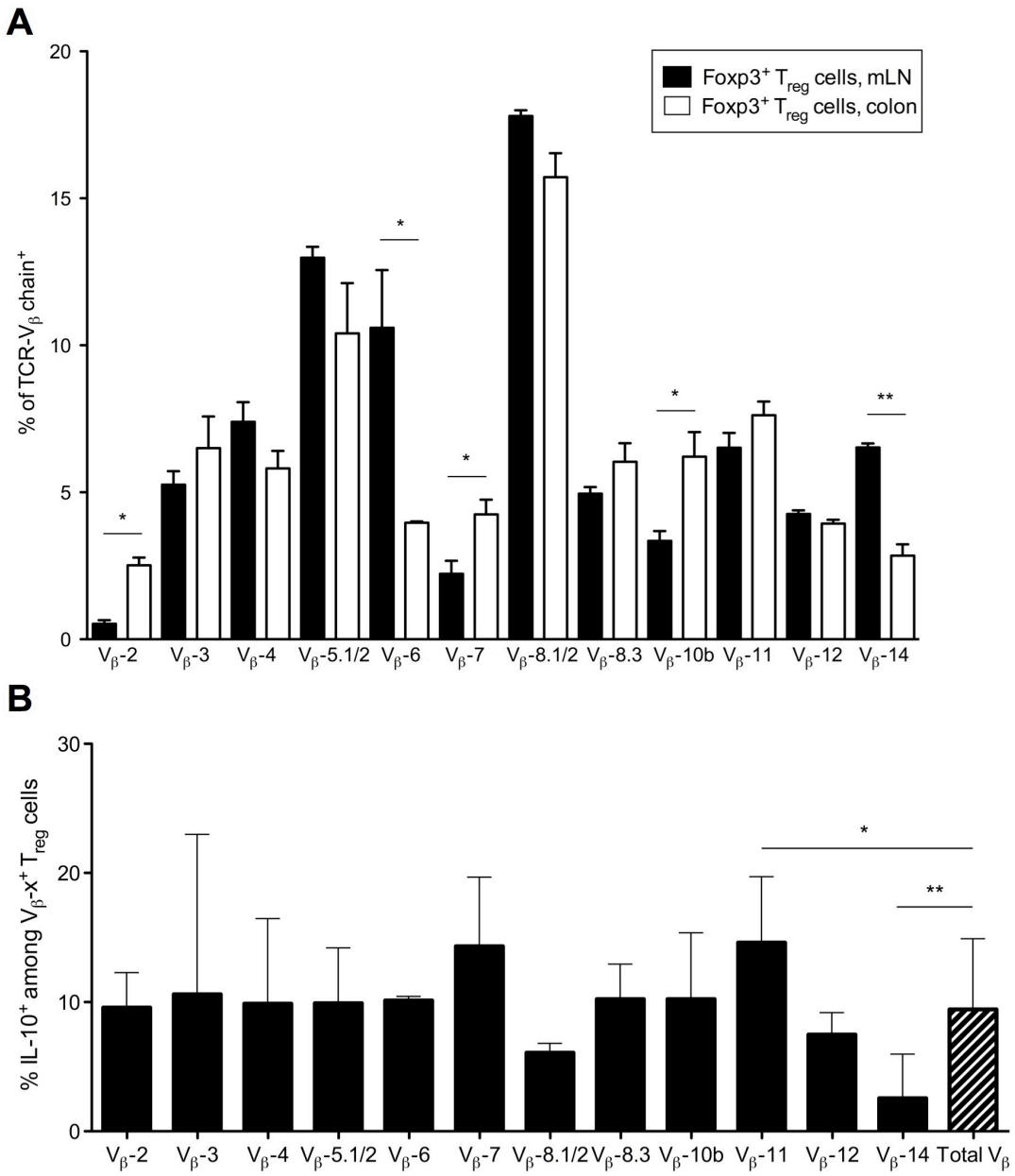

Figure 4.2. Polyclonal production of IL-10 by intestinal T_{reg} cells.

(A) Expression of indicated TCR-V_{β} chains among $CD4^{+}Foxp3^{hCD2+}$ lymphocytes isolated from the mLN and colon LP was assessed using V_{β}-chain-specific monoclonal antibodies. Standard deviation is indicated for each group (n = 5). Groups for which the percentage of TCR-V_{β}-x^{+} T_{reg} cells was found to be significantly different between the mLN and colon LPL using a Mann-Whitney test are indicated (* = $p < 0.05$, ** = $p < 0.01$).

(B) For each group of colon LP $CD4^{+}Foxp3^{hCD2+}$TCR-V_{β}-x^{+} T_{reg} cells, the percentage of IL-10^{gfp+} cells was determined. Standard deviation is indicated for each group (n = 5). Groups for which the percentage of IL-10^{gfp+} cells among $CD4^{+}Foxp3^{hCD2+}$TCR-V_{β}-x^{+} cells was found to be statistically different from the average frequency of IL-10^{gfp+} cells among total $CD4^{+}Foxp3^{hCD2+}$TCRβ^{+} T_{reg} cells using a Mann-Whitney test are indicated (* = $p < 0.05$, ** = $p < 0.01$).

Analysing the expression of cell-surface markers indicative of T cell subsets or activation state allows additional inferences to be made about the characteristics of IL-10-producing $CD4^+$ T cells. For example, in some conditions, a subset of T cells important for helping germinal centre B cell responses, follicular helper T (T_{FH}) cells, can express IL-10 and show a $Cxcr5^+Ccr7^{low}ICOS^{high}PD\text{-}1^{high}$ phenotype (Fazilleau et al., 2009). Also, $Foxp3^+$ T_{reg} cells are a somewhat heterogeneous population that can be subdivided based upon costimulatory molecule and chemokine receptor expression patterns (Huehn et al., 2004; Koch et al., 2009; Sather et al., 2007). In dual-reporter mice, conventional $Foxp3^-IL\text{-}10^-$ $CD4^+$ T (T_{conv}) cells expressed reduced amounts of the costimulatory molecules Ctla-4 in all tissues and both ICOS and PD-1 in the mLN, but intermediate amounts of ICOS and PD-1 in the small intestine LP and colon LP (Figure 4.3). Tr1-like cells in the small intestine LP were indistinguishable from T_{conv} cells, whereas in the mLN they had a $Ctla\text{-}4^-ICOS^{int}PD\text{-}1^{int}$ phenotype (Figure 4.3). Therefore the small intestine LP $Foxp3^-IL\text{-}10^+$ cells have a phenotype characteristic of Tr1-like cells, but expression of Cxcr5 and Ccr7 needs to be analysed in the mLN to conclusively determine whether these $Foxp3^-IL\text{-}10^+$ cells represent T_{FH} or Tr1-like cells.

IL-10-producing $Foxp3^+$ T_{reg} cells showed an intriguing pattern of cell-surface marker expression (Figure 4.3). Ctla-4 and ICOS expression was slightly, but reproducibly higher in $IL\text{-}10^+Foxp3^+$ T_{reg} cells, suggesting that these cells were recently activated. PD-1, a marker characteristic of chronically activated 'exhausted' T cells, was expressed in uniformly large amounts by all $Foxp3^+$ T_{reg} cells, irrespective of IL-10 expression. Thus, there seems to be a set of genes such as PD-1 that are uniformly expressed on all $Foxp3^+$ T_{reg} cells, and others such as Ctla-4, ICOS and IL-10 that could identify activated $Foxp3^+$ T_{reg} cells. Analysis of more markers will be needed to determine whether IL-10 expression is consistently linked to expression of other activation markers such as CD25, CD62L or CD103 in $Foxp3^+$ T_{reg} cells.

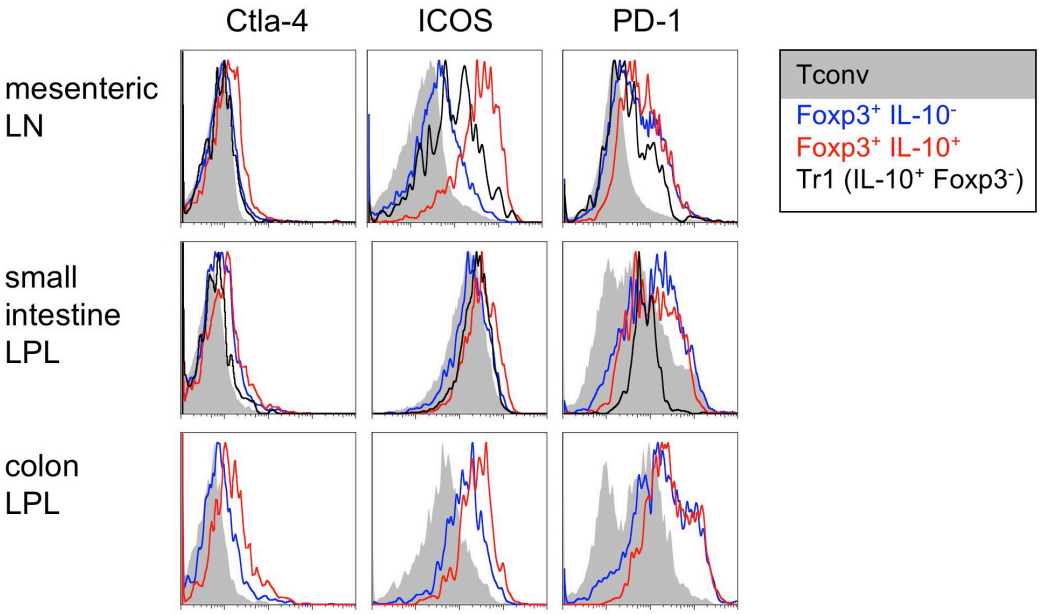

Figure 4.3. Characterisation of cell surface marker expression in IL-10-producing T cells.

Lymphocytes isolated from indicated tissues were analysed for expression of CD4, TCRβ, IL-10 (gfp) and Foxp3 (hCD2). Conventional T (T_{conv}) cells (IL-10^{gfp-}Foxp3^{hCD2-}, shaded), IL-10^{gfp-}Foxp3^{hCD2+} T_{reg} cells (blue), IL-10^{gfp+}Foxp3^{hCD2+} T_{reg} cells (red) and IL-10^{gfp+}Foxp3^{hCD2-} Tr1-like cells (black) were defined among $CD4^{+}TCR\beta^{+}$ T cells. For each cell population, expression of each of the indicated cell surface markers was determined and is shown above in representative histograms. Lymphocyte populations in the colon LP contained very few Tr1-like cells.

4.2.3 Resident bacteria promote systemic expression of IL-10 by T_{reg} cells

The factors that orchestrated the compartmentalised expression of IL-10 in T_{reg} and Tr1-like cells in the GI tract were next investigated. Two major classes of environmental factors were predicted to have the potential to promote IL-10 expression by intestinal $CD4^+$ T cells—dietary molecules and resident intestinal bacteria. The vitamin A metabolite retinoic acid is known to directly promote the induction of Foxp3 expression, but paradoxically also has the capacity to suppress IL-10 expression in effector $CD4^+$ T cells and Tr1-like cells (Maynard et al., 2009). Roles for dietary antigens in promoting IL-10 expression have not been thoroughly investigated and much remains to be learned about how the diet shapes intestinal immunity. The second factor, resident intestinal bacteria, can be ablated by treating mice with a combination of broad-spectrum antibiotics administered in the drinking water. This regimen has been reported to deplete most species of resident intestinal bacteria and to reduce the total number of bacteria by at least ten-fold after just nine days of treatment (Hill et al., 2010). After five weeks of treatment, reductions in the frequency of LP T_H1 and T_H17 effector cell populations have been observed to occur due to depletion of specific bacteria (Hill et al., 2010; Ivanov et al., 2008).

To determine whether resident intestinal bacteria promoted IL-10 expression in addition to the expression of inflammatory cytokines by $CD4^+$ T cells, *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice were treated with the broad-spectrum antibiotic cocktail for five weeks. The caecum of treated mice became enlarged, increasing five to ten times in mass compared to the caecum of control mice, and the caecal contents became dark green (data not shown). Similar effects on the caecum are seen in germ-free mice, due to the absence of bacteria that breakdown dietary components (Loesche, 1969). Strikingly, IL-10 expression by both Tr1-like cells and $Foxp3^+$ T_{reg} cells was abolished by antibiotic treatment (Figure 4.4A). This effect was not limited to the GI tract, as IL-10 expression was also significantly reduced in distal sites such as the inguinal lymph node and spleen, suggesting that intestinal bacteria profoundly affect systemic immune responses. Despite the marked diminution of IL-10

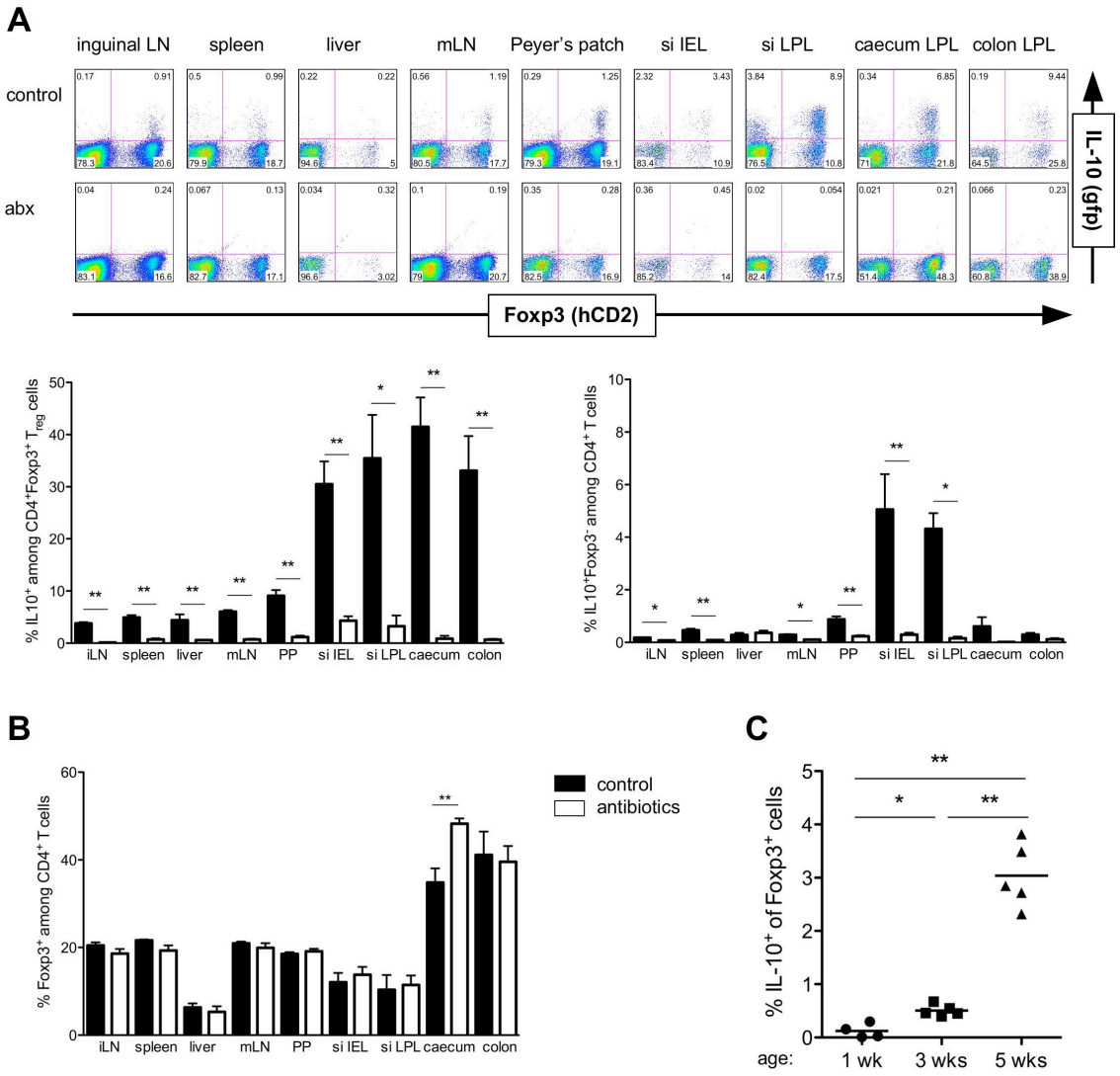

Figure 4.4. Resident intestinal bacteria induce IL-10 expression in Foxp3$^+$ T_{reg} and Tr1-like cells.

(A) To determine the effect of depleting the resident intestinal bacteria on the production of IL-10 by CD4$^+$ T cells, mice were treated with a cocktail of ampicillin (1 g/L), gentamicin (1 g/L), metronidazole (1 g/L), neomycin sulphate (1 g/L) and vancomycin (500 μg/L) in drinking water for five weeks. This regimen has been previously reported to deplete the vast majority of intestinal bacteria and results in enlargement of the caecum, similar to what can be observed in germ-free mice. Lymphocytes were isolated from the indicated tissues and CD4$^+$TCRβ$^+$ T cells were analysed for Foxp3 (hCD2) and IL-10 (gfp) expression. Representative flow cytometry plots are shown (above), along with graphs quantifying the percentage of IL-10^{gfp+} cells among total Foxp3^{hCD2+} T_{reg} cells (lower left) and IL-10^{gfp+}Foxp3^{hCD2-} Tr1-like cells among total CD4$^+$ T cells (lower right).

(B) The percentage of Foxp3^{hCD2+} T_{reg} cells among total CD4$^+$ T cells was quantified in indicated tissues of control mice and mice that received the antibiotic cocktail.

(C) To determine whether microbial colonization of mice during the neonatal period also promotes the induction of IL-10 expression in CD4$^+$ T cells, expression of IL-10 by Foxp3^{hCD2+} T_{reg} cells was measured during ontogeny. Lymphocytes were harvested from the mLN of mice at the ages indicated (1 wk = 7 days old, 3 wks = 21 days old, 5 wks = 35 days old). Each data point represents one mouse.

(A-C) Statistical differences between groups were determined using a Mann-Whitney test (n = 5; * = $p < 0.05$, ** = $p < 0.01$).

expression, the frequency of Foxp3$^+$ T_{reg} cells did not change, except in the caecum where anatomical changes are also observed (Figure 4.4B).

One side effect of the broad-spectrum antibiotic regimen is that in addition to depleting resident intestinal bacteria, it also causes mice to become temporarily dehydrated and alters normal metabolism. An alternative approach for assessing the effect of intestinal bacteria on IL-10 expression is to study mice throughout ontogeny, i.e. at different developmental time points. Foetal mice are essentially germ-free and mice are first exposed to bacteria that will colonise the intestine in the birth canal. Throughout the neonatal period, the abundance and diversity of intestinal bacteria remains low. Between days 21 to 30 of life, as mice begin to stop taking breast milk and maternally-derived IgA antibody concentrations decline, the abundance and diversity of intestinal bacteria increase dramatically (Inoue et al., 2005). Therefore, Foxp3 and IL-10 expression in mLN CD4$^+$ T cells was measured in dual-reporter mice at one, three and five weeks of age. Whereas the frequency of Foxp3$^+$ T_{reg} cells did not change in the mLN during this period (data not shown), the percentage of IL-10 producing Foxp3$^+$ T_{reg} cells increased in correlation with the kinetics of intestinal bacteria colonisation (Figure 4.4C). By five weeks of age (Figure 4.4C), IL-10 expression in Foxp3$^+$ T_{reg} cells was similar to expression in adult mice (Figure 4.1B). These two independent lines of evidence, based upon antibiotic treatment and bacterial colonisation during ontogeny, strongly suggest that resident bacteria induce IL-10 expression in Foxp3$^+$ T_{reg} cells throughout the body.

4.2.4 Intestinal IL-10 expression occurs independently of candidate inflammatory pathways

Many models of the regulation of IL-10 expression predict that an inflammatory stimulus induces the production of IL-10, which then acts to shut down or limit subsequent inflammation. Because MyD88-deficiency has been reported to prevent the development of bacteria-triggered intestinal inflammation in *Il10*$^{-/-}$ mice (Asquith et al., 2010; Rakoff-

Nahoum et al., 2006), TLR and IL-1-related cytokine signalling through MyD88 was predicted to regulate IL-10 expression in Foxp3$^+$ T_{reg} cells. Surprisingly, the frequency of IL-10$^+$ cells did not change in CD4$^+$ T cell populations from the mLN, small intestine LP and colon LP of *Myd88*$^{-/-}$ mice (Figure 4.5A). Similar results have been reported for the development of T_H17 cells, which develop in both *MyD88*$^{-/-}$*Trif*$^{-/-}$ (TLR-signalling deficient) and *Rip2*$^{-/-}$ (NLR-signalling deficient) mice (Atarashi et al., 2008; Ivanov et al., 2009; Ivanov et al., 2008). It might be that redundant, overlapping signals stimulate the innate immune system to promote IL-10 expression in Foxp3$^+$ T_{reg} cells.

Other major candidate signalling pathways for inducing IL-10 expression in intestinal CD4$^+$ T cells include those triggered by the cytokines IL-21, IL-27 and TGF-β. Conflicting data exist as to whether IL-27 induces IL-10 expression only in Tr1-like cells and effector CD4$^+$ T cells, or also in Foxp3$^+$ T_{reg} cells (Batten et al., 2008; Stumhofer et al., 2007). *In vitro* studies suggest that IL-27-signalling induces IL-21 expression, which then acts as a growth factor for IL-10-producing cells (Pot et al., 2009). To examine the relevance of this pathway in the GI tract, *Il10* mRNA expression in CD4$^+$ T cells isolated from the inguinal LN and colon LP of *Il21r*$^{-/-}$ mice was measured. Consistent with other reports that IL-10 producing effector memory CD4$^+$ T cells in the peripheral LNs are reduced in *Il21r*$^{-/-}$ mice (Pot et al., 2009), inguinal LN *Il10* expression was significantly lower in *Il21r*$^{-/-}$ CD4$^+$ T cells (Figure 4.5B). However, in the colon LP, *Il10* expression was not affected by *Il21r*-deficiency (Figure 4.5B). Thus, IL-21-independent pathway(s) control *Il10* expression in intestinal CD4$^+$ T cells.

Additional studies that used a combination of *in vitro* and *in vivo* cell transfer approaches suggested a potential role for TGF-β in the induction of IL-10 expression (Maynard et al., 2007). To investigate the role of TGF-β in the development of IL-10-producing intestinal CD4$^+$ T cells, *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice were treated with anti-TGF-β neutralising antibodies at two and three weeks of age, because IL-10 expression was

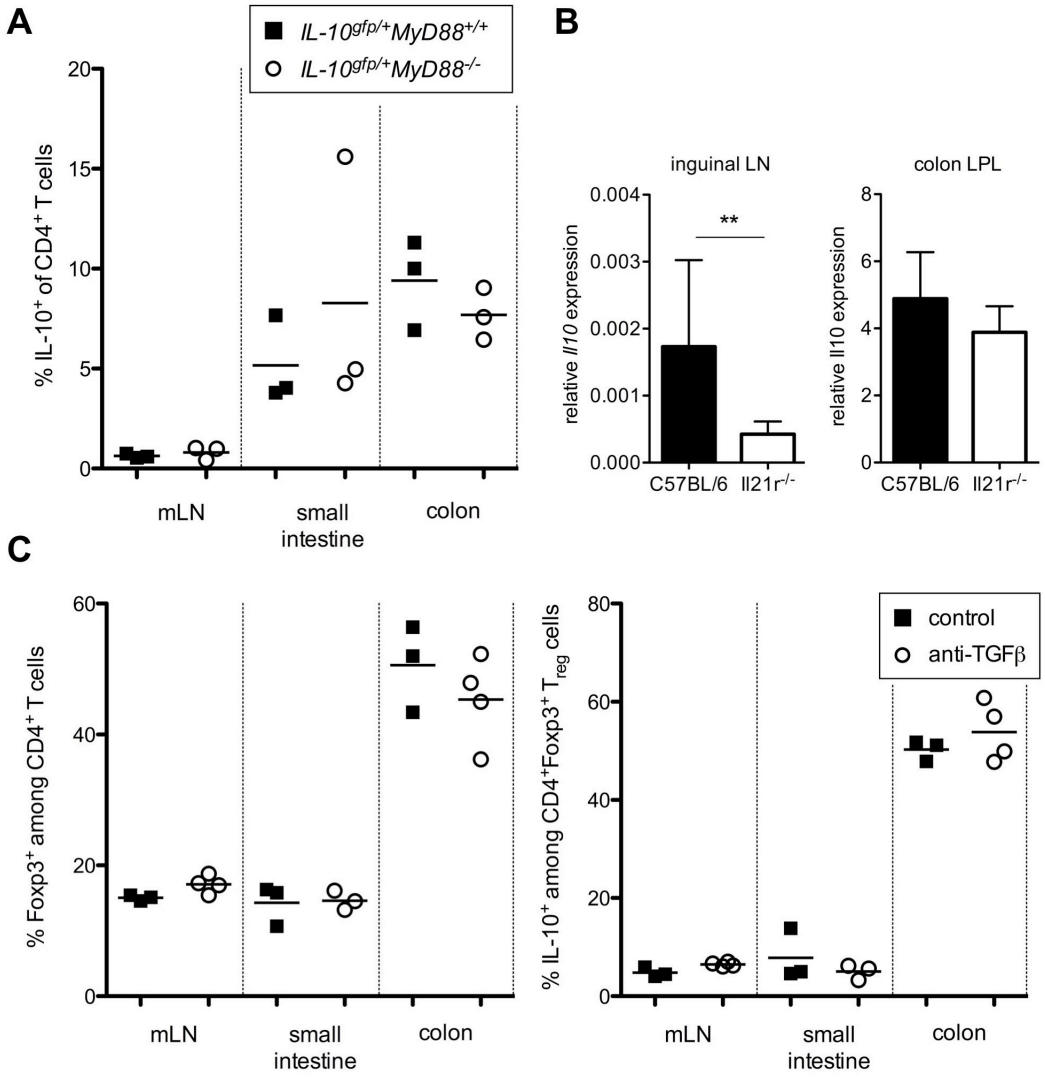

Figure 4.5. Intestinal $CD4^+IL\text{-}10^+$ T cells develop in the absence of IL-21-, MyD88- and TGF-β-dependent signalling.

(A) To determine whether intact MyD88-dependent signalling is required for intestinal IL-10-producing T cells to develop, *Il10*$^{gfp/gfp}$ and *MyD88*$^{-/-}$ mice were bred to generate *Il10*$^{gfp/+}$*MyD88*$^{-/-}$ and *Il10*$^{gfp/+}$*MyD88*$^{+/-}$ littermate control mice. Expression of IL-10 among total $CD4^+$ T cells was determined in eight-week-old mice (n = 3). Each data point represents one mouse. No statistical differences between groups were found using a Student's *t*-test.

(B) To investigate the role of IL-21 in the development of IL-10 producing intestinal T cells, $CD4^+TCR\beta^+$ T cells were isolated by sorting cells to high purity from the inguinal lymph node and colon lamina propria. Groups consisted of pooled tissues from three wild-type or *Il21r*$^{-/-}$ mice. Relative expression of *Il10* mRNA compared to *Hprt*, measured using qPCR, is shown. Error bars represent standard deviation (n = 5). Statistically significant differences between groups were determined using a Mann-Whitney test (** = $p < 0.01$).

(C) To assess the role of TGF-β signalling, two-week-old mice were injected with 1 mg anti-TGF-β monoclonal antibody twice a week for two weeks. The frequency of $Foxp3^{hCD2+}$ T_{reg} cells among total $CD4^+$ T cells (left) and of $IL\text{-}10^{gfp+}$ cells among total $Foxp3^{hCD2+}$ T_{reg} cells (right) in indicated tissues is shown. Each data point represents one mouse. No statistically significant differences were detected using a Student's *t*-test.

negligible in neonatal mice, but detectable by four weeks of age. IL-10 expression was analysed in four-week-old mice to assess whether antibody treatment impaired or enhanced the development of IL-10 producing $CD4^+$ T cells during ontogeny. Tr1-like cells were rare in four-week-old mice, so the role of TGF-β in the development of these cells was difficult to assess using this approach (data not shown). However, no differences in the percentage of $Foxp3^+$ T_{reg} cells or in IL-10-producing T_{reg} cells were observed after anti-TGF-β treatment (Figure 4.5C). Antibody treatment might be more potent in an adoptive cell transfer setting, but it should be noted that previous studies only reported a change in the percentage of IL-10^+Foxp3^+ cells among total $CD4^+$ T cells, not IL-10^+ cells among $Foxp3^+$ T_{reg} cells, as is shown here. This previous study did not account for the probable expansion of $Foxp3^-$ effector cells after anti-TGF-β treatment that would artificially reduce the frequency of IL-10^+Foxp3^+ T_{reg} cells (Maynard et al., 2007). So, IL-10 producing T_{reg} cells might develop independently of TGF-β signalling, although this finding needs to be confirmed using TGF-β1- or TGF-βRII-deficient mice to exclude the possibility that anti-TGF-β antibody treatment was inefficient.

Innate immune stimulation can induce the production of cytokines that have been shown *in vitro*, and in some *in vivo* settings, to affect IL-10 expression by $CD4^+$ T cells. One of the initial descriptions of Tr1 cells showed that exogenous IL-10 could act to differentiate and expand IL-10-producing $CD4^+$ T cells *in vitro* (Groux et al., 1997). However, when 10BiT-$Foxp3^{gfp}$ dual-reporter mice were crossed onto an *Il10*$^{-/-}$ background, no changes in the percentage of "IL-10 competent" Tr1-like or T_{reg} cells were observed (Maynard et al., 2007), although it remained possible that "IL-10 competent" cells did not translate IL-10 protein. Similar to the findings of Maynard and colleagues, no change in IL-10 expression in the GI tract was observed when neonatal *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice were administered anti-IL-10R antibody (Figure 4.6A-C), confirming that exogenous IL-10 is dispensable for the development of intestinal IL-10-producing T_{reg} cells *in vivo*. Another

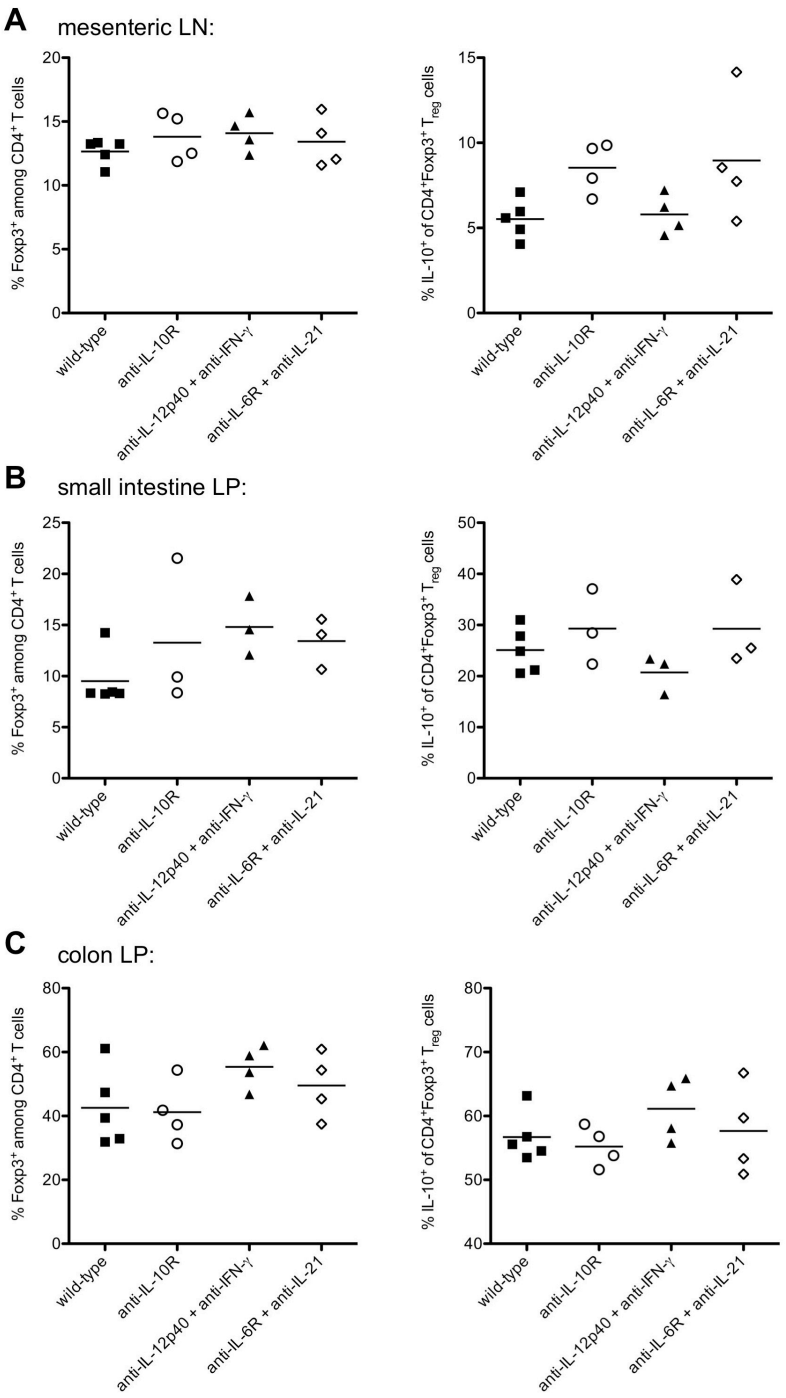

Figure 4.6. Blockade of IL-10R, T_H1 or T_H17 cytokines does not impair the development of intestinal IL-10 producing $CD4^+$ T cells.

(A-C) To test the role of candidate cytokines in the development of IL-10-producing $Foxp3^+$ T_{reg} cells, cytokines were blocked with neutralising antibodies during neonatal development. Two-week-old mice received weekly intra peritoneal injections of anti-IL-10R (1 mg), anti-IL-12p40 (2 mg) and anti-IFN-γ (2 mg), or anti-IL-6R (2 mg) and anti-IL-21 (2 mg) for two weeks. The frequency of $Foxp3^{hCD2+}$ T_{reg} cells among $CD4^+TCR\beta^+$ T cells (left) and $IL\text{-}10^{gfp+}$ cells among $Foxp3^{hCD2+}$ T_{reg} cells (right) from the (A) mLN, (B) small intestine LP or (C) colon LP of four-week-old mice is shown. No statistically significant differences were detected using a Student's *t*-test. The biological activity of each batch of antibody was confirmed in separate colitis experiments in the Powrie lab.

possibility was that both T_H1 and T_H17 cytokines, which are expressed in the intestine, promote IL-10 expression. Previous studies have shown that the T_H1 cytokine IL-12 and the T_H17 cytokine IL-6 can also promote IL-10 expression in $CD4^+$ T cells (Saraiva et al., 2009; Stumhofer et al., 2007). Concomitant blockade of the T_H1 cytokines IFN-γ and IL-12p40 (a component of both IL-12 and IL-23) or the T_H17 cytokines IL-6 (by blocking the IL-6R) and IL-21 resulted in a trend towards increased $Foxp3^+$ T_{reg} cell frequencies in the LP, as might be expected (Izcue et al., 2008), but did not affect IL-10 expression in T_{reg} cells (Figure 4.6A-C). In summary, none of the candidate regulators of IL-10 expression tested showed a non-redundant role in the intestine, where multiple overlapping pathways might operate to ensure that tolerance of resident bacteria is maintained.

4.2.5 Resident bacteria are not an essential co-factor for IL-10 production

Given the striking effect of antibiotic treatment on IL-10 expression, it was considered that resident bacteria might be required to generate an essential co-factor for IL-10 expression. Additionally, it was possible that the IL-10 induced by intestinal bacteria acted as a immunosuppressant that limited the magnitude of systemic immune responses. To test these possibilities, antibiotic treated $Il10^{gfp/+}Foxp3^{hCD2/hCD2}$ dual-reporter mice were infected with a non-lethal dose of MCMV, a virus known to induce IL-10 expression in $CD4^+$ T cells (Arens et al., 2008; Humphreys et al., 2007). Notably, $Il10^{-/-}$ mice infected with MCMV control viral replication, but succumb to fatal immunopathology due to excessive inflammation (Cheeran et al., 2007; Oakley et al., 2008). Eight days after infection of control or antibiotic treated dual-reporter mice with MCMV, robust induction of IL-10 expression in $CD4^+$ T cells compared to uninfected mice (Figure 4.1A) was could be observed in all organs analysed, but did not vary between mice that received antibiotics or not (Figure 4.7A), although it was more variable at mucosal sites of antibiotic-treated mice. A trend towards reduced IL-10 expression could be observed in the colon LP of antibiotic-treated mice (Figure 4.7A), consistent with the absence of bacteria-induced $IL\text{-}10^+Foxp3^+$ T_{reg} cells. To measure the magnitude of the effector response, specific immunodominant MHC class I epitopes have

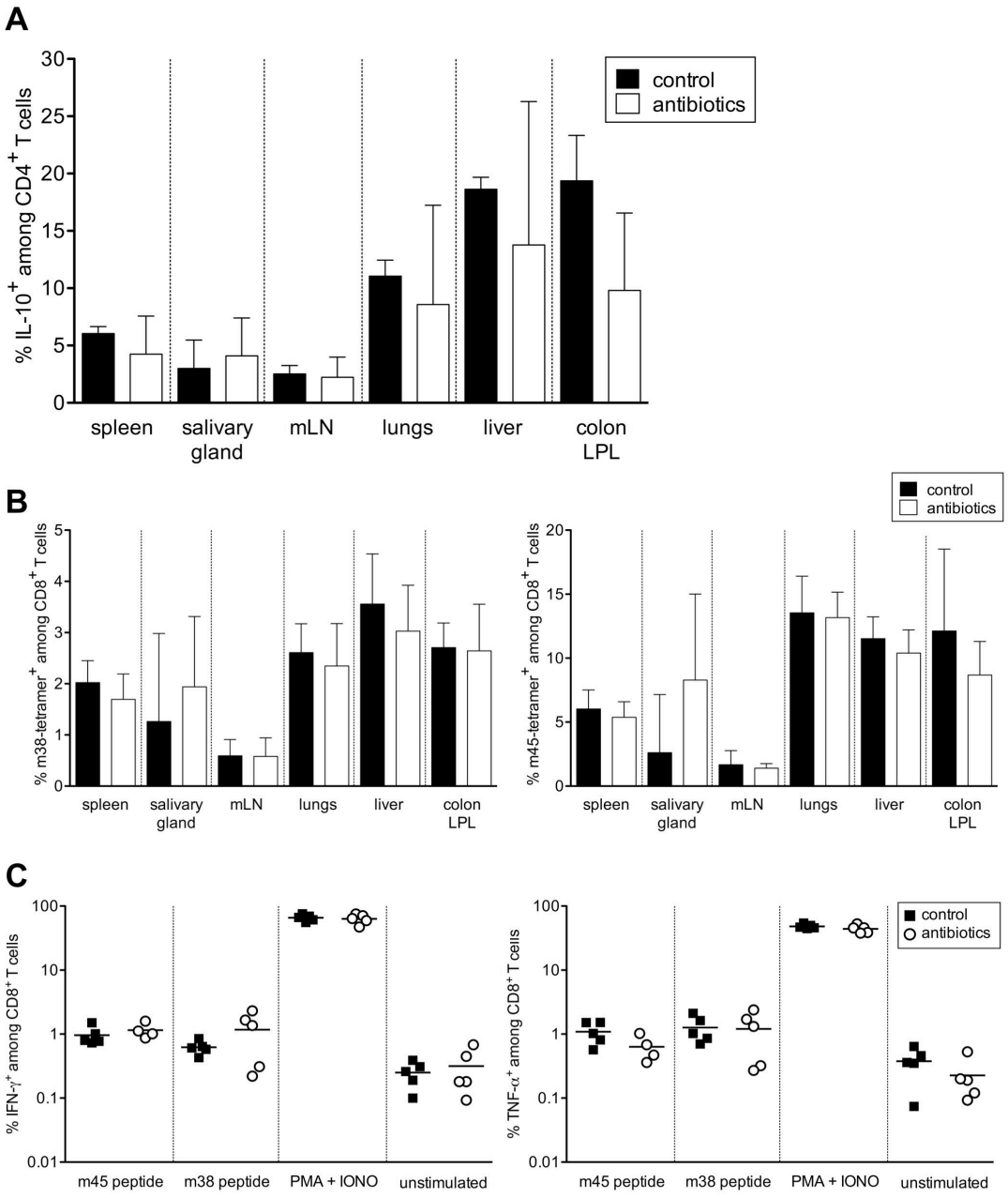

Figure 4.7. Resident bacteria are not required for IL-10 expression during MCMV infection.

(A-C) To investigate the possibility that depletion of resident bacteria would affect systemic immune responses, mice were treated with a cocktail of ampicillin (1 g/L), gentamicin (1 g/L), metronidazole (1 g/L), neomycin sulphate (1 g/L) and vancomycin (500 μg/L) in drinking water for five weeks. *Il10*$^{gfp/+}$ mice that were treated with antibiotics or not were infected with 1 x 10^5 pfu MCMV *intra peritoneally*. The group of mice that received antibiotics was maintained on antibiotics throughout the course of infection. Eight days later, lymphocytes were harvested from indicated tissues and (A) IL-10 expression among $CD4^+$ T cells was measured, as was (B) the frequency of MCMV-specific m38-tetramer$^+$ (left) or m45-tetramer$^+$ (right) cells among $CD8^+$ T cells. (C) Lymphocytes from the mLN were restimulated with m45 peptide, m38 peptide or PMA and Ionomycin for six hours, then were analysed for intracellular IFN-γ (left) and TNF-α (right) expression in $CD8^+$ T cells. IL-17A expression was also analysed, but was expressed by less than 1% of total $CD4^+$ or $CD8^+$ T cells after PMA and Ionomycin stimulation.

been identified that allow the $CD8^+$ T cell response to MCMV to be tracked using tetramer staining and peptide restimulation (Karrer et al., 2003; Munks et al., 2006). In antibiotic treated and control mice, the effector $CD8^+$ T cell response was measured by quantifying $m38^+$ and $m45^+$ MCMV-tetramer specific cells in different organs (Figure 4.7B) and by assessing intracellular IFN-γ and TNF-α production in cells in $CD8^+$ T cells from the mLN that were restimulated with m38- or m45-peptide (Figure 4.7C). Although more variation in the frequency of MCMV-specific $CD8^+$ T cells could be seen in the salivary glands of antibiotic-treated mice (Figure 4.7B), again no significant differences were observed between mice that received antibiotics or not. These data suggest that intestinal bacteria are not required for IL-10 production during viral infection and do not inhibit the systemic immune response to MCMV. It remains possible that in chronic disease models, such as collagen-induced arthritis, resident bacteria-driven production of IL-10 might limit disease similar to the way SFB can promote T_H17 responses that exacerbate disease (Wu et al., 2010).

4.2.6 *Helicobacter hepaticus* induces IL-10 expression in Tr1-like and T_{reg} cells

Finally, the effect of changing the composition of the resident intestinal bacteria was explored. *H. hepaticus* is a gram-negative, flagellated bacterium that predominantly colonises the caecum of mice, but can also be found in the colon, bile duct and liver (Fox et al., 1994). It is closely related to *Helicobacter pylori*, which promotes gastric ulcers and stomach cancer in humans (Marshall, 1995). Importantly, *H. hepaticus* colonisation of *Il10*$^{-/-}$ mice has been shown to break tolerance of resident bacteria and trigger intestinal inflammation (Kullberg et al., 1998), whereas wild-type mice do not develop immunopathology after colonisation. Based on the data presented here, it was hypothesised that IL-10 production driven by the presence of other resident intestinal bacteria could be sufficient to protect a wild-type mouse from intestinal inflammation. Alternatively, *H. hepaticus* might induce additional sources of IL-10 that act to maintain intestinal homeostasis.

To distinguish between these possibilities, adult *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice were colonised with *H. hepaticus*. By two weeks after colonisation, a slight increase in the frequency of Foxp3$^+$ T_{reg} cells in the caecum could be observed (Figure 4.8A), and a ~ 50% increase in the frequency of IL-10$^+$ cells among Foxp3$^+$ T_{reg} cells compared to uninfected controls could be observed at the site of colonisation, the caecum and colon (Figure 4.8B). Additionally, the induction of a population of Tr1-like cells could be detected in the caecum and colon (Figure 4.8C). Although CD4$^+$ T cells remained the only IL-10^{high} population, some CD11b$^+$ myeloid cells also produced low amounts of IL-10 after colonisation (data not shown). Interestingly, the amount of per-cell IL-10^{gfp} expression was highest in Tr1-like and Foxp3$^+$ T_{reg} cells in the caecum (Figure 4.8D), where *H. hepaticus* is most abundant, suggesting that this bacterium acts locally to induce a strong IL-10 response by CD4$^+$ T cells at the site of colonisation.

An important, early study in T_{reg} cell biology showed that CD4$^+$CD45RB^{low}CD25$^-$ T cells from the mLN acquired IL-10-dependent regulatory capacity when mice were colonised with *H. hepaticus* (Kullberg et al., 2002). However, it was not known whether these cells represented activated Foxp3$^+$ T_{reg} cells or IL-10-producing Foxp3$^-$ Tr1-like cells. To distinguish between these possibilities, a modified IL-10-dependent version of the T-cell-transfer colitis model was employed. *Rag2*$^{-/-}$ mice on the 129.SvEv genetic background become colitic when infected with *H. hepaticus* (Li et al., 1998; Ward et al., 1996). If T_{reg} cells that produce IL-10 are adoptively transferred into *H. hepaticus*-induced 129.SvEv *Rag2*$^{-/-}$ mice, intestinal inflammation can be prevented or cured (Maloy et al., 2005; Maloy et al., 2003). To make the requirement for regulatory function more stringent (Cahill et al., 1997), naïve, colitogenic CD4$^+$CD45RB^{high}CD25$^-$ naïve T cells were co-transferred along with potential regulatory T cell populations into *H. hepaticus*-infected 129.SvEv *Rag2*$^{-/-}$ recipient mice at a four to one ratio. In this system, only populations with superior regulatory activity would prevent systemic disease (i.e. splenomegaly or weight loss) or intestinal inflammation.

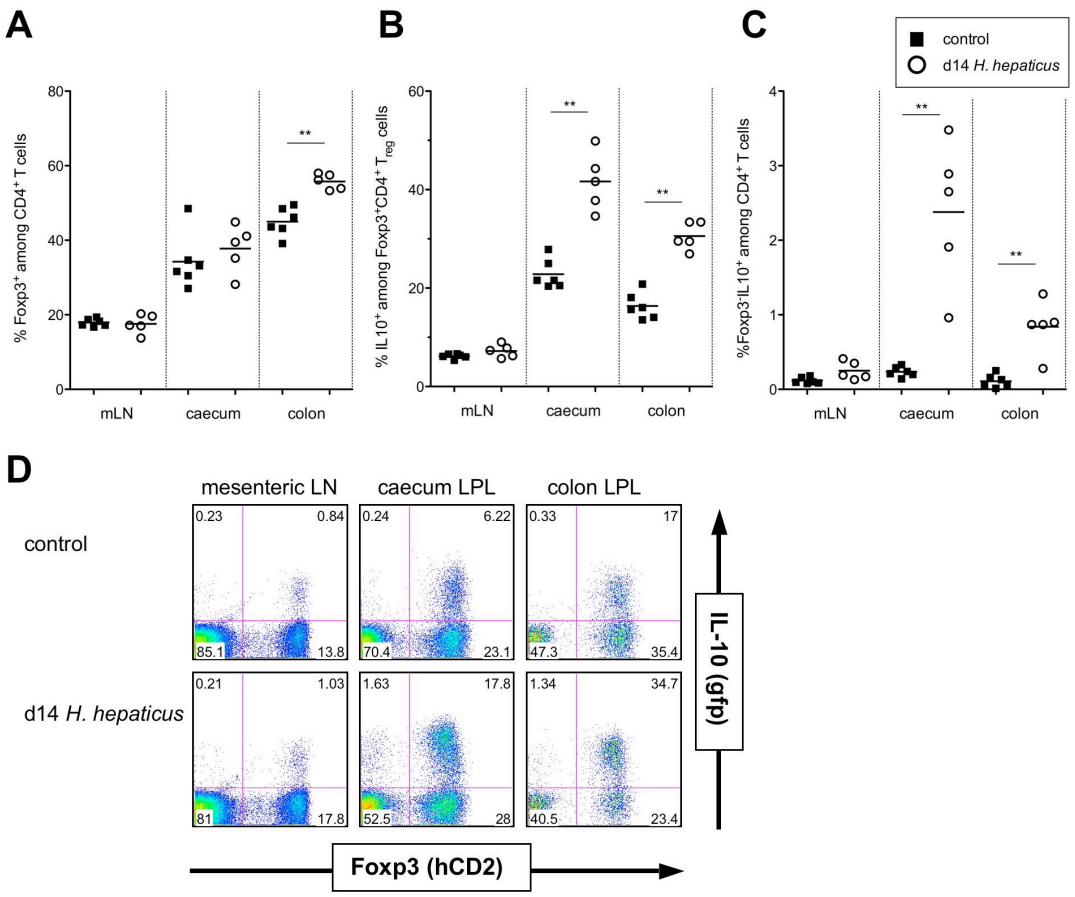

Figure 4.8. *Helicobacter hepaticus* colonisation induces IL-10 expression by Tr1-like and T_{reg} cells.

(A-C) To determine the effect of colonisation with *H. hepaticus* on IL-10 expression, *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice were fed 1 x 10^8 bacteria daily by oral gavage for three consecutive days. Fourteen days after the first feeding, the frequency of (A) Foxp3^{hCD2+} T_{reg} cells among total CD4$^+$ T cells, (B) IL-10^{gfp+} cells among Foxp3^{hCD2+} T_{reg} cells and (C) IL-10^{gfp+}Foxp3^{hCD2-} Tr1-like cells among total CD4$^+$ T cells was determined in lymphocytes isolated from indicated tissues. Each data point represents one mouse. Statistically significant differences between colonised and control groups were determined using a Mann-Whitney test (* = $p < 0.05$, ** = $p < 0.01$).

(D) Representative flow cytometry plots from data presented above (A-C) are shown.

To determine the functional roles of Foxp3$^+$ T_{reg} and Tr1-like cells in this system, a different reporter mouse that expresses a Foxp3-gfp fusion protein (Fontenot et al., 2005b) on the 129.SvEv genetic background was used. If donor Foxp3$^+$ T_{reg} cells ($CD4^+CD45RB^{low}Foxp3^{gfp+}$) or a population that could contain Tr1-like cells ($CD4^+CD45RB^{low}Foxp3^{gfp-}$) were taken from the mLN of a *Helicobacter*-free mouse, no significant amelioration of intestinal inflammation was observed eight weeks after these T cell populations were co-transferred with naïve CD4$^+$ T cells into *H. hepaticus*-infected 129.SvEv *Rag2*$^{-/-}$ mice (Figure 4.9A-D). By contrast, when these cell populations were isolated from reporter mice that had been colonised with *H. hepaticus* five weeks previously, Foxp3$^+$ T_{reg} cells significantly ameliorated colitis and the Tr1-like cell-containing population limited both colitis and typhlitis (inflammation of the caecum) (Figure 4.9A-D). These results suggest that *H. hepaticus* can activate Foxp3$^+$ T_{reg} cells and induce a population of Tr1-like cells, which are both capable of preventing intestinal inflammation.

Colitis induced by naïve T-cell-transfer into Rag-deficient mice or *H. hepaticus* colonisation of 129.SvEv *Rag2*$^{-/-}$ mice causes systemic inflammatory responses in addition to intestinal inflammation. Splenomegaly in these disease models is caused by the accumulation of T cells and Gr1$^+$ myeloid cells (Hue et al., 2006). Foxp3$^+$ T_{reg} cells from *H. hepaticus* colonised mice significantly limited splenomegaly, but Tr1-like cells were less potent in regulating systemic disease (Figure 4.10A). Liver inflammation also occurred in *H. hepaticus* mice that received naïve T cells, and this was similarly prevented by Foxp3$^+$ T_{reg} cells from *H. hepaticus* colonised mice but not Tr1-like cells (Figure 4.10B-C). These findings are consistent with the well-appreciated role for Foxp3$^+$ T_{reg} cells in preventing systemic inflammation (Sakaguchi et al., 2008), and also suggest that Tr1-like cells might act locally within certain tissues. Further studies will be needed to determine whether the tissue-specific actions of Tr1-like cells reflect their recognition of intestine-specific antigens or preferential migration patterns that cause their localisation to the GI tract.

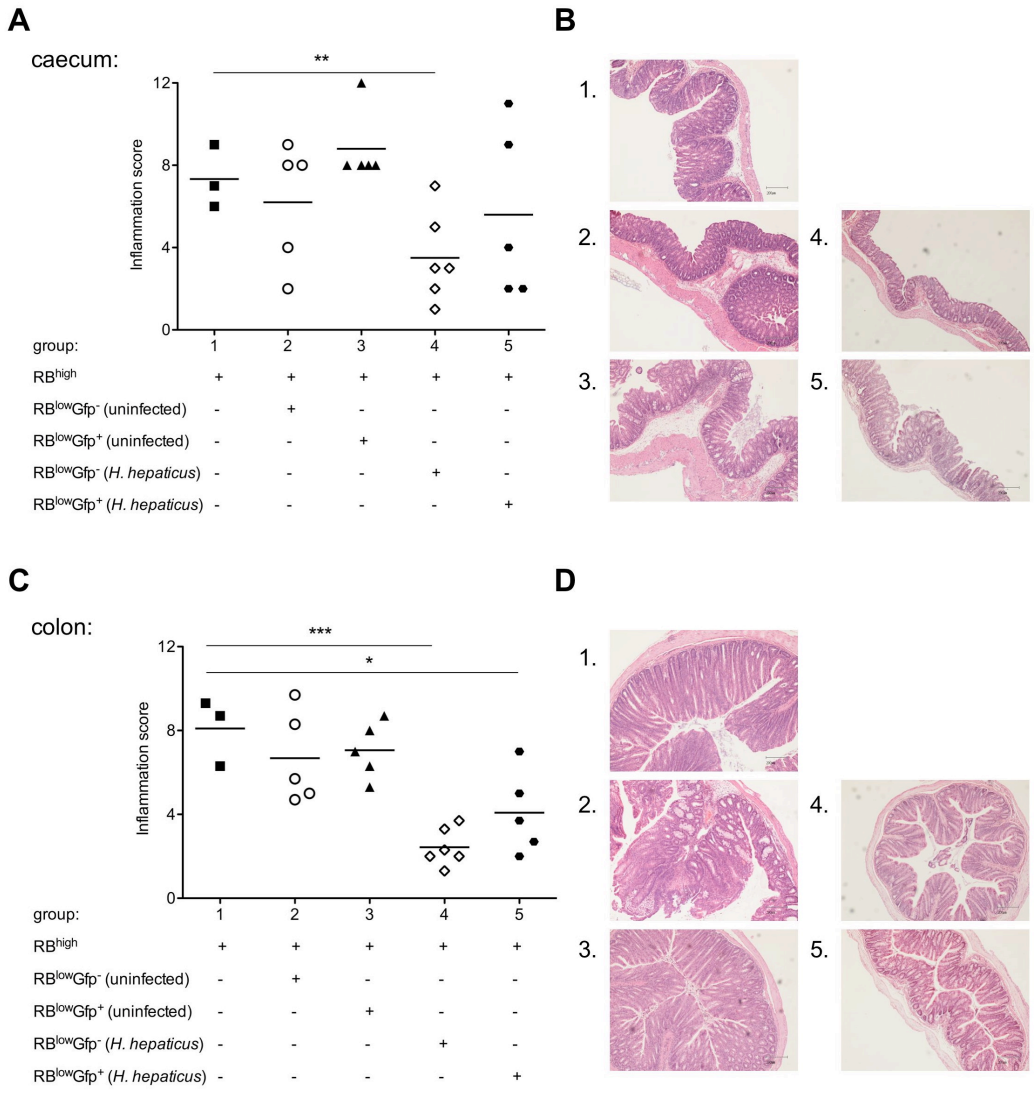

Figure 4.9. *H. hepaticus* induced Tr1-like and T_{reg} cells suppress T-cell-transfer colitis.

(A-D) To compare the functional potency of *H. hepaticus*-induced Tr1-like cells and $Foxp3^+$ T_{reg} cells, *129.Foxp3*$^{gfp/gfp}$ reporter mice were fed 1 x 10^8 *H. hepaticus* daily by oral gavage for three consecutive days. Five weeks later, mLN cells from uninfected or *H. hepaticus* colonised reporter mice were harvested. $CD4^+TCR\beta^+CD45RB^{low}$ T cells were sorted into $Foxp3^{gfp+}$ and $Foxp3^{gfp-}$ populations. 1 x 10^5 of one of these cell populations were co-injected with 4 x 10^5 naïve $CD4^+TCR\beta^+CD45RB^{high}$ T cells into *129.Rag2*$^{-/-}$ mice that had been fed *H. hepaticus* one week previously. Eight weeks later the experiment was terminated and inflammation was assessed in each mouse.

(A-B) Sections from caecal tissues were stained with H & E and (A) slides were scored using a standardised system for assessing caecal inflammation. (B) Representative histology from each experimental group is depicted.

(C-D) Sections from the proximal, mid and distal colon were stained with H & E and (C) slides were scored using a standardised system for assessing colonic inflammation. (D) Representative histology from the mid-colon of a mouse from each experimental group is depicted.

(A, C) Statistically significant differences between groups were determined using a Student's *t*-Test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Each data point represents one mouse.

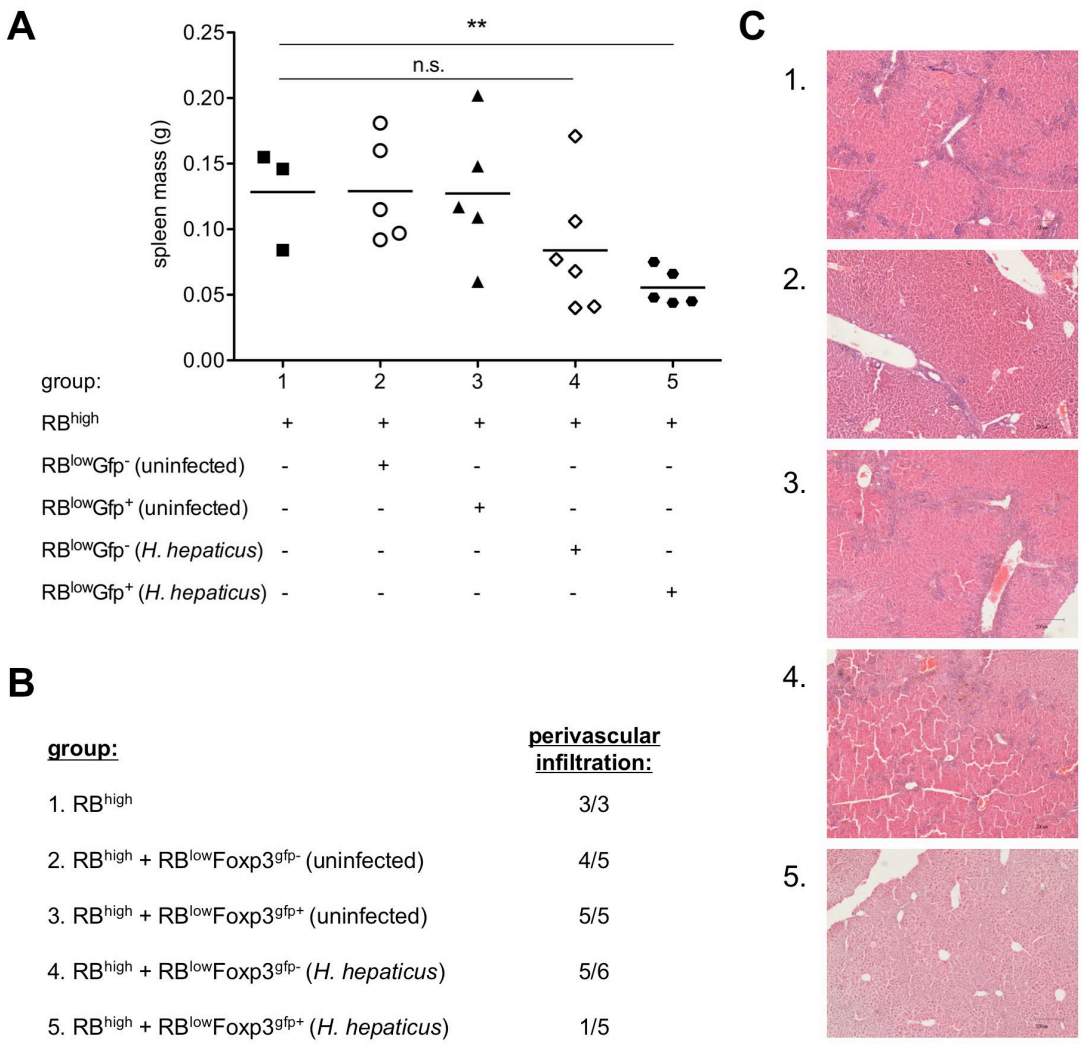

Figure 4.10. *H. hepaticus* induced T_{reg} cells potently suppress systemic inflammation.

(A) To assess an indicator of systemic inflammation, splenomegaly, spleens from *H. hepaticus*-infected *129.Rag2*$^{-/-}$ mice that had been injected with the indicated CD4^{+} T cell populations eight weeks previously (see legend for Figure 4.9) were weighed. Statistically significant differences between groups were determined using a Student's *t*-test.

(B-C) In the same experimental groups, liver sections were analysed for the presence of absence of perivascular infiltrates. (B) Scoring was done in a binary fashion and a liver scored positive if mononuclear cell infiltrates could be observed around two or more venules. (C) Representative H & E stained sections from each experimental group are depicted.

4.3 Discussion

Recent findings that the presence or absence of certain species of resident intestinal bacteria can influence the pathogenesis of autoimmune diseases have captured the attention of immunologists. Using germ-free mice and mice colonised with a limited number of bacterial species, certain intestinal microbes, such as segmented filamentous bacteria, have been shown to exacerbate mouse models of inflammatory bowel disease (Ivanov et al., 2009; Stepankova et al., 2007) and rheumatoid arthritis (Wu et al., 2010). Also, undefined species in the intestinal microbiota can affect the pathogenesis of models of asthma (Blaser and Falkow, 2009; Strickland et al., 2010), Type I diabetes (Wen et al., 2008), multiple sclerosis (in the EAE disease model) (Lee et al., 2010b) and obesity (Turnbaugh et al., 2006). The studies presented here highlight the possibility that another class of intestinal bacteria exists that promotes immune regulation via the induction of IL-10 in $CD4^+$ T cells. Studies of the human intestinal bacterium *Bacteroides fragilis* demonstrated that a carbohydrate, PSA, expressed by this species could enhance IL-10 production by $CD4^+$ T cells (Mazmanian et al., 2008; Round and Mazmanian, 2010). Since the microbiota of mice does not contain this species, the results presented here imply that other species of bacteria must also promote IL-10 expression. Identifying these species and the mechanism by which they induce IL-10 expression may allow this regulatory axis to be manipulated experimentally or perhaps therapeutically. To this end, studies using more selective, single-antibiotic treatments to identify classes of intestinal bacteria (i.e. gram-negative, gram-positive, anaerobic, etc.) that promote IL-10 expression by $CD4^+$ T cells are in progress.

Two published studies have characterised IL-10 expression in germ-free mice. Consistent with the findings reported here, *Il10* mRNA abundance is reduced in colon tissue sections from germ-free mice (Round and Mazmanian, 2010). However, when $CD4^+$ T cells from germ-free mice were restimulated with PMA and ionomycin, only a two-fold reduction in the frequency of $IL\text{-}10^+$ $CD4^+$ T cells could be observed by intracellular cytokine staining

(Gaboriau-Routhiau et al., 2009; Round and Mazmanian, 2010). In contrast, IL-10 expression was totally abrogated by broad-spectrum antibiotic treatment in *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice; it will be important to restimulate CD4$^+$ T cells from these mice in order to test whether restimulation can restore IL-10 expression. If this is the case, it would suggest that a population of CD4$^+$ T cells 'poised' to secrete IL-10 exist in the absence of microbes, but a resident intestinal bacteria-derived signal or antigen is needed to 'trigger' IL-10 expression. At a molecular level, this could mean that 'poised' cells have epigenetic modifications at the *Il10* locus that facilitate transcription, but that a 'trigger' is needed to mobilise relevant transcription factors. Similar modifications control IFN-γ expression by effector T cells and NK cells, and IL-4 expression by T_H2 cells and *i*NKT cells (Wilson et al., 2009).

Alternatively, the germ-free and broad-spectrum antibiotic treatment scenarios may be qualitatively different. In germ-free mice, normal development of the lymphoid architecture in the gut and in 'systemic' organs like the spleen does not occur (Gordon et al., 1997), and an order of magnitude fewer CD4$^+$ T cells populate the small intestine and colon LP (Umesaki et al., 1993). Because CD4$^+$ T cells develop in these mice without exposure to bacterial antigens, they might be skewed towards recognition of dietary antigens. In antibiotic treated mice, T cell development proceeds normally and the microbial stimulus is only removed after normal cohorts of lymphocytes populate the small intestine and colon LP. Determining the antigen specificity of IL-10-producing Foxp3$^+$ T_{reg} and Tr1-like cells is far beyond the scope of this study, but remains an important goal that would significantly aid our understanding of how intestinal homeostasis is maintained. For example, it could be that Tr1 cells develop after repeated exposure to dietary or food antigens to actively prevent intestinal inflammation, whereas Foxp3$^+$ T_{reg} cells might produce IL-10 in response to innate stimuli found in the intestine or could represent induced T_{reg} cells that also recognize gut antigens.

By assessing TCR-V_β chain usage by $CD4^+$ T cells in the colon LP, a few insights into the development and specificity of IL-10^+Foxp3^+ T_{reg} cells can be gained. First, in the context of a normal complement of resident intestinal bacteria, expression of IL-10 occurs among Foxp3^+ T_{reg} cells that express many different TCR-V_β chains. Yet, it is not clear whether a large number of antigens promote IL-10 expression, or, perhaps, a soluble signal directly induces IL-10 expression, similar to the way IL-1β can induce IL-17 expression by γδ T cells independently of TCR stimulation (Sutton et al., 2009). Future studies using germ-free *Il10*$^{gfp/+}$*Foxp3*$^{hCD2/hCD2}$ dual-reporter mice should be informative in this regard. For example, if a dual-reporter mouse was monocolonised with a bacterial species that promoted IL-10 expression in Foxp3^+ T_{reg} cells, analysis of TCR-V_β chain usage should show greater heterogeneity if bacterial antigens promoted IL-10 expression, because the exogenous antigen profile in the gut would be reduced from thousands of bacterial species to a single bacterium plus dietary antigens. Or, if a soluble factor promoted IL-10 expression independently of bacterial antigen recognition, a TCR-V_β chain usage pattern similar to what is reported here would be expected.

A second, unexpected finding was the reduced accumulation of TCR-$V_\beta14^+$ Foxp3^+ T_{reg} cells in the colon LP, and the reduced frequency of IL-10^+ cells within this population. Although others have investigated these cells, previous studies have either focused on the recombination events that form a functional TCR allele in the thymus (Glusman et al., 2001; Ranganath et al., 2008; Senoo and Shinkai, 1998) or the accumulation of TCR-$V_\beta14^+$ effector cells in the periphery (Kai et al., 2005; Kanetake et al., 2003). Ongoing studies seek to determine whether TCR-$V_\beta4^+$, 11^+ or 14^+ cells have a propensity to become a regulatory, IL-10-producing cell or an effector cell using the T-cell-transfer model of colitis. If TCR-V_β chain expression influences eventual $CD4^+$ T cell effector fate, it would suggest that the functional properties of $CD4^+$ T cells begin to be imprinted at a far earlier stage in their development than was expected. Tr1-like cells in the small intestine LP remain to be

characterised in this system and it will be of interest to investigate if their development requires the same bacteria that induce IL-10^{+}Foxp3^{+} T_{reg} cells, and whether the TCR-V_{β} chain usage by this T cell subset is similarly broad.

Apart from TCR-dependent recognition of intestinal antigens, it was predicted that cytokines would also affect IL-10 expression by CD4^{+} T cells. In addition to IL-10-producing regulatory T cells, the intestine includes a large population of T_H1 cells as well as a smaller population of T_H17 cells (Atarashi et al., 2008; Ivanov et al., 2008). One hypothesis was that effector cytokines associated with these cell types could be 'sensed' by Foxp3^{+} T_{reg} cells, which would respond by expressing IL-10. However, it appears that additional pathways regulate the development of IL-10-producing Foxp3^{+} T_{reg} cells, and it should be noted that role of the Stat5-activating cytokines IL-2 and IL-15 remains to be tested and that IL-27 neutralising antibodies are not readily available. Also, the role of TGF-β needs to be further investigated because neutralising antibodies for this cytokine are less effective than for the other anti-interleukin antibodies used here (Carolina Arancibia and Fiona Powrie, unpublished observations).

Developing an *in vitro* system for inducing IL-10 expression in Foxp3^{+} T_{reg} cells, perhaps in response to TGF-β and IL-27, would also be a useful tool. For example, serum from wild-type or antibiotic treated mice could be used in place of FCS to investigate whether soluble factors circulate the serum that might promote IL-10 responses by Foxp3^{+} T_{reg} cells. The influence of the Akt→PI3K→mTor and TCR→Carma1→c-Rel signalling axes, which are important for regulating Foxp3 expression (Feuerer et al., 2009), on IL-10 expression will also be of interest. Co-culture systems using intestinal DCs or macrophages, from control or antibiotic treated mice, and IL-10^{-}Foxp3^{+} T_{reg} cells will be useful way to investigate whether a dedicated antigen presenting cell type promotes IL-10 expression, although ongoing studies have shown that antibiotic treatment has a minimal effect on costimulatory molecule

expression and retinoic acid production in myeloid cells (Andrew Johnson and Fiona Powrie, unpublished observations).

Further insights into the signals that Foxp3$^+$ T_{reg} cells receive in the intestine can be gained by comparing genome-wide transcriptional profiles of Foxp3$^+$ T_{reg} cells isolated from the spleen or colon LP. According to publicly available data sets [(Feuerer et al., 2010); http://cbdm.hms.harvard.edu/TregSubphenotypes/heatmap.html], mRNA transcripts encoding the IL-10-associated transcription factors *Irf1*, *cMaf*, *Stat1* and *Stat5*, as well as *Ahr* and *Prdm1* (which encodes Blimp-1), are selectively enriched in the colon LP. However, a large number of transcripts differ between these two tissues. One simple way to test whether these receptors and transcription factors regulate IL-10 expression *in vivo* would be to measure *Il10* mRNA abundance in CD4$^+$ T cells from knockout mice, as was done for *Il21r*$^{-/-}$ mice. Additionally, using whole-genome microarrays, comparisons of sorted IL-10$^+$ versus IL-10$^-$ Foxp3$^+$ T_{reg} cells and Tr1-like cells from the LP would provide a higher resolution picture of the genes specifically enriched in IL-10-producing T cells.

H. hepaticus colonisation offers another system for manipulating IL-10 expression in Foxp3$^+$ T_{reg} cells, and to study the induction of Tr1-like cells in the caecum and colon. Notably, most experimental mouse facilities have endemic *Helicobacter* in animal colonies, whereas the studies presented here were carried out in a *Helicobacter*-free facility. The presence or absence of *Helicobacter* could account for the differences in Tr1-like cell populations reported in this study and by others (Maynard et al., 2007; Maynard et al., 2009). The induction of Tr1-like cells by *H. hepaticus* is also consistent with the phenotype of *Helicobacter* infected *Foxp3*$^{Cre/+}$*Il10*$^{flox/flox}$ mice that selectively lack IL-10 expression in Foxp3$^+$ T_{reg} cells (Rubtsov et al., 2008). These mice have less severe disease than *CD4*$^{Cre/+}$*Il10*$^{flox/flox}$ mice (Roers et al., 2004). Together with data presented here, these results suggest that Tr1-like cells limit intestinal inflammation in the absence of Foxp3$^+$ T_{reg} cell-derived IL-10, but are not on their own sufficient to maintain intestinal homeostasis. The

dual-reporter mouse offers the additional opportunity to examine where IL-10-producing Tr1-like and Foxp3$^+$ T_{reg} cells localise *in situ*, by immunoflourescence, which might help to explain the different functional roles of these two cell types. Finally, it will be interesting to examine whether MyD88 and inflammatory T_H1 and T_H17 associated cytokines are involved in this system, or whether *H. hepaticus* actively induces IL-10 responses to promote its tolerance independently of inflammation, via tolerogenic Type VI secretion systems (Chow and Mazmanian, 2010) or another mechanism. Considering the stable association of *Helicobacter* species with vertebrates over at least many thousands of years of evolution (Eppinger et al., 2006; Linz et al., 2007), *Helicobacter spp.* could be expected to benefit the host in certain contexts. For example, *Helicobacter*-induced IL-10$^+$ CD4$^+$ T cells might mitigate immunopathology caused by gastrointestinal pathogens that establish chronic infections, which were extremely common in humans before the introduction of sanitation and antibiotics. In contemporary Western societies, *Helicobacter* colonisation of humans has become less common. Testing the influence of *Helicobacter* colonisation in mouse models of 'modern' diseases, such as asthma, diabetes, multiple sclerosis or obesity, also merits investigation.

Chapter V. Gimap5 regulates the survival and proliferation of lymphocytes

5.1 Introduction

In addition to Foxp3$^+$ T_{reg} cells that inhibit immunopathology in *trans*, cell-intrinsic mechanisms also operate within lymphocytes to help maintain self-tolerance. Given the potential of T and B cells to undergo clonal expansion and survive for many weeks, a series of checkpoints exist to limit the survival of auto-reactive clones (Goodnow, 2007). Thymocytes that recognize self-antigens, promiscuously displayed by *Aire*-expressing medullary thymic epithelial cells, die by apoptosis in a process termed negative selection (Palmer, 2003). This process is not completely effective and some self-reactive clones can be found in the peripheral lymphoid organs (Mueller, 2010). The availability of common γ-chain cytokines limits the size and composition of the T cell niche during health, and allows for T cell expansion during infection when innate and adaptive immune stimulation causes the concentrations of these cytokines to increase (Schluns and Lefrancois, 2003; Surh and Sprent, 2008). Notably, IL-2, IL-7 and IL-15 act to promote T cell survival by modulating the expression of Bcl-2-family member proteins (Ma et al., 2006; Marrack and Kappler, 2004). In the bone marrow, B cells are selected for expression of BCRs that do not recognise self-antigen (Goodnow, 2007). Mature B cells populate the bone marrow and peripheral lymphoid organs where their survival requires the cytokine BAFF (Mackay et al., 2010). NK cells also develop in the bone marrow and are 'educated' to remain tolerant of self-tissues, but to become activated upon encountering cells that express surface markers characteristic of stress, DNA damage or intracellular infection (Orr and Lanier, 2010). The peripheral survival of NK cells requires the cytokine IL-15 (Kennedy et al., 2000).

Mutation or deletion of genes involved in lymphocyte apoptosis also alters the size of the peripheral lymphocyte niche. One important apoptotic pathway is triggered when the death receptor, Fas/CD95, binds its ligand, FasL/CD95L (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). Loss of either Fas or FasL causes lupus-like autoimmunity in mice on some

genetic backgrounds (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992) and in humans with mutations in either gene (Fisher et al., 1995; Rieux-Laucat et al., 1995). Other death and survival signals act upon a cell-intrinsic pathway that is regulated by the Bcl-2-family member proteins, which are intimately associated with triggering or inhibiting mitochondrial associated effector-caspase activation (Adams and Cory, 1998). Combined deficiency in the cell-intrinsic and -extrinsic pathways, in *Bim*$^{-/-}$*Fas*$^{lpr/lpr}$ mice, causes severe lymphoproliferation and autoimmune disease (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008), whereas deletion of the survival regulator common to both pathways, *Bcl-2*, causes the progressive death of all lymphocytes (Nakayama et al., 1993). Furthermore, cellular 'housekeeping' functions, such as cytoskeletal organisation and autophagy, must be intact in order to maintain peripheral lymphocyte populations (Pua et al., 2007; Shiow et al., 2008; Snapper and Rosen, 1999).

Homozygosity for the *lyp* mutation in rats has been known to cause severe T cell lymphopaenia by a poorly characterised mechanism and has been shown to predispose rats to the development of autoimmune diabetes (Ramanathan and Poussier, 2001) and intestinal inflammation (Cousins et al., 2006). However, lymphopaenia caused by the *lyp* mutation alone is insufficient to trigger immunopathology, which requires the presence of additional modifier alleles at other loci in the genome (Cousins et al., 2006; Jackson et al., 1984; Jacob et al., 1992; Moralejo et al., 2003; Wallis et al., 2009). In both susceptible and disease-free genetic backgrounds, homozygous *lyp* rats largely lack $CD8^+$ T cells and have reduced $CD4^+$ T cell and NKR-$P1^+$ NKT cell numbers (Elder and Maclaren, 1983; Iwakoshi et al., 1999; Moralejo et al., 2003), whereas deficiencies in conventional B cells have not been reported (Tullin et al., 1997). Positional cloning efforts identified the *lyp* mutation as a frameshift mutation in the gene *GTPase of the immunity associated protein 5* [*Gimap5*, also known as *Immune-associated nucleotide binding protein 4* (*Ian4*), *Ian5* or *inhibitor of radiation- and okadaic acid-induced apoptosis* (*Irod*)] (MacMurray et al., 2002).

The *Gimap* genes comprise a family of genes that are physically clustered in the genome and predominantly expressed in lymphocytes (Nitta et al., 2006). All Gimap proteins share a GTP-binding AIG1 homology domain (Poirier et al., 1999), which was originally identified as an important component of anti-bacterial immune responses in plants (Reuber and Ausubel, 1996). Expression of *Gimap5* has been reported in mammalian NK cell, $CD4^+$ T cells, $CD8^+$ T cells, monocytes, hematopoietic stem cells (HSCs) and common lymphocyte precursors (www.biogps.org; Ng et al., 2009). *Gimap5* is also expressed in mouse B cells and mast cells, however this transcript does not appear to be expressed in the equivalent human cell types. The Gimap proteins contain several conserved domains that remain poorly functionally characterised. In Gimap5, this includes a putative GTP-binding domain, an IAN motif, a coiled-coiled domain and a predicted hydrophobic region. A recent study suggests that Gimap5 is a 35 kD protein that is localised to the lysosome and multivesicular bodies in Jurkat T cells (Wong et al., 2010).

In mammals, Gimap1, Gimap3 and Gimap5 have been reported to promote both thymocyte and T cell survival (Hernández-Hoyos et al., 1999; Nitta et al., 2006; Pandarpurkar et al., 2003; Ramanathan et al., 1998; Saunders et al., 2010), whereas Gimap4 has been shown to limit T cell viability (Carter et al., 2007; Schnell et al., 2006). Recently, the phenotype of *Gimap5*$^{-/-}$ mice were reported (Schulteis et al., 2008). Like the *lyp* rat, *Gimap5*$^{-/-}$ mice have decreased numbers of peripheral T cells, including an abundance of mature $CD44^{high}$ T cells. Additional phenotypes were observed in *Gimap5*$^{-/-}$ mice that were not present in the *lyp* rat. These included disrupted development of NK cells, reduced survival of $V_{\alpha}14$ TCR-expressing invariant NKT (*i*NKT) cells, liver abnormalities, anaemia and early mortality. Thus, the effect of Gimap5-deficiency in mice is much more severe than the truncation of Gimap5 by the *lyp* mutation in rats.

These discrepancies open several new avenues for investigation into the function of Gimap5 *in vivo*. First, although liver failure caused by hepatocyte apoptosis has been suggested

(Schulteis et al., 2008), the role of the haematopoietic system in the early morbidity in *Gimap5*$^{-/-}$ mice remained unclear. In addition, despite the report of decreased B1 cell accumulation in the peritoneum of *Gimap5*$^{-/-}$ mice (Schulteis et al., 2008), the effect of complete Gimap5-deficiency on B cell development was not examined, nor was the role of Gimap5 in thymocyte selection or peripheral T cell and B cell function explored. In this chapter, a recessive ENU–induced mutation in Gimap5 is described, which was termed *sphinx*. Like *Gimap5*$^{-/-}$ mice, *sphinx* homozygotes are lymphopaenic, anaemic and exhibit liver abnormalities. Using the *sphinx* mouse, previously uncharacterized cell-intrinsic roles for Gimap5 in lymphocyte survival and antigen-receptor induced proliferation are described.

5.2 Results

5.2.1 *sphinx*: an ENU-induced germline mutation causing severe lymphopaenia

The *sphinx* mutation was identified in a forward genetic screen designed to detect defective *in vivo* cytotoxic NK cell and $CD8^+$ T cell responses in G_3-ENU mutagenised mice (see section 2.1.1). The original *sphinx* founder mouse that was previously immunized with OVA-expressing cells (Ehst et al., 2003), was unable to reject adoptively transferred NK cell or $CD8^+$ T cell target cells (Figure 5.1A). The mice had a near absence of $CD8^+$ T cells and NK cells in the peripheral lymphoid organs (Figure 5.1B-C) and a marked reduction in total T cell numbers. However, relatively normal thymocyte development occurred, including the $CD4^+$ T cell, $CD8^+$ T cell, γδ T cell, $Foxp3^+$ T_{reg} cell and *i*NKT cell lineages (Figure 5.2A). By contrast, most NK cells in the bone marrow expressed the marker CD69, suggesting that they might be activated or impaired in their ability to exit the bone marrow (Figure 5.2B).

Most strikingly, *sphinx* livers exhibited an abnormal morphology that presented itself as extramedullary haematopoiesis with associated foci of mononuclear cells (Figure 5.1D-E). Hyperplastic nodules developed in adult livers that contained well-differentiated hepatocytes bordered by clusters of mononuclear cells. Hepatocytes from *sphinx* mice showed no growth advantage in cell culture and no dysplastic liver cells were observed throughout this study (unpublished observations). In addition, when I crossed *sphinx* mice (C57BL/6 genetic background) with mice of one of five different genetic backgrounds (BALB/c, C3H/HeN, DBA/2J, FVB/NJ or NOD/ShiLtJ) for two generations and then intercrossed, the liver phenotype remained consistent and dysplastic cells were not observed in any other organs (data not shown). Thus, it appeared that *sphinx* livers exhibited an abnormal morphology caused by extramedullary haematopoiesis and reactive hyperplasia of hepatocytes, rather than a hepatic neoplasia. With age, *sphinx* mice progressively developed normocytic anaemia (Figure 5.3A-B), with increased variation in RBC width (Figure 5.3C) and thrombocytopaenia (Figure 5.3D). The onset of anaemia was correlated with a rapid reduction in circulating blood lymphocytes (Figure 5.3E) and neutrophilia (Figure 5.3F).

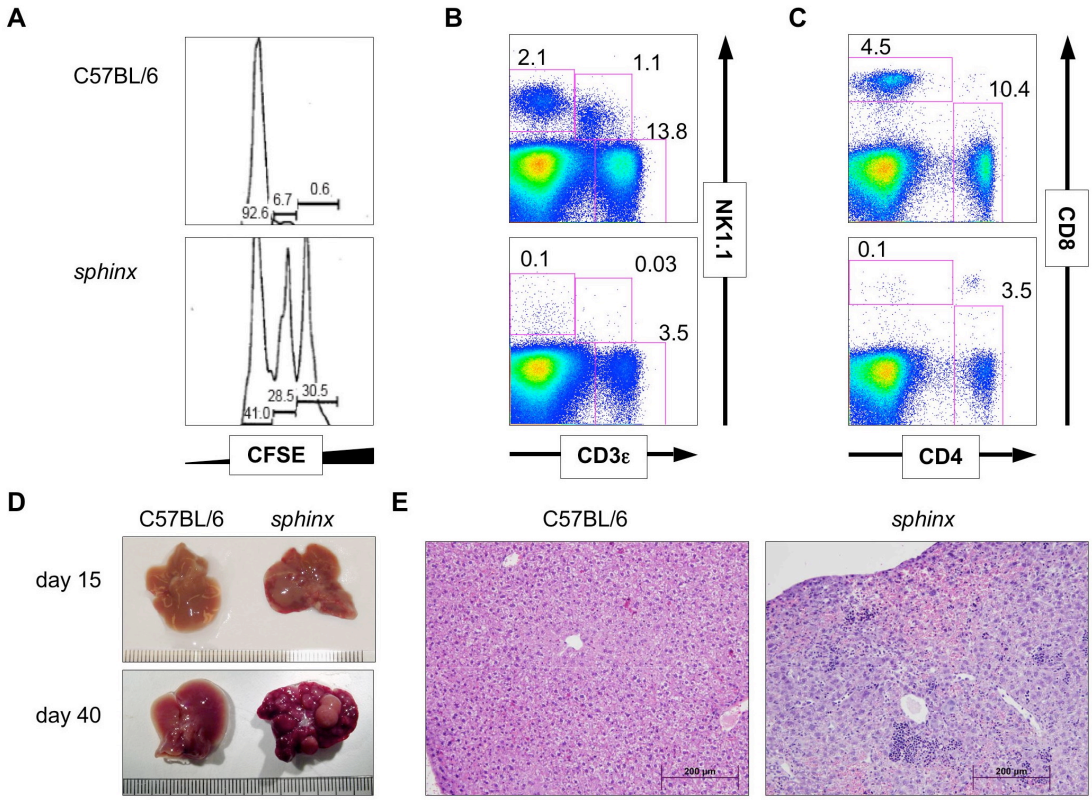

Figure 5.1. Identification of *sphinx*, an ENU germline mutant exhibiting severe lymphopaenia and hepatic extramedullary haematopoiesis.
(A) $CD8^+$ T cell and NK cell cytotoxicity were tested in ENU mutagenised mice that had been immunised with 1 x 10^8 γ-irradiated (1,500 rad) *act-mOVA* cells. Cytotoxic function was assessed by injecting CSFE-labelled *β2m*$^{-/-}$ ($CFSE^{int}$) NK cell and SIINFEKL-loaded ($CFSE^{high}$) $CD8^+$ T cell target cells, along with C57BL/6 control cells ($CFSE^{low}$) that were used as a reference population, and then measuring removal of target cells populations from the blood two days later. Histograms show recovered target cells and the frequency of each target cell population
(B-C) The percentage of (B) $CD3\varepsilon^+$ T cells and $NK1.1^+$ NK cells and (C) $CD4^+$ and $CD8^+$ T cells were quantified among splenocytes from six-week-old mice. Representative flow cytometry plots are shown of one of more than five independent analyses.
(D) Whole livers from 15-day-old (upper) and 40-day-old (lower) mice are shown.
(E) H&E stained liver sections from 40-day-old mice are shown.

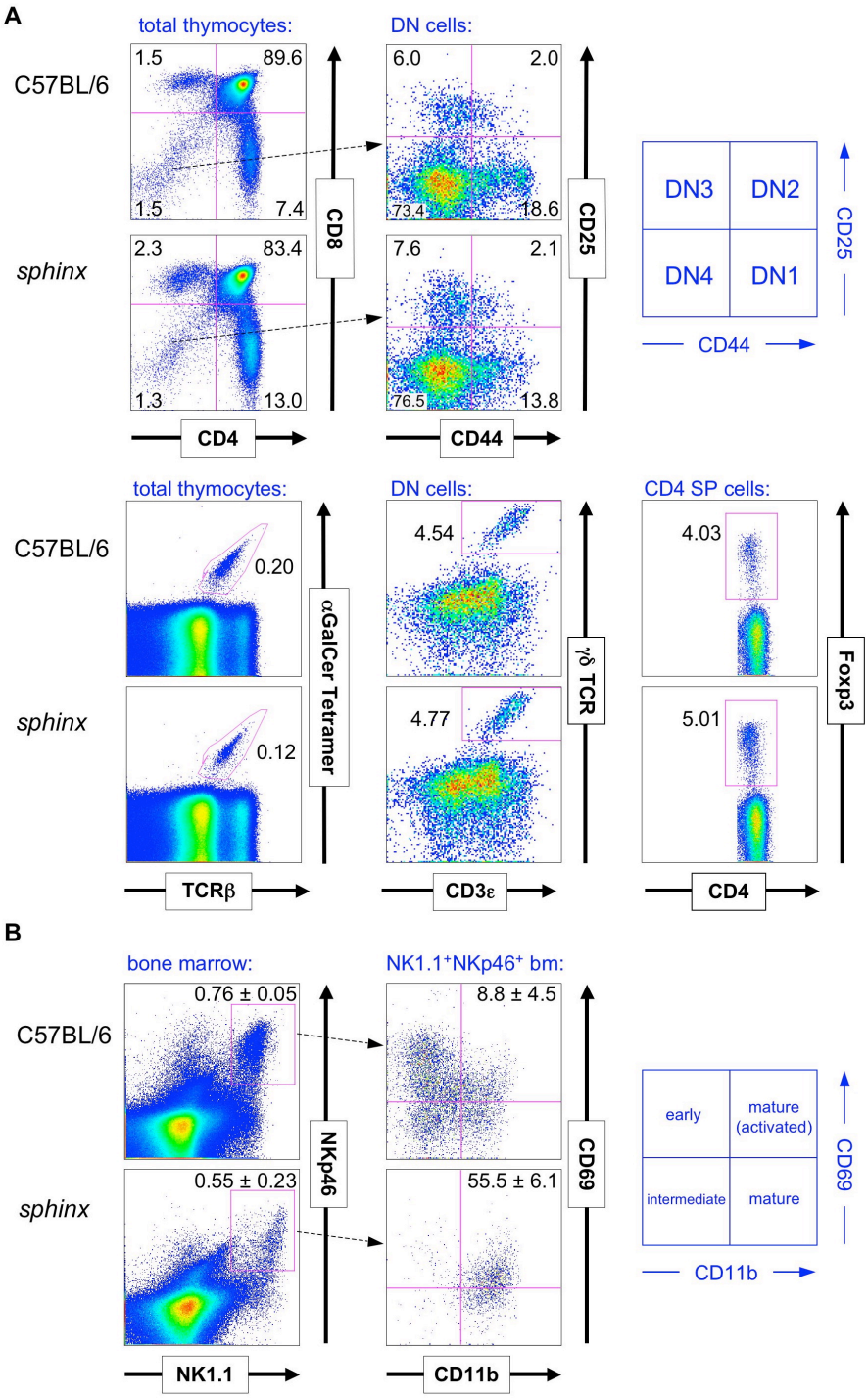

Figure 5.2. Thymocyte and bone marrow cell development in *sphinx* mice.
(A) Thymocyte development was assessed in six-week-old mice. Representative flow cytometry plots show cell frequency among total (upper left), DN (upper centre), total (lower left), DN (lower centre) or CD4 SP (lower right) thymocytes. Stages of DN thymocyte development are diagrammed (upper right). Representative data from more than five independent stainings is shown.
(B) To analyse NK cell development, expression of NK cell precursor markers was measured in bone marrow cells from six-week-old mice. The percentage of cells expressing the pan-NK cell markers NK1.1 and NKp46 is shown (left). Among these cells, expression of CD11b and CD69 was tracked and the percentage of double-positive cells is shown (centre), along with standard deviation (n = 3; *** = $p<0.001$). Stages of bone marrow NK cell development are diagrammed (right).

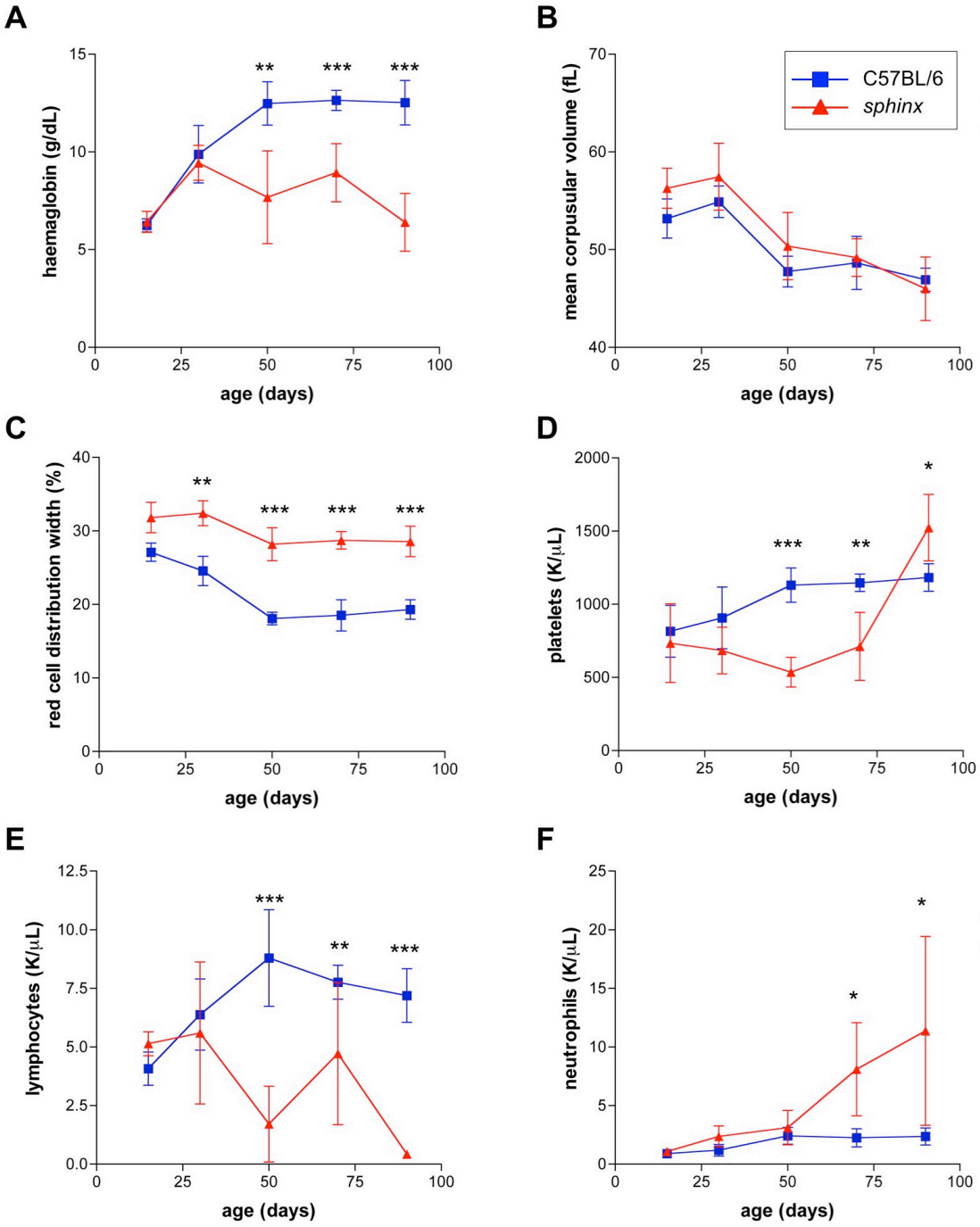

Figure 5.3. Anaemia, thrombocytopaenia, lymphopaenia and neutrophilia in *sphinx* mice. (A-F) To monitor haematological parameters in neonatal and adult mice, blood samples were obtained when mice were 15, 30, 50, 70 and 90 days old. Plots represent (A) haemaglobin concentration, (B) mean corpuslar volume, (C) red blood size variation, (D) platelet concentration, (E) lymphocyte concentration or (F) neutrophil concentration. Error bars indicate standard deviation (n = 5; * = p< 0.05, ** = p<0.01, *** = p<0.001).

These observations indicated that the *sphinx* mutation affected a gene essential for lymphocyte maintenance and normal haematopoiesis.

5.2.2 Positional cloning of the *sphinx* mutation

To identify the causative mutation, a positional cloning strategy was used (Beutler et al., 2007). Using thirteen meioses from *sphinx* mice backcrossed to the C3H/HeN strain, all phenotypes observed in *sphinx* mice were linked to chromosome 6 with a peak Lod score of 4.0 (Figure 5.4A). Further fine mapping with 572 meioses derived from F_1 intercrosses confined the mutation to a 2.9-megabase critical region, bounded by the microsatellite markers D6mit315 and D6mit276. All annotated and predicted coding base-pairs in the critical region were amplified and sequenced, resulting in the generation of high-quality sequence for 89.9% of total base-pairs in predicted coding regions and 100% of coding base-pairs for genes in the *Gimap* gene cluster. Among 1.74×10^5 base pairs sequenced, a single G→T point mutation in the *Gimap5* coding region differed between the *sphinx* and C57BL/6 genomes (Figure 5.4B-C). *Gimap5* is predominantly transcribed in lymphocytes (Nitta et al., 2006), but is also expressed in HSCs (Ng et al., 2009). The *sphinx* mutation appeared to destabilize the Gimap5 protein in haematopoietic tissues, including total bone marrow cells and splenic B cells, resulting in a lack of detectable protein (Figure 5.4D), whereas mRNA expression of *Gimap5* remained unaffected (Figure 5.4E).

The *sphinx* mutation resulted in the amino acid substitution G38C, which changed an amino acid that is highly conserved within all mouse Gimap homologs and within all annotated orthologous genes containing an AIG1 domain, in kingdoms as distant as Planta and Protista (Figure 5.4F). This residue is located in the P-loop of the AIG1 domain of Gimap5, in the pocket that is predicted to bind and hydrolyze GTP→GDP. Previous studies of the p21Ras GTPase have shown that mutation of the analogous Gly residue to Val (G15V) resulted in a protein that was unable to hydrolyze GTP or to transform NIH 3T3 cells when overexpressed (Clanton et al., 1987). The G38C mutation most likely destabilises Gimap5 by preventing the

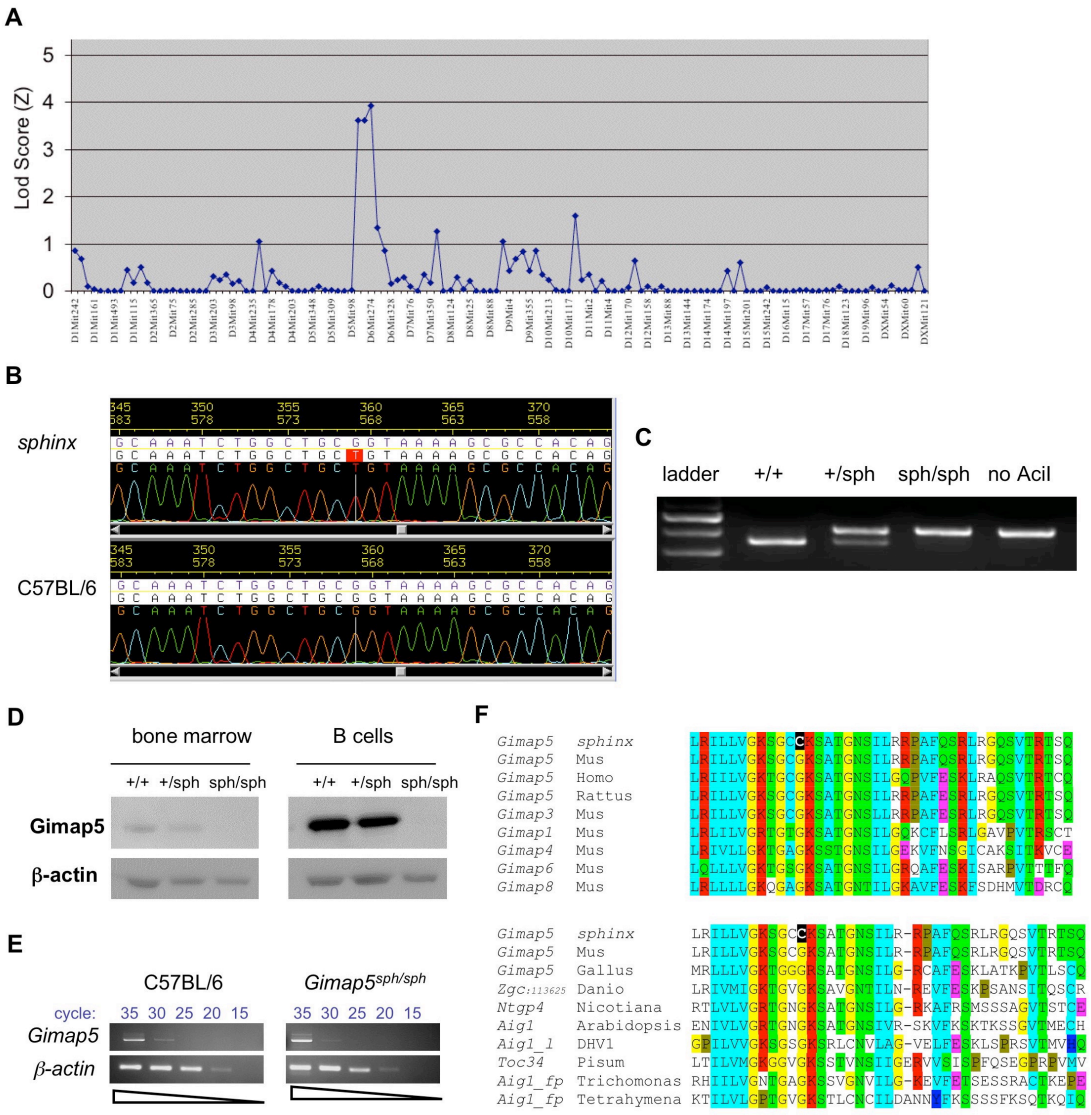

Figure 5.4. Positional cloning of the *sphinx* mutation.
(A) Using thirteen meioses and a panel of 130 microsatellite markers covering the entire genome, the *sphinx* phenotype was linked to chromosome 6.
(B) A G→T nucleotide transition in the coding region of *Gimap5* results in a G38C amino acid change in the middle of the Gimap5 AIG1 homology domain.
(C) The G→T nucleotide change abolishes an existing AciI restriction site in the C57BL/6 genome allowing for genotyping of *sphinx* (sph) mice by restriction enzyme digestion.
(D) Gimap5 protein expression was measured in lysates of total bone marrow and purified splenic $CD19^+$ B cells.
(E) *Gimap5* mRNA abundance in purified splenic $CD19^+$ B cells isolated from C57BL/6 and $Gimap5^{sph/sph}$ mice was measured by RT-PCR.
(F) The evolutionary conservation of the Gly residue altered by the *sphinx* mutation was assessed by creating multiple amino acid sequence alignments (ClustalW) of mouse Gimap5 with *Mus musculus* Gimap homologues and orthologous AIG1 domain-containing proteins from different species. The *sphinx* G38C mutation is indicated in black.

proper folding of the mutant Gimap5^{sphinx} protein. Although no Gimap5 protein was detected by immunoblotting, any residual intact protein below the limit of detection would be predicted to be nonfunctional because of the mutation of the conserved Gly residue. Overall, the *sphinx* phenotype mirrors the phenotype of previously described *Gimap5*$^{-/-}$ mice (Schulteis et al., 2008), indicating that the *sphinx* mutation (hereafter *Gimap5*$^{sph/sph}$) effectively results in a Gimap5-null animal.

5.2.3 Cell-intrinsic haematopoietic defects in *Gimap5*$^{sph/sph}$ mice

To investigate which aspects of the *Gimap5*$^{sph/sph}$ phenotype were due to cell-intrinsic requirements for Gimap5, radiation bone marrow and foetal liver chimaeric mice were generated. Similar to what was reported for *Gimap5*$^{-/-}$ mice (Schulteis et al., 2008), *Gimap5*$^{sph/sph}$ bone marrow cells poorly reconstituted the haematopoietic compartment of lethally irradiated congenic recipient mice (data not shown), suggesting that they were functionally impaired. To exclude the possibility that defective HSC function in bone marrow cells from adult *Gimap5*$^{sph/sph}$ mice was a secondary consequence of the immunopathology that occurs in *Gimap5*$^{sph/sph}$ mice, lethally irradiated *Rag2*$^{-/-}$*Il2rγ*$^{-/-}$ recipients were reconstituted with *Gimap5*$^{sph/sph}$ or *Gimap5*$^{sph/+}$ littermate control foetal liver cells from embryonic day (ED) 19 of development foetuses. The recipients were chosen because they lacked endogenous T, B and NK cells, but could support the development of a normal lymphocyte compartment from wild-type HSCs. Six weeks after transfer, mice reconstituted with *Gimap5*$^{sph/sph}$ foetal liver cells were severely lymphopaenic and had substantially fewer thymocytes than mice reconstituted with *Gimap5*$^{sph/+}$ foetal liver cells. In the spleen, *Gimap5*$^{sph/sph}$ CD8^{+} T, B and NK cell numbers were markedly reduced compared with recipients of control foetal liver cells, whereas CD4^{+} T cells were present at normal numbers (Figure 5.5). *Gimap5*$^{sph/sph}$ foetal liver cell recipients exhibited weight loss and wasting, whereas control foetal liver cell recipients appeared healthy (data not shown). In *Gimap5*$^{sph/sph}$ foetal liver cell recipients, CD11b^{+}Gr1^{+} myeloid cells accumulated in the spleen (Figure 5.5), whereas liver abnormalities were not observed. The transfer of weight loss and

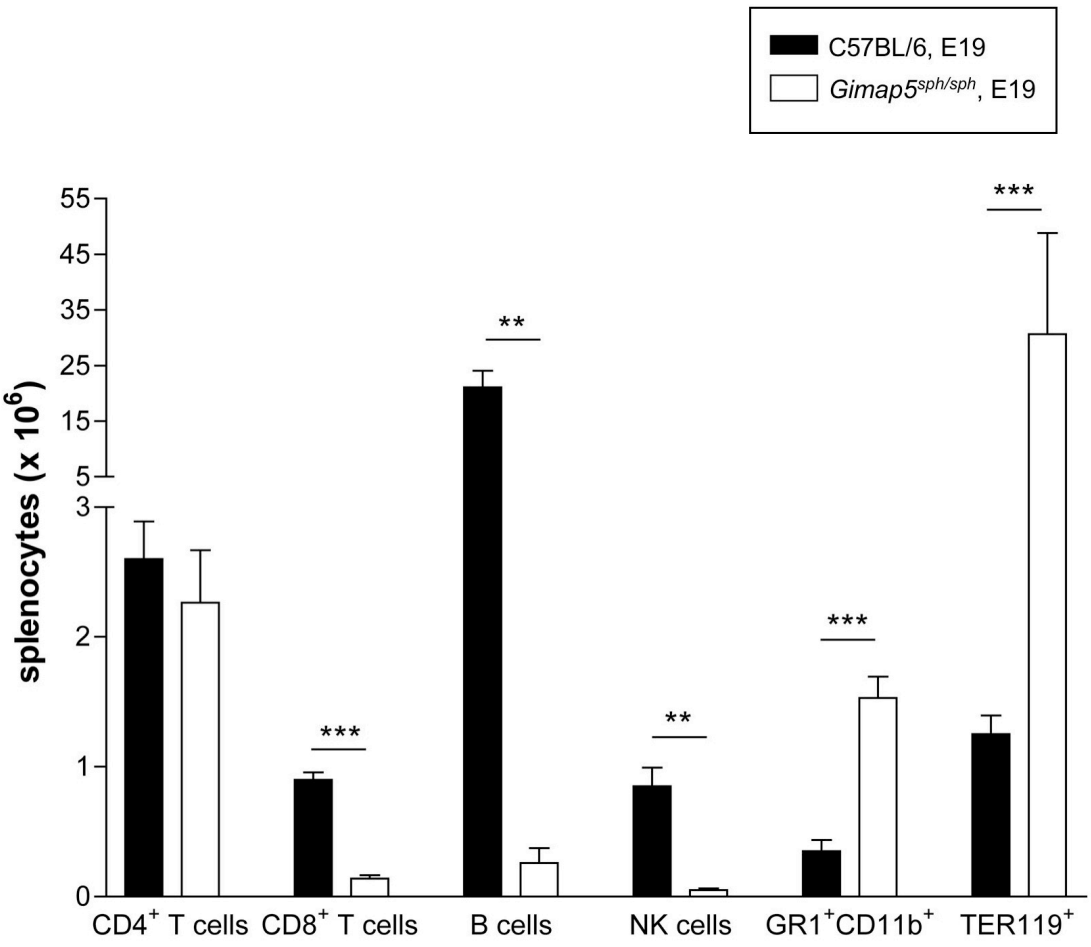

Figure 5.5. Gimap5 has a cell-intrinsic role in haematopoiesis.
To determine whether the haematopoietic abnormalities in *Gimap5*$^{sph/sph}$ mice were caused by a cell-intrinsic requirement for Gimap5, foetal liver cell chimaeras were created. *Rag2*$^{-/-}$ *Il2rγ*$^{-/-}$ recipient mice were γ-irradiated (1,000 rad) and reconstituted with ED 19 foetal liver cells from *Gimap5*$^{sph/sph}$ or C57BL/6 littermate foetuses. Six weeks later, splenocyte populations in chimaeric mice were quantified by flow cytometry. Error bars represent standard deviation and statistically significant differences were determined using a Student's *t*-test (n = 3; ** = $p < 0.01$, *** = $p < 0.001$).

wasting disease, but not liver abnormalities, by *Gimap5*$^{sph/sph}$ foetal liver cells suggested that the early mortality observed in *Gimap5*$^{sph/sph}$ mice is at least partially caused by defective haematopoiesis. Furthermore, the *Gimap5*$^{sph/sph}$ foetal liver cells exhibited an intrinsic defect in lymphopoiesis, affecting CD8$^+$ T, B and NK cells.

5.2.4 Reduced survival of *Gimap5*$^{sph/sph}$ T cells

To better understand the cell-intrinsic role for Gimap5 in lymphocytes, T cells were studied first. Roles for Gimap5 in T cells have been previously proposed for thymic selection, thymic egress and survival in the periphery (Ramanathan and Poussier, 2001). Like *Gimap5*$^{lyp/lyp}$ rats and *Gimap5*$^{-/-}$ mice, six-week-old *Gimap5*$^{sph/sph}$ mice exhibited normal thymic cellularity and frequencies of DN1-4, DP, CD4 SP and CD8 SP thymocyte subsets (data not shown). To examine thymic selection in *Gimap5*$^{sph/sph}$ thymocytes, the mutation was bred onto the HY-TCR transgenic background. The HY-TCR recognizes an MHC class I-restricted endogenous peptide that is derived from the HY antigen, which is expressed only in male mice. Consequently, HY-TCR$^+$ thymocytes are normally deleted by negative selection in male mice, whereas they develop into mature CD8$^+$ T cells in female mice. In *Gimap5*$^{sph/sph}$ males, HY–reactive thymocytes were deleted, implying that the mutation did not impede negative selection (Figure 5.6A). In contrast, the thymus of *Gimap5*$^{sph/sph}$ females contained normal numbers of HY-TCR$^+$ DP thymocytes, but significantly fewer HY-TCR$^+$ CD8 SP thymocytes (Figure 5.6A), suggesting that positively selected thymocytes became more sensitive to proapoptotic negative selection cues in the absence of Gimap5. In the periphery, HY-TCR$^+$ CD8$^+$ T cells were found at reduced numbers and frequencies in the spleen of both male and female HY-TCR-expressing *Gimap5*$^{sph/sph}$ mice (Figure 5.6B), similar to the lymphopaenia observed in *Gimap5*$^{sph/sph}$ mice.

The expression of molecules important for thymic egress and peripheral accumulation of T cells was examined next. Normally, when TCRβ^+ CD4 SP and CD8 SP thymocytes become terminally differentiated, they increase CD5 and IL-7Rα (CD127) expression and decrease

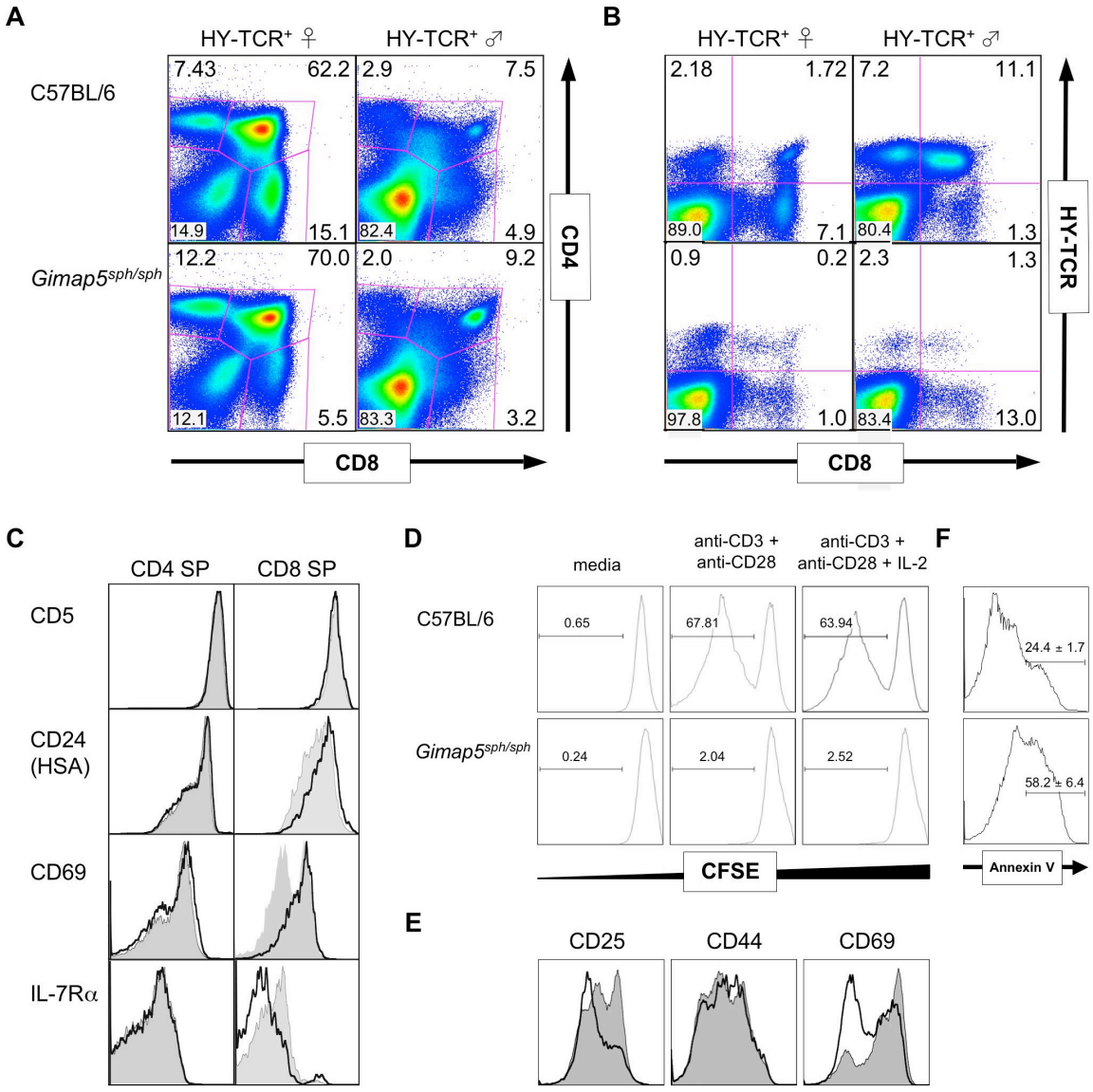

Figure 5.6. Altered T cell maturation and survival in *Gimap5*$^{sph/sph}$ mice.
(A-B) To assess positive and negative selection, (A) thymocyte and (B) splenocyte populations from HY-TCR^{+} transgenic mice of both genders were quantified and the average percentage of cells in each quadrant is shown in representative stainings of three independent experiments.
(C) Expression of terminal thymocyte maturation markers was compared between C57BL/6 (shaded, grey) and *Gimap5*$^{sph/sph}$ (open, dark line) TCRβ^{+} CD4 SP and CD8 SP thymocytes.
(D) The proliferative capacity of CD4^{+} T cells isolated from eight-week-old mice was assessed 96 hours after stimulation with plate-bound anti-CD3ε (2 μg/mL) and anti-CD28 (2 μg/mL) antibodies, with or without exogenous IL-2 (100 U/mL) by measuring CFSE dilution.
(E) Expression of T cell activation markers was examined 24 hours after purified CD4^{+} T cells were activated with plate-bound anti-CD3ε (2 μg/mL) and anti-CD28 (2 μg/mL) antibodies from C57BL/6 (shaded, grey) or *Gimap5*$^{sph/sph}$ (open, dark line) mice.
(F) *Ex vivo* apoptosis induction was monitored by culturing purified CD4^{+} T cells in complete media for 12 hours and then measuring the percentage of Annexin V^{+} cells. Standard deviation is indicated (n = 5).
(C-F) Histograms are representative of at least three independent experiments.

CD24 (HSA) and CD69 expression (Egawa et al., 2007; Palmer, 2003). *Gimap5*$^{sph/sph}$ CD8 SP, but not CD4 SP, thymocytes failed to modulate CD24, CD69 or IL-7Rα expression (Figure 5.6C). This finding suggests that Gimap5-deficiency either blocked terminal CD8 SP thymocyte maturation, caused premature egress of immature thymocytes or impaired the survival of mature CD8 SP cells. Decreased CD69 expression has been associated with the ability of thymocytes to exit the thymus (Nakayama et al., 2002; Shiow et al., 2006) and it is possible that egress of CD8 SP thymocytes is impaired in *Gimap5*$^{sph/sph}$ mice. Alternatively, thymocytes could be exiting the thymus prematurely during their development. It is interesting that $CD4^+CD8^+$ T cells can be observed in the *Gimap5*$^{sph/sph}$ spleen (Figure 5.1C) and lymph nodes, similar to mice in which immature thymocytes are prematurely 'forced' out of the thymus by overexpression of *S1P*$_1$ (Zachariah and Cyster, 2010). In addition, as IL-7 promotes naive T cell survival by modulating expression of anti-apoptotic molecules (Akashi et al., 1997), reduced IL-7Rα expression in *Gimap5*$^{sph/sph}$ CD8 SP thymocytes could contribute to the failure of $CD8^+$ T cells to survive and accumulate in the periphery.

$CD4^+$ T cells isolated from the spleen of six-week-old *Gimap5*$^{sph/sph}$ mice were unable to proliferate upon TCR stimulation (Figure 5.6D). Expression of the activation markers CD25 and CD69, but not CD44 was also impaired in *Gimap5*$^{sph/sph}$ $CD4^+$ T cells (Figure 5.6E). After overnight culture *ex vivo*, more *Gimap5*$^{sph/sph}$ than C57BL/6 $CD4^+$ T cells expressed cell surface phosphatidyl serine and bound the apoptosis marker Annexin V (Figure 5.6F). Impaired TCR-induced activation and proliferation, as well as reduced survival represent potential contributing factors to the $CD4^+$ T cell lymphopaenia that occurs as *Gimap5*$^{sph/sph}$ mice age.

Similar to what occurred in conventional $CD4^+$ T cells, peripheral *Gimap5*$^{sph/sph}$ *i*NKT cells rapidly declined in number with age (data not shown) and expressed reduced amounts of NK1.1 (Figure 5.7A). NK1.1 expression occurs at a late step in *i*NKT cell differentiation,

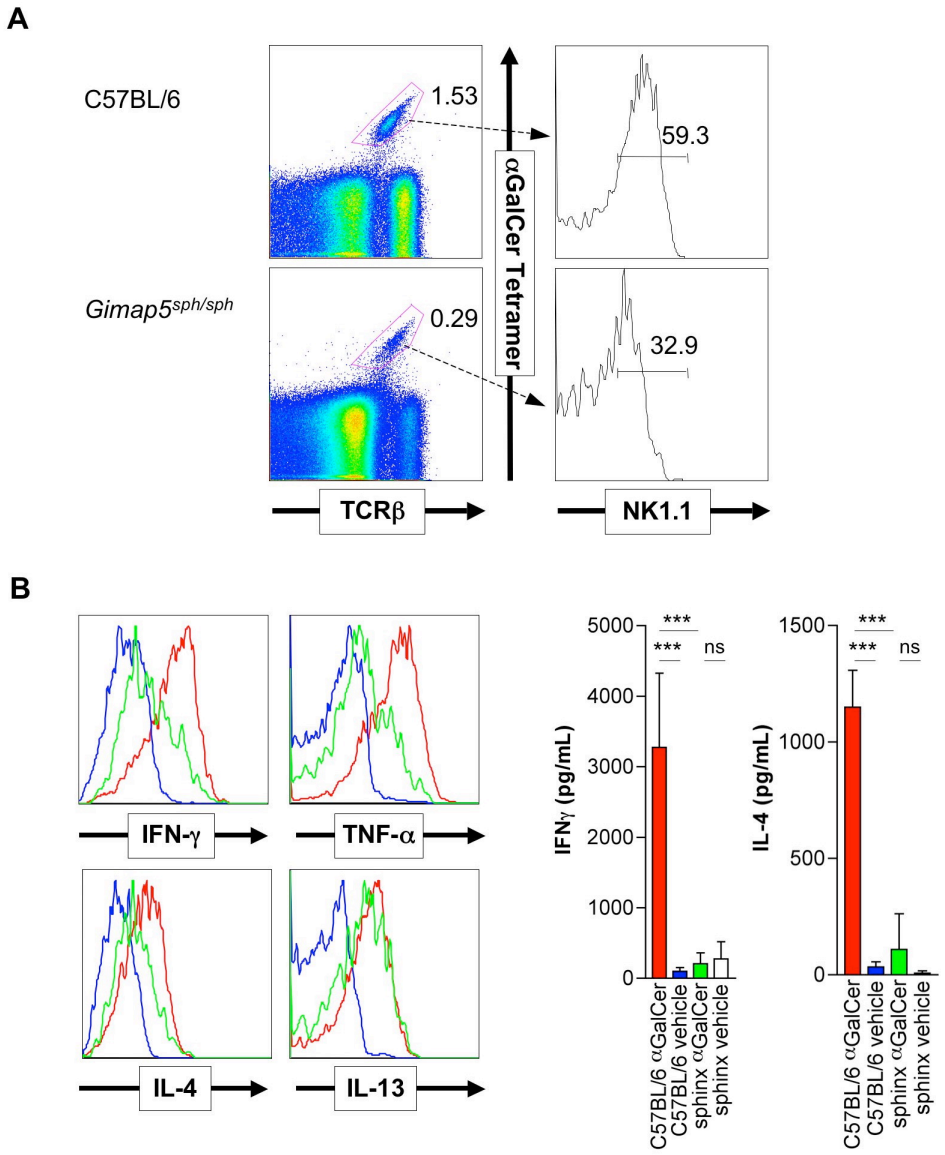

Figure 5.7. Reduced *i*NKT cell survival and aberrant αGalCer-induced cytokine responses in *Gimap5*$^{sph/sph}$ mice.
(A) *i*NKT cells in the spleen of six-week-old *Gimap5*$^{sph/sph}$ mice were identified by αGalCer-loaded CD1d-tetramer binding and quantified by flow cytometry. The percentages of CD1d-tetramer^{+} *i*NKT cells among total splenic lymphocytes (left) and NK1.1 expressing CD1d-tetramer^{+} cells (right) are shown in representative plots.
(B) Cytokine production was measured 90 minutes after injection of the *i*NKT cell agonist αGalCer on a per cell basis by intracellular staining for IFN-γ, TNF-α, IL-4 or IL-13 expression among αGalCer-loaded CD1d-tetramer^{+} *i*NKT cells (blue, unstimulated C57BL/6; red, stimulated C57BL/6; green, stimulated *Gimap5*$^{sph/sph}$). Serum cytokine concentrations of IFN-γ and IL-4 were also measured by ELISA 90 minutes after injection of αGalCer. Error bars represent standard deviation (n = 3; *** = $p < 0.001$).
(A-B) Flow cytometry plots are representative of two independent experiments (n = 3).

after export from the thymus, and is thought to require engagement of the invariant *i*NKT cell TCR with the CD1d antigen-presenting molecule (McNab et al., 2005). Upon *in vivo* activation of *i*NKT cells with the agonist αGalCer, little intracellular IFN-γ or TNF-α, reduced amounts of IL-4 and normal amounts of IL-13 were detected on a per cell basis (Figure 5.7B). These results were confirmed by ELISA (Figure 5.7B). Distinct T_H1 (TNF-α and IFN-γ) and T_H2 (IL-4 and IL-13) cytokine producing subsets of *i*NKT cells have been described in humans (Lee et al., 2002), and in mice T_H2 skewing has been proposed to occur after repeated priming (Singh et al., 1999). The impaired survival of *i*NKT cells and aberrant *i*NKT cell cytokine response that occurs in *Gimap5*$^{sph/sph}$ mice suggests roles for Gimap5 in both lymphocyte survival and function.

5.2.5 Functional defects in *Gimap5*$^{sph/sph}$ B cells

At six weeks of age, splenic CD19$^+$ B cells in *Gimap5*$^{sph/sph}$ mice, unlike T cells and NK cells, were less reduced in number compared with C57BL/6 littermates. However, the B cell compartment contained fewer mature B cells (Figure 5.8A), whereas the percentages of both marginal zone B cells (Figure 5.8B) and follicular B cells (Figure 5.8C) were similar to C57BL/6 littermates. Between six-to-ten weeks of age, splenic B cell numbers declined in *Gimap5*$^{sph/sph}$ mice (Figure 5.9A). In the peritoneal cavity, B1 cells were absent in neonatal and adult *Gimap5*$^{sph/sph}$ mice (data not shown), suggesting that B1 cells require Gimap5 to either develop or survive. Additionally, the serum of eight-week-old *Gimap5*$^{sph/sph}$ mice contained ~ 75 % less IgM and ~ 50 % less total IgG than heterozygous littermates (Figure 5.9B). The reduced total IgG reflected significant reductions in concentrations of the isotypes IgG1, IgG2b and IgG3 and a non-significant trend toward reduced IgG2a (Figure 5.9B). These abnormalities suggested that B cell function, in addition to survival, was impaired in *Gimap5*$^{sph/sph}$ mice. To assess B cell function, six-week-old mice were immunized with T-dependent (NP-CGG + alum + LPS) or T-independent (NP-ficoll) antigens. *Gimap5*$^{sph/sph}$ mice failed to produce either T-dependent NP-specific IgG1 antibodies (Figure 5.8D) or T-

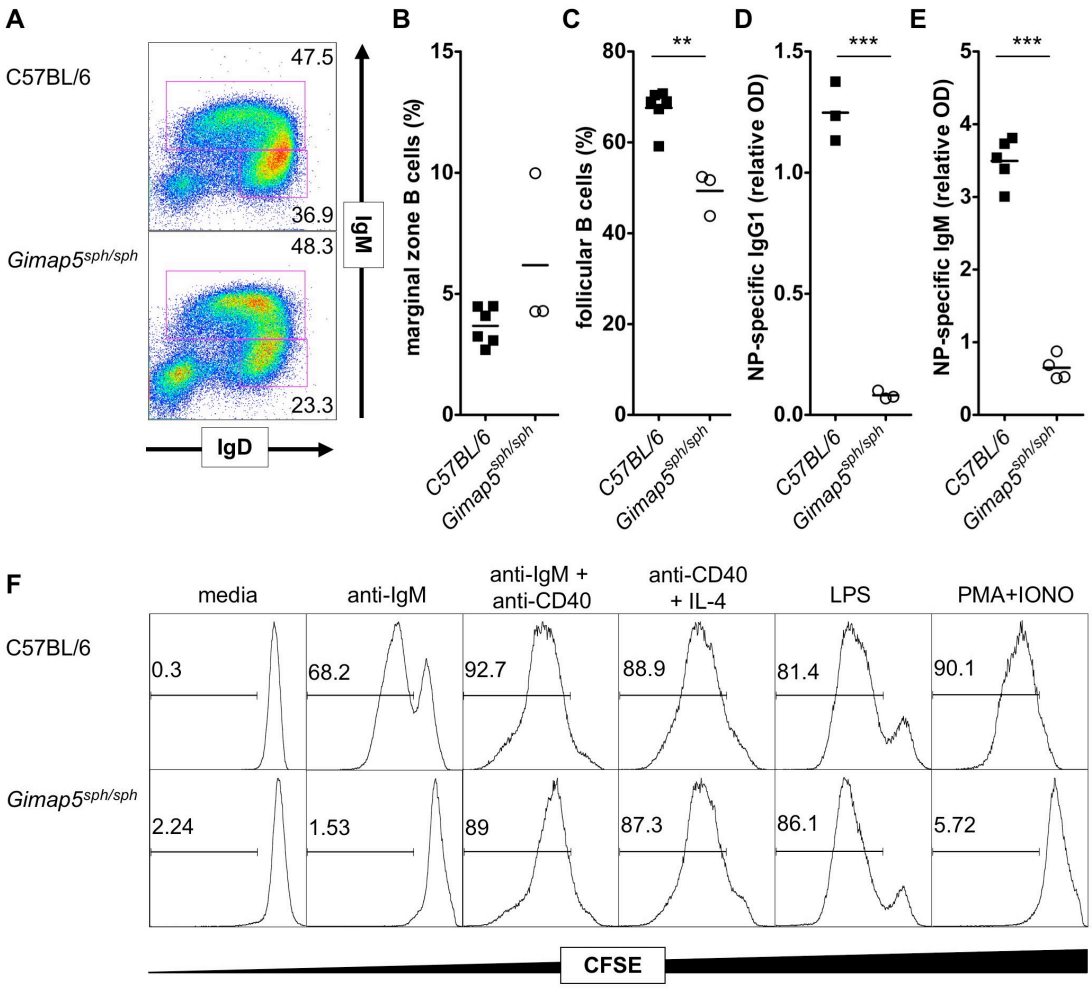

Figure 5.8. Impaired BCR-dependent responses in *Gimap5*$^{sph/sph}$ B cells.
(A-C) Among CD19^{+} splenocytes from six-week-old mice, the percentage of (A) IgM^{high}IgD^{int} naive and IgM^{low}IgD^{high} mature B cells, (B) CD21^{high}CD23^{low} marginal zone B cells and (C) CD21^{high}CD23^{high} follicular B cells was determined.
(D-E) Six-week-old mice were immunized with 50 µg NP36-CGG with alum and LPS or 50 µg of NP50-Ficoll to assess (D) T-dependent or (E) T-independent antibody responses. The concentration of NP-specific serum antibodies was measured by ELISA fourteen days after immunisation.
(F) To assess B cell proliferation, CFSE-labelled CD19^{+} splenocytes were cultured *in vitro* for 90 hours with media alone or with soluble anti-IgM-Fab (10 µg/ml), LPS (2 µg/ml), soluble anti-CD40 (10 µg/ml) plus IL-4 (10 ng/ml) or PMA (50 ng/ml) and ionomycin (500 ng/ml). Representative histograms of three independent experiments are shown.

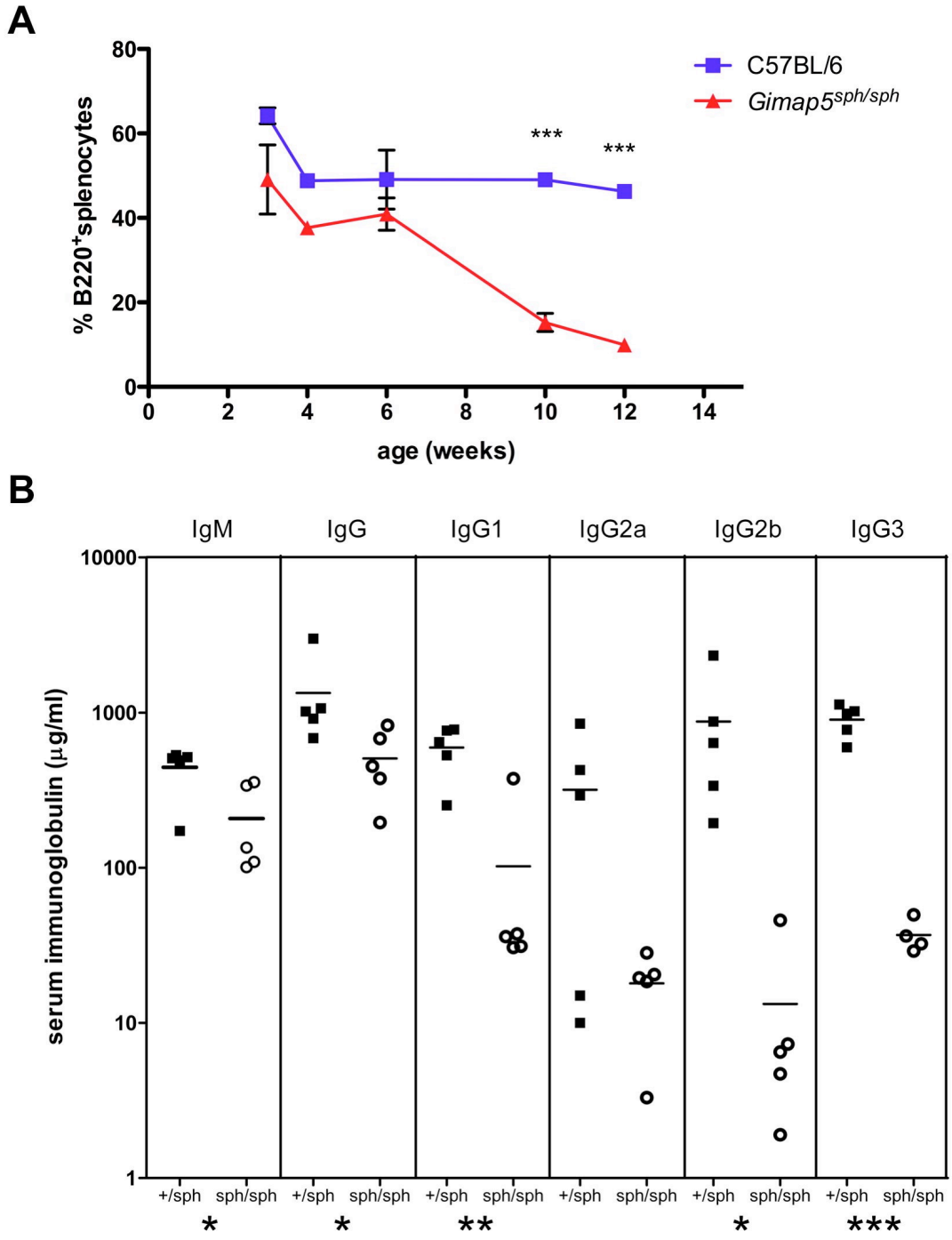

Figure 5.9. Reduced B cell survival and immunoglobulin production in *Gimap5*$^{sph/sph}$ mice.
(A) The percentage of B220^{+} splenocytes from three- to twelve-week-old mice was measured by flow cytometry (n = 5; *** = $p < 0.001$).
(B) The concentration of basal serum Ig isotypes was measured in eight-week-old mice by ELISA (n = 5; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

independent NP-specific IgM responses (Figure 5.8E), suggesting that *Gimap5*$^{sph/sph}$ B cells might have an intrinsic functional defect. To investigate B cell-intrinsic signalling, splenic B cells were isolated from six-week-old *Gimap5*$^{sph/sph}$ and C57BL/6 mice and their capacity to proliferate upon activation *in vitro* was assessed. *Gimap5*$^{sph/sph}$ B cells failed to proliferate after BCR stimulation, or treatment with the diacylglycerol mimetic, PMA, and the Ca^{++} mobilizing agent, ionomycin (Figure 5.8F). However, other pathways triggered by LPS stimulation or CD40 ligation induced normal *Gimap5*$^{sph/sph}$ B cell proliferation (Figure 5.8F). Activation of the NF-κB, MAP kinase and Akt pathways are essential for either BCR or PMA and ionomycin induced B cell proliferation. The activation of these pathways was examined in *Gimap5*$^{sph/sph}$ B cells stimulated with PMA and ionomycin. Normal degradation of the NF-κB inhibitor, IκB, and phosphorylation of the Erk, Jnk and p38 MAP kinases occurred (Figure 5.10A). In addition, normal activation of Akt, marked by phosphorylation of serine and threonine residues, and other proximal molecules in the Akt pathway was observed (Figure 5.10B). Nonetheless, *Gimap5*$^{sph/sph}$ B cells failed to proliferate upon PMA and ionomycin activation.

5.2.6 Mitochondrial accumulation in *Gimap5*$^{sph/sph}$ CD8 SP thymocytes

Autophagy is another cellular process that is important for lymphocyte proliferation. Specifically, T cells lacking Atg5, a critical inducer of autophagosome formation in lymphocytes, exhibit impaired proliferation after TCR stimulation *in vitro* (Pua et al., 2007), while the role of autophagy in B cell proliferation has yet to be determined (McLeod and He, 2010). One role of autophagy is the removal of excess mitochondria, which occurs in terminally differentiated thymocytes and in the periphery in mature $CD4^+$ and $CD8^+$ T cells (Pua et al., 2009). Since a hallmark of Atg5- and Atg7-deficient thymocytes and T cells is increased mitochondrial content, thymocytes and T cells from *Gimap5*$^{sph/sph}$ and wild-type littermate mice were stained with MitoTracker Green, a dye that binds mitochondria irrespective of mitochondrial oxidation state. Although the mitochondrial content of

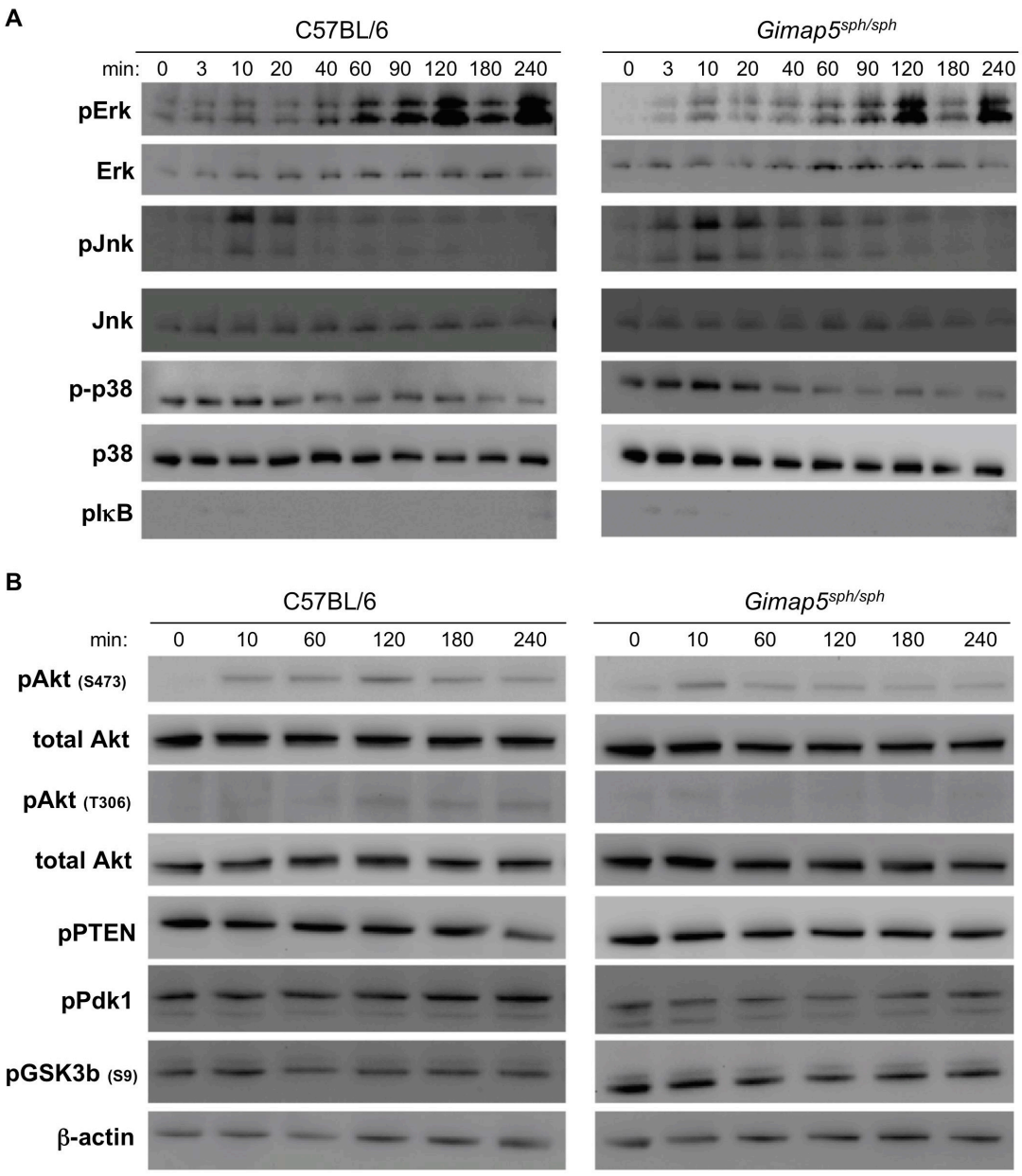

Figure 5.10. Normal *in vitro* activation of proximal B cell signaling pathways in *Gimap5*$^{sph/sph}$ B cells.
(A-B) Purified CD19^{+} B cells from C57BL/6 or *Gimap5*$^{sph/sph}$ mice were stimulated *in vitro* with PMA (50 ng/mL) and ionomycin (500 ng/mL) for the indicated number of minutes. Immunoblotting was used to assess (A) MAP kinase activation and IκB degradation and (B) phosphorylation of Akt, PTEN, Pdk1 and GSK3b. The blots shown are representative of three independent experiments.

Gimap5$^{sph/sph}$ CD4 SP thymocytes was slightly decreased, CD8 SP thymocytes had increased mitochondrial content compared to wild-type controls (Figure 5.11). This result could indicate that autophagy is impaired in CD8 SP thymocytes, however it is also consistent with an absence of terminally differentiated CD8 SP thymocytes in the *Gimap5*$^{sph/sph}$ thymus. Intriguingly, rare CD8^{+} T cells in the mLN of *Gimap5*$^{sph/sph}$ mice also had increased mitochondrial content compared to wild-type littermates (Figure 5.11).

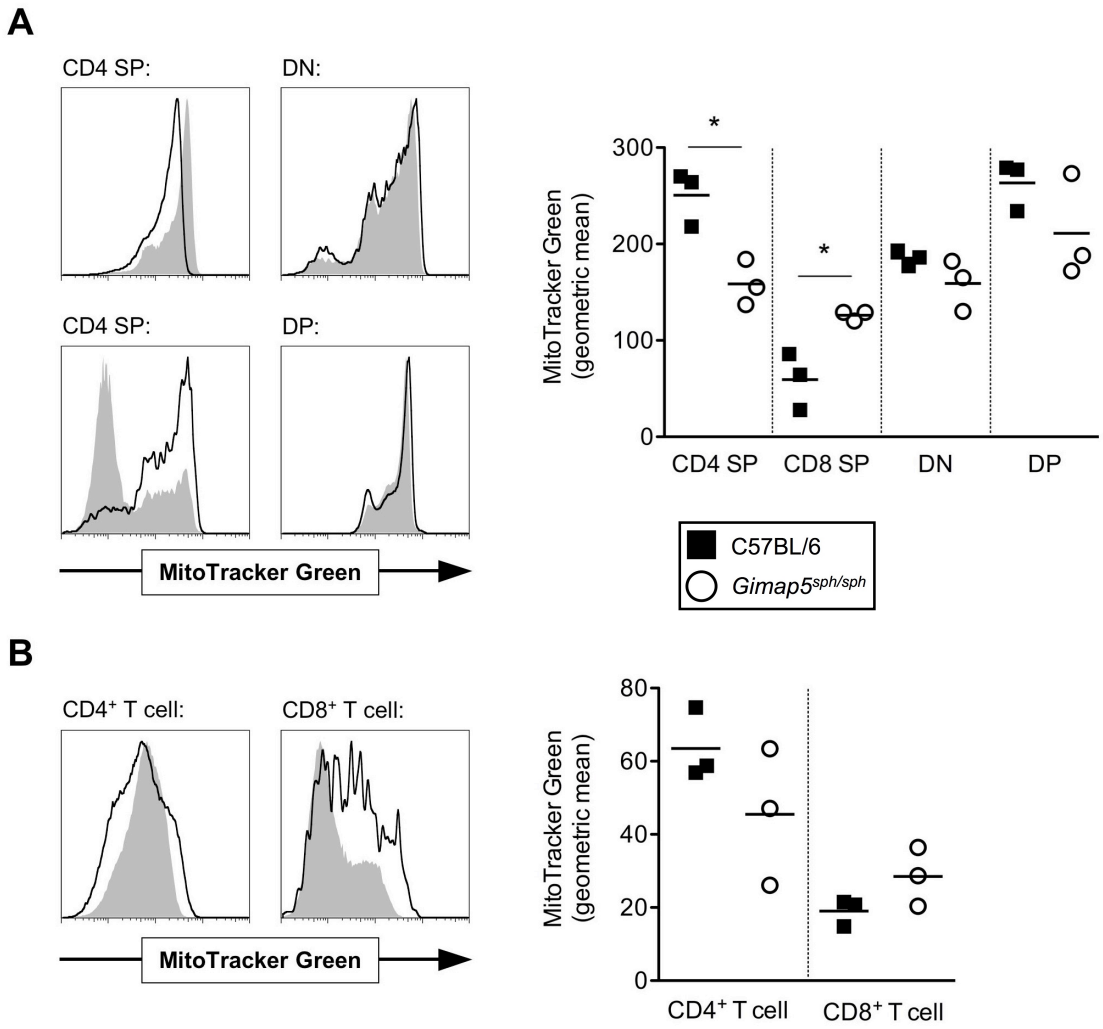

Figure 5.11. Mitochondrial content in *Gimap5*$^{sph/sph}$ thymocytes and T cells.
(A) To determine the per cell mitochondrial mass, thymocytes from six-week-old mice were stained with CD4, CD8 and MitoTracker Green (40 nM), a dye that stains mitochondria irrespective of oxidation state. Representative histograms are shown (left) comparing C57BL/6 (grey, shaded) and *Gimap5*$^{sph/sph}$ (black, open) cells, as well as the geometric mean of MitoTracker Green fluorescence intensity (right). Statistically significant differences were determined using a Student's *t*-test (n = 3, * = $p < 0.05$).
(B) Mitochondrial content was also examined in $CD4^+$ and $CD8^+$ T cells from the mLN of six-week-old mice. No statistically significant differences were detected using a Student's *t*-test.

Discussion

The identification and subsequent characterization of the *sphinx* mutation confirms the non-redundant role of Gimap5 in lymphocyte survival and normal haematopoiesis (Schulteis et al., 2008). The *Gimap5*$^{sph/sph}$ mouse exhibits a number of haematological abnormalities. From birth, peripheral CD8$^+$ T cells and NK cells fail to accumulate, suggesting that Gimap5 has a direct role in controlling these processes. The finding that CD4$^+$ T cell numbers declined with age, but were normal in *Gimap5*$^{sph/sph}$ foetal liver chimaeric mice might indicate that Gimap5-deficiency only limits the survival of CD4$^+$ T cells in contexts such as lymphopaenia that induce cell proliferation, such as in the *Gimap5*$^{sph/sph}$ mouse. Although the requirement for Gimap5 in TCR-dependent T cell proliferation and survival has been known for some time (Elder and Maclaren, 1983), this study identifies several important mechanisms that fail to operate in *Gimap5*$^{sph/sph}$ T cells. In the thymus, developing *Gimap5*$^{sph/sph}$ CD8 SP thymocytes show impaired survival after positive selection, suggesting that Gimap5 may help set survival thresholds for developing thymocytes. Also, either as a direct consequence of Gimap5-deficiency, or because mature Gimap5-deficient CD8 SP thymocytes fail to survive during development, reduced expression of the critical cytokine receptor for T cell survival, IL-7Rα, is seen on CD8 SP thymocytes. This finding might help explain why the survival of Gimap5-deficient CD8$^+$ T cell is impaired more than CD4$^+$ T cells.

The immunopathologies that can develop in *Gimap5*$^{lyp/lyp}$ rats, diabetes and intestinal inflammation, and in *Gimap5*$^{sph/sph}$ mice (see Chapter 6) suggest that an immunoregulatory mechanism also requires Gimap5 to operate. Although *Gimap5*$^{sph/sph}$ Foxp3$^+$ T_{reg} cells can function in an *in vitro* suppression assay and are present at normal frequencies among total CD4$^+$ T cells (unpublished data), it is not known whether they function *in vivo*. A recent study suggests that in *Gimap5*$^{lyp/lyp}$ rats, conventional CD4$^+$ T cells are predisposed to become T_H17 effector cells and T_{reg} cells have a skewed TCR repertoire (van den Brandt et al., 2010). It will be interesting to investigate *Gimap5*$^{sph/sph}$ T_{reg} cell function *in vivo* using the T-cell-transfer colitis model (or other disease models that do not involve a lymphopaenic

environment), as well as the role of T_H17-related cytokines in Gimap5-depedent immunopathologies.

The observations that *Gimap5*$^{sph/sph}$ foetal liver cells were unable to reconstitute the B cell compartment of irradiated recipients and that *Gimap5*$^{sph/sph}$ B cells were unable to undergo antigen-receptor induced proliferation or generate antigen-specific IgG or IgM responses were especially surprising. In the *lyp* rat model, *Gimap5*$^{lyp/lyp}$ bone marrow cells are able to normally reconstitute the B cell compartment of irradiated recipients (Ramanathan et al., 1998) and gross B cell functional defects are not observed (Tullin et al., 1997). This discrepancy might reflect different requirements for Gimap proteins in rats and mice. The *Gimap* gene cluster has undergone rapid divergent evolution between rodent species (Carter et al., 2007; Nitta et al., 2006), including changes in the number of *Gimap* genes, amino acid sequence variation between homologous Gimap proteins and differential *Gimap* gene expression patterns in lymphocytes (Nitta et al., 2006; Saunders et al., 2009). To better characterize the consequences of Gimap5-deficiency in mouse B cells, it was first considered that Gimap5 might be involved in the activation of the NF-κB and MAP kinase signalling pathways after BCR stimulation, because others had reported that these pathways are dysregulated in rat *Gimap5*$^{lyp/lyp}$ T cells (Kupfer et al., 2007). However, *Gimap5*$^{sph/sph}$ mice have normal percentages of marginal zone B cells, which require NF-κB signalling to develop (Casola, 2007), and display normal IκB degradation and MAP kinase phosphorylation upon B cell activation. Normal activation of the PI3K/Akt pathway, which is similarly required for lymphocyte proliferation, also occurs in *Gimap5*$^{sph/sph}$ B cells. Therefore, Gimap5 seems to impair BCR-induced proliferation and Ig responses independently of activation of the canonical BCR signalling pathways.

A previous study reported that Gimap5 might mediate its effects in part by interacting directly with Bcl-2 and a related anti-apoptotic protein Bcl-x_L (Nitta et al., 2006). IL-7Rα signalling

promotes expression of Bcl-2, which can directly inhibit the proapoptotic activity of Bim (Marrack and Kappler, 2004), and indeed ablation of *Bim* rescues the T cell lymphopaenia in that occurs in *Il7r*$^{-/-}$ mice (Pellegrini et al., 2004). Therefore, it was hypothesised that *Gimap5*$^{sph/sph}$*Bim*$^{-/-}$ mice might exhibit less lymphopaenia if reduced IL-7Rα expression prevented T cell survival and accumulation. However, combined Gimap5- and Bim-deficiency did not affect lymphopaenia (H. Aksoylar and K. Hoebe, personal communication), suggesting that additional survival pathways are perturbed in Gimap5-deficient lymphocytes. Similarly, ablation of the cell-extrinsic death pathway did not affect lymphopaenia in *Gimap5*$^{sph/sph}$*Fas*$^{lpr/lpr}$ mice (data not shown). Gimap5 might be more than just a prosurvival protein, and could help maintain the quiescent state in resting lymphoid cells. Lymphocyte 'quiescence' refers to the resting, non-cycling state normally observed in naïve lymphocytes that can be overcome by stimulation through the TCR for T cells and *i*NKT cells, via the BCR, CD40 or TLRs for B cells and by altering the balance of inhibitory and activating receptor signalling for NK cells. In support of the possibility that quiescence is affected in the absence of Gimap5, negative selection thresholds appear to be reduced in *Gimap5*$^{sph/sph}$ thymocytes, bone marrow NK cells have an activated phenotype and *i*NKT cells show a T_H2 cytokine secretion bias characteristic of cells that have been repeatedly primed or stimulated. Among B cells, Gimap5 deficiency might in some ways mimic B cell anergy, which can be induced by constant low-affinity BCR stimulation (Gauld et al., 2005). Indeed, *Gimap5*$^{sph/sph}$ B cells can proliferate in response to LPS or CD40 stimulation, but, like anergic B cells, fail to respond to BCR stimulation alone. At this point, the cellular mechanism that causes these effects remains unidentified.

The deletion or mutation of genes in the same pathway often produces similar phenotypes at the organismal or cellular level. For example, in fission yeast many of the 'wee' mutations that cause cells to have a smaller size were found to be involved in control of cell cycle progression via cdc25 (Nurse, 2004). In mice, deletion of *Carma1*, *Bcl10* or *Malt1* all result in the inactivation of antigen receptor signalling to NF-κB and cause similar lymphocyte

developmental defects; the products of these genes were subsequently found to exist in a multiprotein complex (Thome et al., 2010). The similarities between mice lacking essential autophagy genes in the haematopoietic system and *Gimap5*$^{sph/sph}$ mice highlight the possibility that Gimap5 might regulate autophagy in some haematopoietic cell types. Both *Gimap5*$^{sph/sph}$ mice and *Vav*^{iCre}*Atg7*$^{flox/flox}$ mice (that lack *Atg7* expression specifically in haematopoietic cells) become anaemic (Mortensen et al., 2010; Zhang et al., 2009). Similar to what occurs in *Gimap5*$^{sph/sph}$ mice, B cell-specific deletion of *Atg5* prevents the accumulation of peritoneal B1 cells but not splenic follicular or marginal zone B cells, whereas *Atg5*$^{-/-}$ foetal liver cells poorly reconstitute the splenic B cell compartment of lethally γ-irradiated recipient mice (Miller et al., 2008). During T cell development, others have shown that constitutive autophagy occurs in the thymus (Mizushima et al., 2004; Nedjic et al., 2008). Yet, the development of DN, DP and SP thymocyte populations occurs relatively normally in Atg5- or Atg7-deficient thymocytes (however, expression of the terminal CD8 SP maturation markers CD69 and IL-7Rα has not been reported). Akin to Gimap5-deficiency, genetic ablation of *Atg5* or *Atg7* causes a pronounced deficiency in peripheral CD8$^+$ T cells and a less severe CD4$^+$ T cell lymphopaenia (Mortensen et al., 2010; Pua et al., 2007; Pua et al., 2009; Stephenson et al., 2009). It is thought that lymphopaenia is caused in large part due to impaired mitophagy (the degradation of damaged mitochondria by autophagy), which causes depolarized mitochondria to accumulate within T cells and results in the generation of reactive oxygen species and subsequent cell death.

An intriguing study in *Gimap5*$^{lyp/lyp}$ rats showed that mitochondrial membrane potential remained normal among thymocytes, but declined in peripheral T cells, indicative of mitochondrial depolarization (Pandarpurkar et al., 2003). Together with the data presented here, indicating that mitochondrial content is increased in *Gimap5*$^{sph/sph}$ CD8 SP thymocytes and CD8$^+$ T cells, a potential link between Gimap5 and mitophagy can be proposed. Gimap5 has a transmembrane domain and can be observed by confocal microscopy to exist in punctate intracellular domains that do not co-localise with markers for the ER, Golgi or

mitochondria (Keita et al., 2007). This finding was unexpected, given the strong mitochondrial phenotype observed in *Gimap5*$^{lyp/lyp}$ rat T cells (Pandarpurkar et al., 2003) and the previously reported interactions between Gimap5 and the mitochondrial proteins Bcl-2 and Bcl-x_L that were found using overexpression approaches (Nitta et al., 2006). It will be important to determine by microscopy whether Gimap5 co-localizes with $LC3^+$ autophagosomes and to assess by immunoblotting whether lipidation of unconjugated LC3 (LC3-I) to LC3-II, a biochemical hallmark of autophagy, occurs in *Gimap5*$^{sph/sph}$ lymphocytes. Defective autophagy could potentially explain the link between Gimap5-deficiency and the accumulation of depolarized mitochondria, which ultimately results in lymphopaenia.

Chapter VI. Spontaneous extramedullary haematopoiesis and intestinal bacteria-triggered immunopathology occurs in Gimap5-deficient mice

6.1 Introduction

In addition to lymphocyte survival defects, Gimap5-deficiency has been associated with various forms of immunopathology. Initially, the BB *Gimap5*$^{lyp/lyp}$ rat was found to develop spontaneous diabetes on certain genetic backgrounds (Ramanathan and Poussier, 2001) and eosinophilic gastroenteritis on other genetic backgrounds (Cousins et al., 2006). When *Gimap5*$^{-/-}$ or *Gimap5*$^{sph/sph}$ mice were created, they were found to develop spontaneous liver inflammation (Barnes et al., 2010; Schulteis et al., 2008). In the diabetic BB rat model, regulatory T cell function, perhaps among a Foxp3^{-} population, is impaired by Gimap5-deficiency and adoptive transfer of blood from a wild-type non-lymphopaenic rat could prevent disease (Ramanathan and Poussier, 2001; Rossini et al., 1983). However, although aberrant T_H17 responses have been reported, none of these three forms of immunopathology have been conclusively linked to excessive effector T cell responses.

Recent studies have highlighted the role of innate lymphoid cells (ILCs) in pathways associated with immunopathology. Cells with a lymphoid size that expressed no major lineage marker other than Thy-1 could produce the T_H2 effector cytokines IL-4 and IL-13 in response to either IL-25 administration or helminth worm infection (Hurst et al., 2002; Moro et al., 2010; Neill et al., 2010; Price et al., 2010). These cells did not develop in Rag-deficient common-γ chain knockout mice, suggesting that their survival is closely related to pathways used by T cells. Importantly, reconstituting Rag-deficient, common-γ chain knockout mice with lineage-negative, Thy-1^{+} ILCs restored the ability of these mice to expel worm infections, suggesting that they are a functionally important cell type. A similar population of lineage-negative cells that produced the T_H17 effector cytokine IL-17 in response to IL-23 stimulation was identified in wild-type and Rag-deficient mice (Buonocore et al., 2010).

These cells accumulated in 129.SvEv Rag-deficient mice infected with *H. hepaticus* and in C57BL/6 Rag-deficient mice administered an agonistic anti-CD40 antibody. In both settings, ILCs were required for the development of intestinal inflammation, because anti-Thy-1 depleting antibody abrogated disease.

Three recent studies suggest that the function of these cells might be in part related to coordinating extramedullary haematopoiesis. In chronic murine malaria infection, a lineage-negative IL-7R^{+}c-Kit^{high} cell type, with many similarities to ILCs, emerged in response to IFN-γ that could differentiate into myeloid cells and help to clear infected erythrocytes (Belyaev et al., 2010). Second, after administration of IL-25, a population of cells that may be related or identical to ILCs emerged that could also differentiate into myeloid cells and granulocytes (Saenz et al., 2010). Finally, in addition to lacking most T cells, athymic *nude* mice are neutropaenic and lack lineage-negative Thy-1^{+} IL-23-responsive IL-17-producing cells (Smith et al., 2009). Adoptive transfer of these ILC-like cells into *nude* mice could restore the normal production and circulation of neutrophils. Here, the liver phenotype of Gimap5-deficient mice is characterised during ontogeny and reconsidered in light of these new data.

6.2 Results

6.2.1 Spontaneous liver inflammation in *Gimap5*$^{sph/sph}$ mice

The initial characterisation of *Gimap5*$^{-/-}$ mice reported that a high frequency of hepatocytes stained Tunel-positive, indicative of cells undergoing apoptosis (Schulteis et al., 2008). However, despite the abnormal liver morphology, normal concentrations of serum bilirubin and albumin in eight-week-old *Gimap5*$^{sph/sph}$ mice were measured along with normal concentrations of a panel of serum biomolecules (Table 6.1), suggesting that the metabolic functions of the liver remained largely intact. Of the panel of biomolecules analysed, aspartate aminotransferase (AST) but not alanine aminotransferase (ALT) concentration was found to be elevated, indicative of chronic hepatitis. This finding suggested that Gimap5-deficiency might cause a primary haematopoietic cell defect that then resulted in secondary inflammation-dependent apoptosis of hepatocytes, consistent with the abundant expression of *Gimap5* mRNA in haematopoietic cells but not hepatocytes (http://biogps.gnf.org).

To better understand the onset of liver abnormalities, the development of the embryonic and neonatal liver was investigated. At embryonic day (ED) 16 of development and at birth, livers from *Gimap5*$^{sph/sph}$ mice and heterozygous littermates appeared similar (Figure 6.1). However, haematopoietic cells remained in *Gimap5*$^{sph/sph}$ livers eight days after birth. By day fourteen, extramedullary haematopoiesis could be observed prior to the formation of the nodules observed in adult mice. Extramedullary haematopoiesis persisted in adult mice and was limited to the liver (Figure 6.1), although aberrant splenic architecture including reduced follicle size and granulocytosis was observed (data not shown). Analysis of leukocyte cells in the liver of young *Gimap5*$^{sph/sph}$ mice revealed that most lymphoid cells lacked expression of lineage markers, and included cells expressing markers characteristic of precursors for all haematopoietic lineages, of which IL-7R^{+}Sca-1^{+} cells were the most abundant [(Barnes et al., 2010); K. Hoebe personal communication]. These cells expressed markers characteristic of both common lymphocyte progenitors and ILCs, making their identity unclear.

Table 6.1. Normal metabolic biomarker expression in the serum of *Gimap5*$^{sph/sph}$ mice.

	C57BL/6	*Gimap5*$^{sph/sph}$	normal	S.D.	unit
Bicarbonate	11	11	18.5	1.9	mEq/L
Chloride	106	113	112.1	2	mEq/L
Sodium	148	150	154.4	1.6	mEq/L
Potassium	4.4	4.5	4.6	0.5	mEq/L
Calcium	9	8.8	9.6	0.3	mg/dL
Bilirubin, direct	0.1	0.1	0.1	0.1	mg/dL
Bilirubin, total	0.3	0.3	0.4	0.1	mg/dL
Albumin	1.5	1	1.6	0.1	gm/dL
Total protein	3.9	3.1	4.4	0.2	gm/dL
Phosphorous	9.2	7	7.4	1.2	mg/dL
Creatinine	<0.2	<0.2	0.1	0.1	mg/dL
Urea nitrogen	20	20	14.8	1.7	mg/dL
AST (SGOT)	68	***166***	67.6	16.8	IU/L
Alkaline phos	190	202	146	34.5	IU/L
ALT (SGPT)	26	36	28.5	6.6	IU/L
Cholesterol	60	46	86.1	9.2	mg/dL
Triglycerides	152	52	45.8	9.2	mg/dL
Lipase	42	40	47.6	6.8	unit/L

Values shown represent the average of values obtained for two eight-week-old mice. Larger cohorts of mice were not analysed because of the cost associated with the analysis and the absence of a clear metabolic defect in the mice analysed.

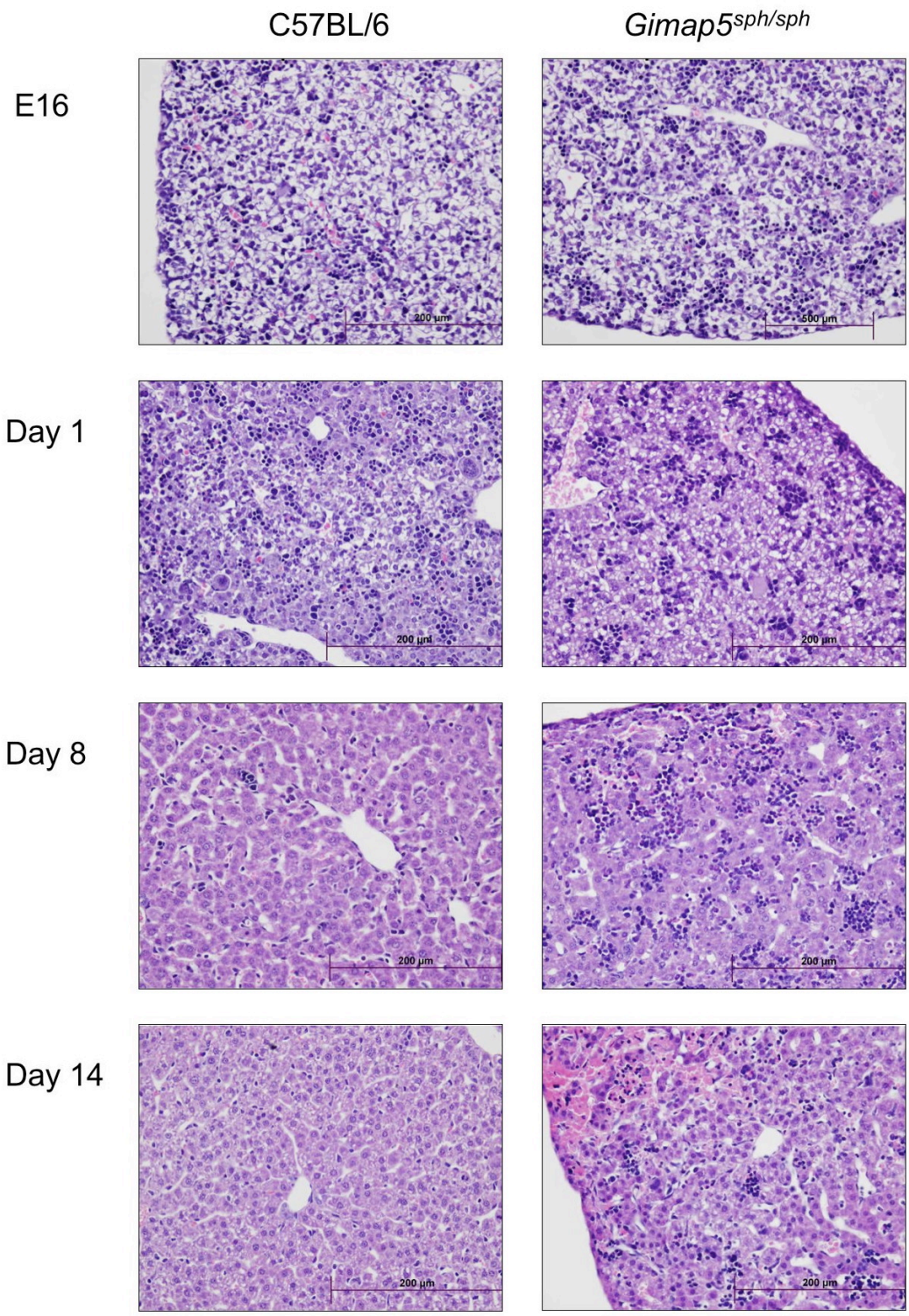

Figure 6.1. Haematopoietic cell accumulation in the neonatal *Gimap5*$^{sph/sph}$ liver.

Haematopoietic cell egress from the neonatal liver was monitored by examining H&E-stained sections from livers obtained at indicated developmental time-points. At least three livers were analyzed for each time point with consistent results and representative sections are shown.

6.2.2 IL-23 responsive ILCs accumulate in the *Gimap5*$^{sph/sph}$ foetal liver

The most abundant lineage-negative leukocyte cell type in the *Gimap5*$^{sph/sph}$ neonatal liver had an IL-7R^{+}Sca-1^{+}Thy-1^{+} phenotype, similar to previously described ILCs that produced IL-17 in response to IL-23 stimulation (Buonocore et al., 2010). To determine whether these cells possessed ILC function, liver leukocytes from three-week-old *Gimap5*$^{sph/sph}$ mice were stimulated with IL-23. Indeed, preliminary studies suggest that between 10 - 20% of lineage-negative Thy-1^{+}Sca-1^{+} cells expressed IL-17 after overnight culture. An intriguing possibility is that other types of ILCs exist in the *Gimap5*$^{sph/sph}$ neonatal liver and it will be interesting to examine whether, for example, another subset of cells expresses IL-4 or IL-13 in response to IL-25 stimulation.

Resident intestinal bacteria, particularly *H. hepaticus*, are associated with ILC accumulation and activation, ultimately driving intestinal inflammation on certain genetic backgrounds. Interestingly, when *Gimap5*$^{sph/sph}$ mice were maintained on a C57BL/6 background at TSRI, no intestinal inflammation was observed (data not shown). After *Gimap5*$^{sph/sph}$ mice were crossed onto a Balb/c background, using an in-house colony, mice developed intestinal inflammation (data not shown). Initially, this suggested that Balb/c alleles controlled genetic susceptibility to colitis. However, *Gimap5*$^{sph/sph}$ on the C57BL/6 background developed spontaneous colitis that could be ameliorated by antibiotics when transferred to another facility at Cincinnati Children's Hospital (Barnes et al., 2010), suggesting that common component(s) of the resident intestinal bacteria in the Balb/c colony and Cincinnati colony triggered colitis, not Balb/c alleles. Rederivation of Balb/c *Gimap5*$^{sph/sph}$ mice into a *H. hepaticus*-free facility at the University of Oxford abrogated colitis and reduced weight loss in *Gimap5*$^{sph/sph}$ mice (data not shown). Preliminary results suggest that colonisation of *Gimap5*$^{sph/sph}$ mice at four weeks of age with *H. hepaticus* causes weight loss and intestinal inflammation (2 of 3 mice), but this needs to be repeated with larger cohorts of mice. It will be especially interesting to colonise *Gimap5*$^{sph/sph}$*Rag2*$^{-/-}$ mice, which retain the inflamed liver phenotype, to test whether the colitis can be driven solely by innate immune activity.

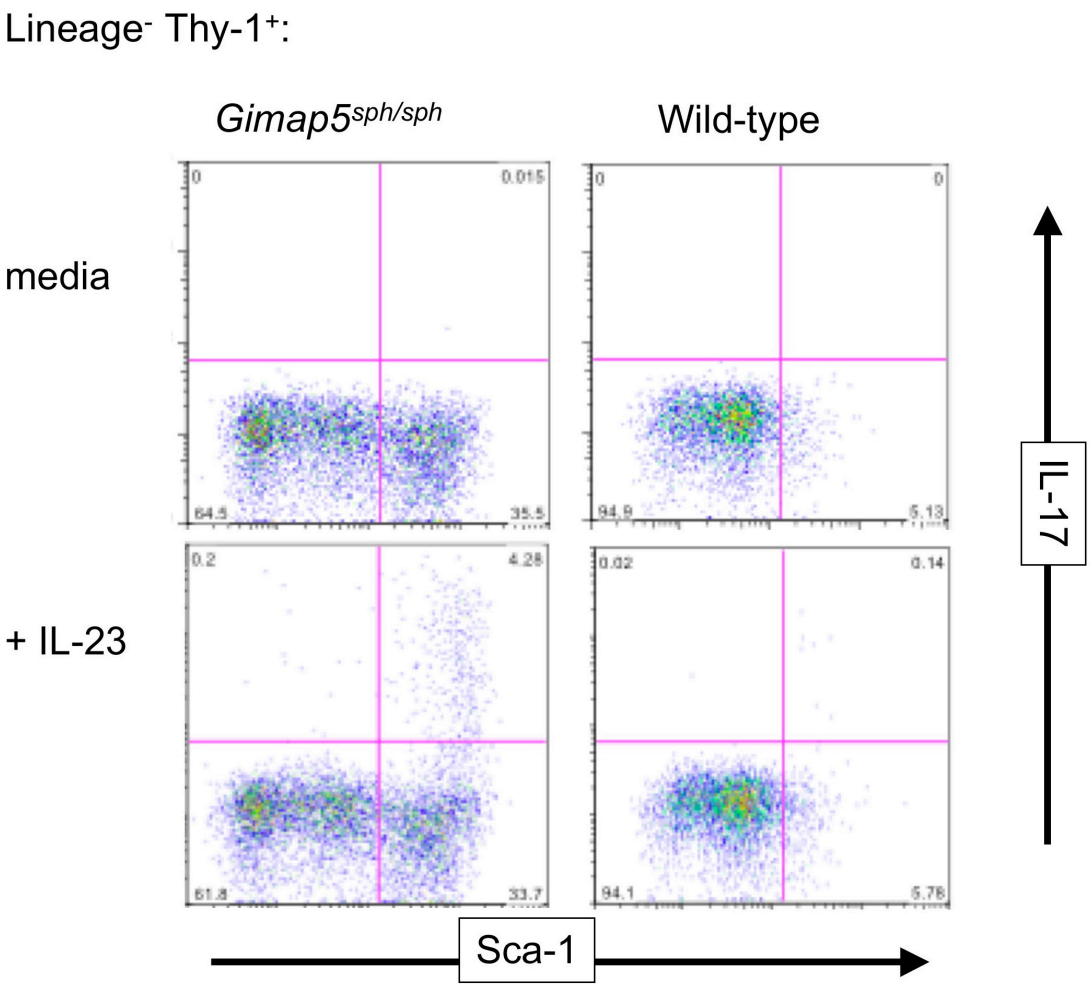

Figure 6.2. Liver leukocytes in the *Gimap5*$^{sph/sph}$ liver have ILC activity.

Liver leukocytes were isolated from three-week-old mice and stimulated overnight with recombinant IL-23 (10 ng/mL). Cells were treated with brefeldin and then stained for cell-surface lineage markers (CD3, CD19, Cd11b, CD11c, DX5, Gr1, Ter119), Sca-1 and Thy-1, and intracellular IL-17. Data are representative of three independent experiments in which one or two *Gimap5*$^{sph/sph}$ mice were analysed.

6.2.3 Adoptively transferred lymphocytes prevent early mortality in *Gimap5*$^{sph/sph}$ mice

Similar to reports for *Gimap5*$^{-/-}$ mice, *Gimap5*$^{sph/sph}$ mice lost weight after weaning and became moribund by 14 weeks of age. Given the ability of adoptively transferred $CD4^+CD25^+$ T_{reg} cells to suppress ILC-dependent colitis in *H. hepaticus* infected 129.SvEv *Rag2*$^{-/-}$ mice (Maloy et al., 2003), and of blood transfusions from wild-type rats to prevent diabetes in BB *Gimap5*$^{lyp/lyp}$ rats (Rossini et al., 1983), it was considered that wild-type lymphocytes might prevent early mortality in *Gimap5*$^{sph/sph}$ mice. To assess the importance of lymphocytes in the immunopathology observed in *Gimap5*$^{sph/sph}$ mice, 1 x 10^7 wild-type C57BL/6 splenocytes were transferred into young *Gimap5*$^{sph/sph}$ mice before the onset of wasting disease. Strikingly, recipient *Gimap5*$^{sph/sph}$ mice did not succumb to early death (Figure 6.3) or develop wasting disease (data not shown). Additionally, after adoptive transfer of C57BL/6 splenocytes, the accumulation of lineage-negative leukocytes in the liver of *Gimap5*$^{sph/sph}$ mice was significantly reduced [(Barnes et al., 2010); K. Hoebe, personal communication]. Finally, no reduction in mortality or wasting disease was observed when *Gimap5*$^{sph/sph}$ mice received *Rag2*$^{-/-}$ splenocytes, indicating that a lymphocyte population is required to prevent wasting disease and early mortality (Figure 6.3).

Therefore, at least two Gimap5-dependent activities contribute to the immunopathology that occurs in *Gimap5*$^{sph/sph}$ mice. First, inflammatory cells, including ILCs, accumulate in the liver. Second, lymphocyte-mediated immune regulation is impaired due to a role for Gimap5 in regulatory cell function or survival. These combined defects result in spontaneous liver inflammation and early mortality in conventionally housed *Gimap5*$^{sph/sph}$ mice.

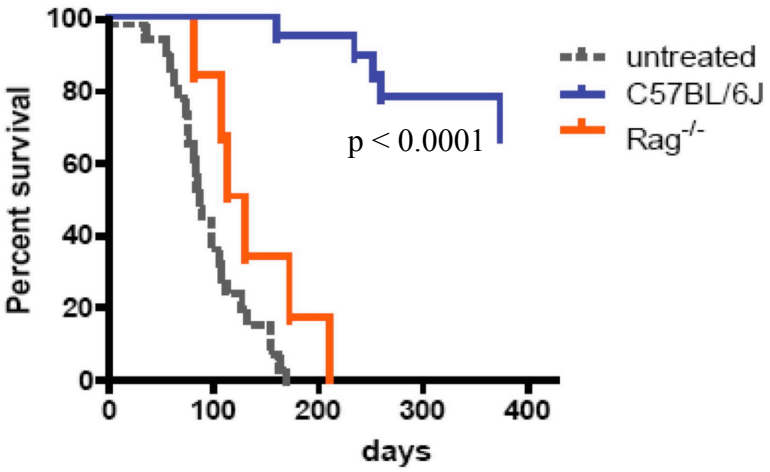

Figure 6.3. Prevention of wasting disease by adoptive transfer of lymphocytes.

25-to-35-day-old *Gimap5*$^{sph/sph}$ mice were injected with 1 x 10^7 splenocytes from C57BL/6 or *Rag2*$^{-/-}$ donors. The survival of recipient mice was monitored for up to nine months ($n \geq 6$ per group) and statistically significant differences were measured using the Gehan-Breslow-Wilcoxon test.

6.3 Discussion

The *Gimap5*$^{lyp/lyp}$ diabetes-prone BB rat served as the main animal model of type I diabetes before use of the NOD-mouse became common (Ramanathan and Poussier, 2001). Because of the profound lymphopaenia identified in these rats (Elder and Maclaren, 1983), the vast majority of immunological studies in this animal model focused on $CD4^+$ T cells and led to some of the early evidence for a dedicated subset of regulatory T cells (Greiner et al., 1986; Greiner et al., 1987). However, impaired T cell regulation might be only one piece of the puzzle in this animal model and it remains possible that lineage-negative ILCs contribute to diabetes and intestinal inflammation in *Gimap5*$^{lyp/lyp}$ rats. The *Gimap5*$^{-/-}$ and *Gimap5*$^{sph/sph}$ mouse have similar liver and anaemia phenotypes on a Rag-deficient background, suggesting that much or all of the Gimap5-dependent immunopathology in mice occurs in the absence of lymphocytes.

Further study is needed to establish whether the Gimap5-deficient mouse is a spontaneous model of ILC activity. Initially, ILCs should be depleted from the liver of *Gimap5*$^{sph/sph}$*Rag2*$^{-/-}$ mice using anti-Thy-1 antibodies to determine whether this treatment abrogates the extramedullary haematopoiesis that occurs in the liver. Molecular approaches will also be needed to determine whether Gimap5 has an intrinsic function in ILCs or whether the ILC accumulation is secondary to the actions of Gimap5 in another cell type. It is interesting that a very recent study, using fate-mapping mice, showed that RORγt$^+$ ILCs capable of secreting IL-17 developed from foetal liver precursors (Sawa et al., 2010). These cells comprised ~ 60% of $CD3^-$ leukocytes in the liver at day three of life and declined in frequency throughout the neonatal liver period. A role for Gimap5 could be envisioned in this process based on the analysis of liver sections throughout ontogeny—in *Gimap5*$^{sph/sph}$ mice leukocytes did not decline in the liver during the neonatal period. Since *Gimap5*$^{sph/sph}$ bone marrow NK cells show a spontaneously activated phenotype, it is reasonable to speculate that ILCs become similarly activated in the neonatal *Gimap5*$^{sph/sph}$ liver, preventing the decline in foetal liver ILC populations and triggering extramedullary haematopoiesis. If this hypothesis is correct,

the *Gimap5*$^{sph/sph}$*Rag2*$^{-/-}$ mouse could serve as a powerful tool for dissecting the genetic and cellular pathways that result in ILC development and activation.

Chapter VII. General Discussion

If I have learned one lesson while studying immunology, it is that conclusions are rarely completely correct and even the most black-and-white findings can turn out to be the net product of incredible complexity. The work presented here adds to the reservoir of immunological knowledge a new gene important for T_{reg} cell development, a role for intestinal bacteria in promoting their own tolerance via the induction of IL-10 and a new phenotype for an 'old' gene, *Gimap5*. These findings all have the potential to inform future studies on lymphocytes and tolerance that will hopefully one day have beneficial consequences. However, it is perhaps more interesting to consider these studies in the light of established 'dogma' in immunology.

Study of the thymus dominated T cell biology in the 1980's and 1990's, leading to the cloning of the TCR, the definition of positive selection based upon productive TCR rearrangements, the definition of negative selection based upon self-reactivity of that TCR and the identification of markers to track thymocytes on their journey from precursor to mature T cell. Recently, the bulk of the field has shifted its attention to the peripheral tissues and on the role of lymphocytes in host defence and immunopathology. Implicit in this change in focus was the assumption that antigen specificity dictated whether a T cell became activated and that the innate immune system, principally DCs, guided $CD4^+$ T cells to adopt the appropriate specialised effector phenotype.

Several pieces of evidence have suggested that these cells emerging from the thymus are less homogenous than assumed. The discovery of $CD4^+CD25^+$ SP thymocytes and later of $CD4^+Foxp3^+$ thymocytes with regulatory potential negated the assumption that mature CD4 SP thymocytes only varied with respect to the antigens they recognised (Read et al., 2000; Sakaguchi et al., 2008). However, it was initially assumed that T_{reg} cells recognised a distinct set of self-antigens. TCR sequencing has demonstrated that such a dichotomy does not exist; rather $Foxp3^+$ thymocytes show a bias towards expressing certain TCR sequences, but no

clear distinction between the T_{reg} cell and conventional T cell TCR repertoire (Feuerer et al., 2009; Josefowicz and Rudensky, 2009). Even when a T_{reg} cell-derived TCR transgenic T cell precursor is used to repopulate a thymus at a very low precursor frequency, at most only 50% of cells become $Foxp3^+$ (Bautista et al., 2009). Thus, additional, undescribed signals in the thymus might influence the fate of a developing thymocyte. In fact, these TCR-transgenic thymocytes do not undergo TCR rearrangement, a process during which a developing thymocyte might receive additional signalling inputs.

Considering Carma1-deficiency in this light, differences in DN thymocyte populations prior to β-selection are observed in *Carma1*$^{tut/tut}$ mice, including premature apoptosis (Egawa et al., 2003). Based on the characterised role of Carma1 as a mediator of TCR signalling, this is unexpected. It maybe that in developing DN thymocytes, a TCR-independent pathway uses Carma1 to signal into DN cells. It should be noted that Carma1 is part of a TCR-independent signalling pathway in NK cells that is important for the production of effector cytokines (Gross et al., 2008). Another outstanding question is why infection with LCMV or *Listeria monocytogenes* does not trigger autoimmunity in *Carma1*$^{tut/tut}$ mice, since both infections lead to protective immune responses without triggering immunopathology or T_{reg} cell expansion. It is possible that self-reactive thymocytes, maybe cells analogous to recently described thymus derived T_H17 cells (Marks et al., 2009), also require Carma1 to survive. This raises a further question, if Carma1 is not required for protective immune responses to pathogens, why did a scenario evolve in which T_{reg} cells were needed in the first place?

The finding reported here that $V_\beta14^+$ T cells give rise to fewer IL-10-producing regulatory T cells in the colon LP is perhaps preliminary at the moment and shouldn't be over-interpreted, however, previous studies implicate these cells in colitogenic T_H17-related effector responses (Kai et al., 2005; Kanetake et al., 2003). It could be simply that this population of T cells is more likely to react with intestinal antigens. Molecular studies of TCR rearrangement suggest that something unique might be going on in this population of cells (Glusman et al.,

2001; Ranganath et al., 2008; Senoo and Shinkai, 1998). Because thymocytes expressing this *Vb* allele are not subject to allelic exclusion (silencing of transcription of other *Vb* alleles), they might receive a qualitatively different type of signal during their development that ultimately influences their eventual propensity to become an effector or regulatory type cell. The heterogeneity of DN and maturing thymocytes could be investigated using a combination of approaches, but it will be simplified when single cell microarrays or epigenetic profiling become routinely available. If these cells are found to be more variable than currently appreciated, understanding the factors that generate this heterogeneity would add a new conceptual layer to our understanding of immune tolerance.

Roles in the immune response once assigned to thymus-derived T cells might sometimes be carried out by lineage-negative ILCs. The phenotype of the *Gimap5*$^{sph/sph}$ mouse is in agreement with developmental studies that showed T_H17-cytokine-producing ILCs derive from a foetal liver precursor (Sawa et al., 2010). Therefore, it is particularly interesting that these cells may be absent from athymic *nude* mice, which have a mutation in the *Foxn1* gene (Smith et al., 2009). These cells might begin as HSCs in the foetal liver, migrate to the foetal thymus and then migrate back to the foetal liver during development. Or there could be an unappreciated role for *Foxn1* outside the thymus. This finding will be important to dissect further, because it could point to an additional role for the thymus as a source of ILCs that might be host protective in some cases, but contribute to chronic inflammatory disease in others.

Finally, it is worth considering the role of genetics in these studies and the possibilities for genetics to continue to inform basic immunology in the future. Reverse genetics and cell-type specific deletion of certain genes will continue to provide the basis for advances in basic immunology and our understanding of how specific cells and genes function. It would be extremely valuable to generate a mouse with a *Gimap5* 'floxed' allele in this regard. However, classical or forward genetics offers better chances to define new therapeutic targets

for treating human disease. Inflammatory bowel disease represents one of the few major successes of the 'HapMap' project that used SNPs common in human populations to link risk alleles to disease susceptibility loci. Yet, this approach has only to date captured a small fraction of the genetic predisposition to IBD. Screens using mouse genetics offer one potentially useful way forward, although humans have a much different lifestyle (and history of potential immunopathology-triggering infections) than a laboratory mouse! Given the advent of inexpensive whole-genome sequencing, humans might become the subjects of a new era of forward genetic studies. By analysing many families and micro pedigrees (Roach et al., 2010), a new set of genes important for maintaining self-tolerance could be revealed.

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