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Vitamin A Metabolism and Commensal Stimulationi in the Promotion of Mucosal Immunity

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Abstract
The gastrointestinal tract is replete with commensal microbes and dietary nutrients that provide homeostatic signals. Antigen presenting dendritic cells (DC) residing in the underlying lamina propria (Lp) respond to these signals; however, how they contribute to intestinal T cell homeostasis is unclear. In Chapter 2, LpDC are revealed to uniquely induce naïve T cell differentiation into the Foxp3+ regulatory T cell (Treg) subset. Further, the molecular mechanisms controlling this capacity both in vitro and in vivo are shown to hinge on the vitamin A metabolite, retinoic acid (RA), which LpDC are equipped to synthesize, and the cytokine, TGF-beta. T cell expression of retinoic acid receptor alpha (RARalpha) is shown to be critical for RA to induce enhanced Foxp3+ Treg induction. Chapter 3 extends upon these findings and addresses the influence of the commensal microbiota in the regulation of this pathway. A Toll like receptor (TLR) 9 ligand, commensal derived DNA, is identified as a potent adjuvant in the gut mucosa, which shapes T cell homeostasis in the GI tract. Accordingly Tlr9–/– mice display an intestinal site-specific increase in Foxp3+Treg concomitant with a decrease in TH cells. Dysregulation in Foxp3+ Treg/TH homeostasis results in mucosal-specific impaired immune responses in Tlr9–/– animals, which can be reversed upon partial depletion of Foxp3+ Treg. Chapter 4 builds upon findings from Chapter 2. The role of vitamin A metabolism in the regulation of mucosal immunity is examined. Vitamin A insufficient (VAI) mice, which lack vitamin A and metabolic derivatives, mount impaired mucosal TH-1 and TH-17 responses. These defects are reversed upon administration of RA. Moreover, Rara–/– mice recapitulate the homeostatic and immune defects observed in VAI mice. Strikingly, loss of basal RA/RARalpha signaling hinders early T cell activation events. Cumulatively, the data argue that steady-state cues from microbiota and nutrients shape the inflammatory tone of the Lp to prime mucosal TH responses. These data also identify a fundamental role for vitamin A metabolism in T cell activation and suggest this pathway may have evolved with the development of adaptive CD4+ T cell responses to coordinate host protection.

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Abstract

Vitamin A metabolism and commensal stimulation in the promotion of mucosal immunity

Jason A. Hall

Yasmine Belkaid

The gastrointestinal tract is replete with commensal microbes and dietary nutrients that provide homeostatic signals. Antigen presenting dendritic cells (DC) residing in the underlying lamina propria (Lp) respond to these signals; however, how they contribute to intestinal T cell homeostasis is unclear. In Chapter 2, LpDC are revealed to uniquely induce naïve T cell differentiation into the Foxp3$^+$ regulatory T cell (T_{reg}) subset. Further, the molecular mechanisms controlling this capacity both *in vitro* and *in vivo* are shown to hinge on the vitamin A metabolite, retinoic acid (RA), which LpDC are equipped to synthesize, and the cytokine, TGF-β. T cell expression of retinoic acid receptor alpha (RARα) is shown to be critical for RA to induce enhanced Foxp3$^+$ T_{reg} induction. Chapter 3 extends upon these findings and addresses the influence of the commensal microbiota in the regulation of this pathway. A Toll like receptor (TLR) 9 ligand, commensal derived DNA, is identified as a potent adjuvant in the gut mucosa, which shapes T cell homeostasis in the GI tract. Accordingly *Tlr9*$^{-/-}$ mice display an intestinal site-specific increase in Foxp3$^+$ T_{reg} concomitant with a decrease in T_H cells. Dysregulation in Foxp3$^+$ T_{reg}/T_H homeostasis results in mucosal-specific impaired immune responses in *Tlr9*$^{-/-}$ animals, which can be reversed upon partial depletion of Foxp3$^+$ T_{reg}. Chapter 4 builds upon findings from Chapter 2. The role of vitamin A metabolism in the regulation of mucosal immunity is examined. Vitamin A insufficient (VAI) mice, which lack vitamin A and metabolic derivatives, mount impaired mucosal T_H-

1 and T_H-17 responses. These defects are reversed upon administration of RA. Moreover, *Rara*$^{-/-}$ mice recapitulate the homeostatic and immune defects observed in VAI mice. Strikingly, loss of basal RA/RARα signaling hinders early T cell activation events. Cumulatively, the data argue that steady-state cues from microbiota and nutrients shape the inflammatory tone of the Lp to prime mucosal T_H responses. These data also identify a fundamental role for vitamin A metabolism in T cell activation and suggest this pathway may have evolved with the development of adaptive $CD4^+$ T cell responses to coordinate host protection.

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2. Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., **Hall, J.**, Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-{beta}- and retinoic acid-dependent mechanism. J Exp Med.
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4. Hill, J.A.; **Hall, J.A.**; Sun, C.M.; Cai, Q.; Ghyselinck, N.; Chambon, P.; Belkaid, Y.; Mathis, D., and Benoist, C. (2008). Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity *29*, 758-770.
5. **Hall, J.A.;** Cannons, J.L.; Grainger, J.R.; Dos Santos, L.M.; Naik S; Wohlfert, E.A.; Oldenhove, G.; Robinson, M.; Kastenmayer, R; Grigg, M.E.; and Belkaid Y. (2011) Essential role for retinoic acid in the promotion of $CD4^+$ T cell effector responses via retinoic acid receptor alpha. Under Revision**.**

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CHAPTER 1: Intestinal immune homeostasis: influential contributions by the commensal microbiota and vitamin A metabolism

The intestinal microbiota establishes a commensal relationship with the host

The mucosal surfaces are comprised of the oronasopharyngeal, urogenital, and gastrointestinal tracts and serve as the primary interface between the outside environment and the body. Although they are opportunistic sites for pathogen entry, they are also home to a dense array of microbial populations. The total number of these microorganisms exceeds the number of cells in the body by at least an order of magnitude (Garrett et al.). Far from bystanders, these microorganisms typically forge a commensal relationship with the host. Nowhere is this more apparent than in the gastrointestinal tract, which contains, by far, the densest microbial niche in the body. Studies using germ-free or gnotobiotic, as well as, antibiotic treated animals have highlighted the crucial role of the microbiota in the regulation of a broad range of processes, which include: 1) *Metabolism* via degradation of otherwise indigestible products and nutrient absorption (Hooper and Gordon, 2001; Hooper et al., 2002). 2) *Intestinal tissue development* via the elaboration of underlying microvasculature and isolated lymphoid follicles, respectively (Bouskra et al., 2008; Stappenbeck et al., 2002). 3) *Intestinal tissue repair and maintenance* via regulation of growth factor expressing cells (Brown et al., 2007; Rakoff-Nahoum et al., 2004). 4) *Immunity* via growth and proper development of secondary lymphoid tissue (Pollard and Sharon, 1970; Stepankova et al., 1980). 5) *Protection against opportunistic microbial invasion* via elicitation of microbicidal proteins from epithelial cells, most prominently, Paneth cells, which reside at the base of intestinal crypts (Vaishnava et al., 2008); (Brandl et al.,

2008). Release of these microbicidal proteins also regulates the composition of the commensal microbiota under steady-state conditions (Salzman et al.).

Despite the plethora of benefits conferred onto the host by the presence commensal microbiota, it's important to bear in mind that its compartmentalization is central to maintaining a long-term advantage to the host, with impairments provoking an immunological imbalance that can drive aberrant local immune responses, such as inflammatory bowel disease (Nenci et al., 2007), and in other instances, distal or even systemic immune responses, the most extreme example being sepsis (Cohen, 2002).

While the commensal barrier is maintained, in part, through goblet cell secretion of an overlaying mucus layer and a dynamic and resilient epithelial layer (Johansson et al., 2008; Turner, 2009), other cell populations interdigitated within or beneath this barrier also play an essential role. These populations, which include cells of both the innate and adaptive arms of immunity, have evolved in close association with the commensals and in contact with their stimulatory ligands. Consequently, immune cells protecting this region, and by extension other mucosal sites, have acquired specialized functions to facilitate coexistence with the commensals, while maintaining the capacity to induce a potent immune response in the event of a pathogenic encounter. The execution of these dichotomous functions also contributes to the commensal composition, which is a critical factor in the establishment and maintenance of intestinal immune homeostasis (Fagarasan et al., 2002; Garrett et al.; Garrett et al., 2007).

In addition to aberrant immune reactivity against the commensals, aberrant reactivity against dietary antigen also poses a substantial risk to intestinal immune homeostasis and can promote a variety of outcomes, ranging from tissue destruction in the case of celiac

disease to immunoglobulin E mediated anaphylaxis in the case of peanut allergy (Izcue et al., 2006; Jabri and Ebert, 2007; Li et al., 2000). Thus, the orchestration of tolerance to food-borne antigen likely hinges on similar, if not, redundant immunological mechanisms with those that are in place to regulate the presence of commensals.

The seemingly disparate tasks of the immune system protecting the GI tract, then, are to maintain a state of détente with commensals and food antigens, while retaining the capacity to mount a swift immune response upon pathogen encounter. Further, this must be achieved in a manner that avoids grossly jeopardizing host tolerance to commensals and food antigen. To begin to consider the cells and interactions that are involved in carrying out these duties it is important to understand the basic anatomy and organization of the GI tract and its associated lymphoid tissues. For conciseness and simplicity, the description to follow is intended to provide a framework for considering the complex and dynamic regulation of immunity in the small intestine. This tissue is of primary focus in the research to be described herein.

The intestinal immune system is composed of inductive and effector sites

Broadly, the immune system of the gastrointestinal (GI) tract and the associated lymphoid tissues (GALT) can be separated into inductive sites and effector sites (**Figure 1**).

I. **Inductive sites**, including the mesenteric lymph nodes (mln), which drain the GI tract, and organized lymphoid aggregates called Peyer's patches (Pp). These structures increase in frequency as the density of the commensal microbiota increases from the proximal duodenum to the

terminal ileum, underscoring their dependence on interactions with commensals (Neutra et al., 2001).

II. **Effector sites**, which include the intestinal epithelial lymphocyte (IEL) and lamina propria (Lp) compartments (Mowat, 2003; Newberry and Lorenz, 2005).

I. Lymphocyte activation in inductive sites

Under steady-state conditions, naive T cells constitutively enter into the Pp and mln from blood via high endothelial venules (HEV) (Jalkanen et al., 1987). Upon exit from HEVs, these cells may come into contact with dendritic cells (DC), which have captured and processed their cognate antigen (Ag) (Bajenoff et al., 2003). Ag is delivered into Pp usually via microvilli-lacking, specialized epithelial cells, called M cells. Upon trancytosis or endocytosis, these antigens are captured by DCs residing within the sub-epithelial dome (Cerutti and Rescigno, 2008). While Ag may also be captured in this manner within the Lp, upon entry into isolated lymphoid follicles (Jang et al., 2004; Vallon-Eberhard et al., 2006), Ag can also be actively captured by DC that extend protrusions into the intestinal lumen; however, the physiological importance of this latter mode of capture remains controversial (Chieppa et al., 2006; Niess and Reinecker, 2005; Rescigno et al., 2001). Ag-bearing DCs from the Lp, then, migrate via the afferent lymphatics into the mln, where a cognate T cell potentially lies in wait. Intriguingly, the subset of LpDC that undergo migration ($CD103^+$) is reportedly different from those capable of extending processes into the intestinal lumen ($CX3CR1^+CD103^-$) (Bogunovic et al., 2009; Schulz et al., 2009); however, it remains unclear whether $CD103^+$ LpDC are also capable of this function (Chieppa et al., 2006).

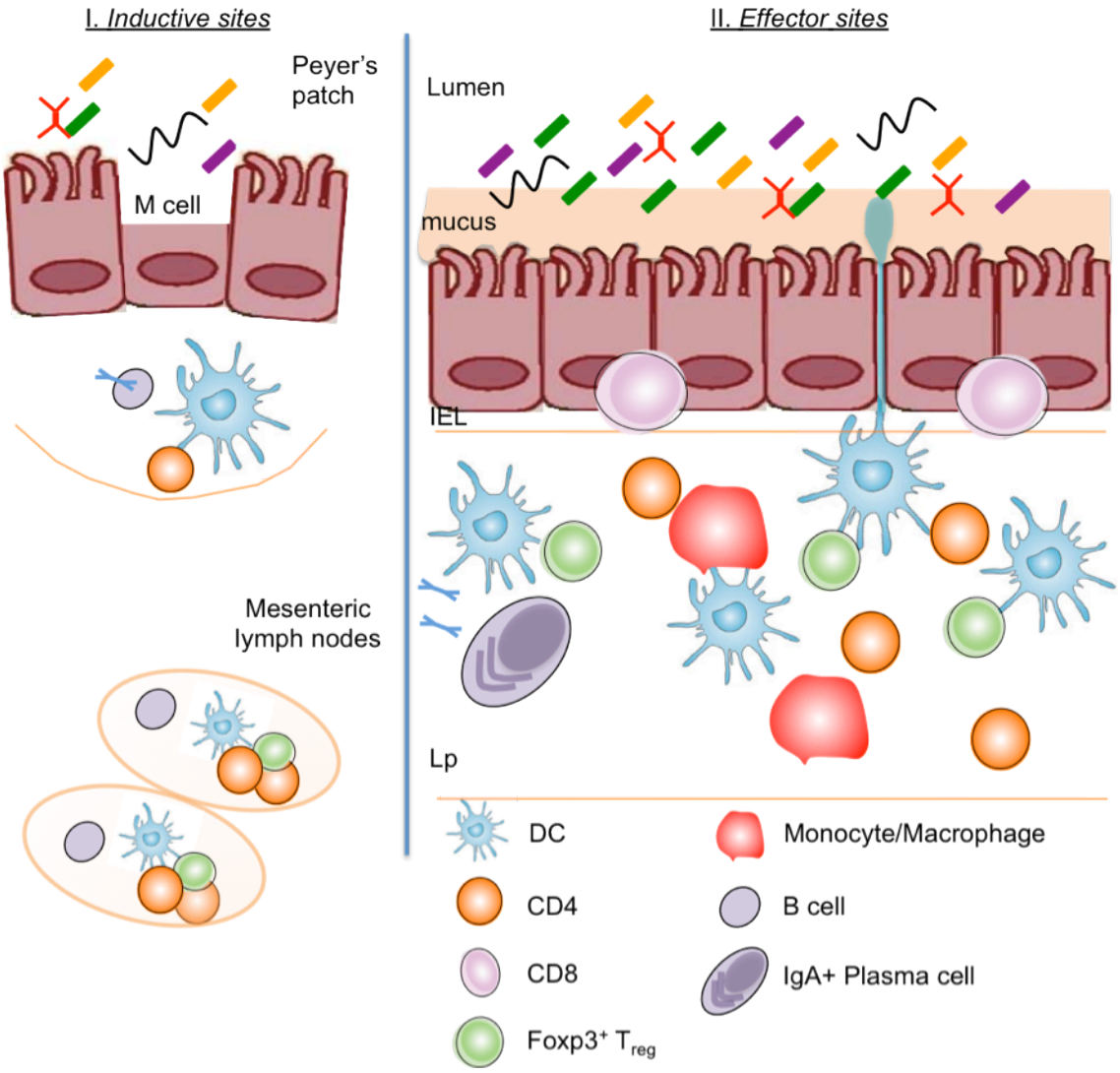
During activation in the Pp and mln, $CD4^+$ T cells receive signals that result in the upregulated expression of mucosal homing receptors including the chemokine receptor CCR9 and the integrin heterodimer, $\alpha_4\beta_7$, which facilitate their migration to and retention in the Lp (Berlin et al., 1993; Iwata et al., 2004; Johansson-Lindbom et al., 2003; Kunkel et al., 2000; Mora et al., 2003). Activated $CD4^+$ T cells within Pp can also undergo directed migration from T cell areas toward B cell areas and, in turn, induce immunoglobulin A (IgA) class switching in B cells, which then migrate into the Lp to undergo full differentiation into IgA secreting plasma cells (Allen and Cyster, 2008; Fagarasan et al.). This process also occurs independently of T cells *in situ*, specifically within ILFs (Tsuji et al., 2008). Although they were not investigated in the studies to be described herein, IgA^+ B cells have potent regulatory capacity and shape the luminal composition of the commensal microbiota (Fagarasan et al., 2002).

II. Lymphocyte organization in effector sites

In spite of their adjacent locations, the IEL and Lp compartments are, in fact, comprised of quite distinct lymphocyte populations during steady-state. One obvious distinction is the presence of $\gamma\delta$T cells in the IEL, which are largely absent in the Lp (Bucy et al., 1988; Bucy et al., 1989). These cells were previously shown to promote intestinal epithelial cell (IEC) survival and maturation via incompletely defined mechanisms (Chen et al., 2002; Komano et al., 1995). Another striking distinction between the IEL and Lp is the MHC class restriction of $\alpha\beta$ T cells: while most in the IEL are MHC I or MHC I-like restricted and are $CD8^+$, the majority in the Lp are MHC II restricted and $CD4^+$ (Bonneville et al., 1988; Chiba et al., 1986; Park et al., 1999). Nevertheless, MHC II restricted cells, such as $Foxp3^+$ regulatory T cells, are still present in this compartment and serve important function. The immunological basis for this

dichotomy has several potential explanations. Notably, $CD8^+$ cells are known to engage in a vivid dialogue with the IEC barrier and can dispose of infected/damaged IECs via the release of cytolytic granules without, necessarily, the concomitant release of pro-inflammatory cytokines (Hayday et al., 2001; Jabri and Ebert, 2007). This feature implies that these cells are ideal sensors of tissue distress during steady-state conditions. However, in situations of extreme tissue distress, for instance, upon encounter with an alimentary allergen or infectious pathogen, resident $CD8^+$ T cells may expand and become potent producers of pro-inflammatory cytokines (Jabri and Ebert, 2007). Moreover, such scenarios also foster the activation and recruitment of naive $CD8^+$ $\alpha\beta$ T cells from inductive sites.

Figure 1. Representation of the intestinal mucosal environment and several of the important mediators of innate and adaptive immune responses



LpDC actively sample luminal content:

While one could argue that the primary duty of the IEL compartment is to avoid direct engagement with antigen, the Lp compartment appears poised for active sampling of the luminal environment during steady-state conditions. Indeed, the Lp is host to a sizable population of antigen presenting cells (APC), which include resident macrophages, as well as $CCR7^{-}$ and $CCR7^{+}$ resident and migratory DCs, respectively (Bogunovic et al., 2009; Chirdo et al., 2005; Jang et al., 2006; Johansson-Lindbom et al., 2005; Mayrhofer et al., 1983; Schulz et al., 2009). Previous studies have shown that specific subpopulations of $CD11c^{+}$ MHC II^{+} cells, which may also encompass macrophages in this tissue (Denning et al., 2007; Uematsu et al., 2008), are able to extend balloon-like protrusions into the luminal space (Chieppa et al., 2006; Niess and Reinecker, 2005). In this regard, the resident $CX3CR1^{+}$ subpopulation ($CCR7^{-}$ $CD103^{-}$) which was the first population characterized to display this property in an intact tissue, was revealed to readily take up both fluorescently-labeled non-pathogenic bacteria and food antigen from the intestinal lumen (Niess and Reinecker, 2005; Schulz et al., 2009). Although migratory $CCR7^{+}CD103^{+}$ LpDC are less phagocytic than their $CX3CR1^{+}CCR7^{-}$ counterparts (Brian Kellsall, personal communication), this sub-population was found to possess inclusions of apoptotic cells, including epithelia, upon transit into the mln (Huang et al., 2000). It was recently speculated that migratory LpDC also acquire luminal-captured antigen from resident $CX3CR1^{+}CCR7^{-}$ DC via a mechanism that remains to be clarified (Rescigno, 2009). Cumulatively, these findings indicate that the Lp compartment, via resident and migratory APC, constantly surveys the luminal environment. These processes likely play a significant role in the composition of $CD4^{+}$ T cells residing within the Lp.

APC are influenced by IEC interactions at the barrier interface:

Another element to consider is the effect of the luminal content on the exchange of information between the IEC and Lp compartment. This conversation is likely dependent on the sensing of microbial components relayed by pattern recognition receptors, including: 1) intracellular nucleotide-binding-oligomerisation domains (Nod), which recognize muramyl dipeptides derived from bacterial cell wall components, and 3) extracellular and intracellular toll-like receptors (TLR), which recognize an array of structurally conserved molecules derived from microbes (Garrett et al.; Lavelle et al.). Nods, in particular Nod2, may influence microbiota composition via constitutive Paneth cell secretion of antimicrobial peptides (Kobayashi et al., 2005; Petnicki-Ocwieja et al., 2009). TLRs signal through the adaptor proteins TRIF and/or MyD88 (Yamamoto et al., 2004). While TLR3 and TLR4 can utilize TRIF, all other TLRs, including TLR4, utilize MyD88, which is recruited following recognition of: lipopeptides (TLR2/1 and TLR2/6) peptidoglycans (TLR2), lipopolysaccharides (TLR4), flagellin (TLR5), ssRNA (TLR7/8) bacterial and viral DNA (TLR9) (Kawai and Akira). Importantly, the localization, expression, and regulation of each of these TLRs vary in the IEC and, therefore, may differentially contribute to intestinal immune homeostasis (Artis, 2008). Nevertheless, using a more encompassing strategy, Germain and colleagues found that the ability of LpDC to sample the luminal small bowel was dependent on MyD88 signaling in the epithelia (Chieppa et al., 2006). Thus, the rate at which LpDC sample the luminal environment is likely tailored to the types of constituents present in the microbiota. As such, one could imagine this mechanism especially coming into play when compositional shifts in the commensals occur, such as during infection or inflammation (Finlay and

Medzhitov, 2007; Heimesaat et al., 2006). Another example of IEC/Lp cross talk is the influence of IEC secreted thymic stromal lymphopoietin (TSLP). This cytokine inhibits the capacity of LpDC to induce proinflammatory cytokines (Rimoldi et al., 2005; Zaph et al., 2007). Notably, the role of the commensal microbiota on the regulation of TSLP production remains unclear. However, one could speculate that it may inversely correlate with the pathogenicity of the microbiota.

GALT activated lymphocytes are imprinted to traffic to intestinal effector sites:

A general feature of activation in Pp and mln is T and B lymphocyte upregulation of $\alpha_4\beta_7$ and CCR9, which equips these cells with the superior capacity to migrate into intestinal effector tissues (Mora et al., 2008; Sigmundsdottir and Butcher, 2008). Specifically, $\alpha_4\beta_7$ enables activated lymphocytes to bind to mucosal vascular addressin cell-adhesion molecule-1 (MadCAM-1) present on post capillary venules in the intestinal Lp (Bargatze et al., 1995; Wagner et al., 1996). CCR9 reinforces adherence to post capillary venules via binding to the chemokine, TECK (CCL25), which is generated and secreted by small intestinal epithelia (Hieshima et al., 2004; Kunkel et al., 2000). Strikingly, the upregulation of $\alpha_4\beta_7$ and CCR9 is critically dependent on nutrient metabolism of vitamin A, which will be discussed in greater detail further on (Iwata et al., 2004; Mora et al., 2006).

$CD4^+$ T cell composition in the Lp:

The composition of $CD4^+$ T cells in the small intestinal Lp compartment is quite heterogeneous and strongly reflects: I) the cytokines produced by the local tissue and II) the types of antigens constantly available for survey in the intestinal lumen, which likely

also influence the local cytokine milieu. Indeed, antigen sampling features heavily in this region, and plays a probable role in the control of both steady-state tolerance and responsiveness to pathogenic insult. In order to proceed with this crucial function intact, the Lp tissue requires T cells with both immunoregulatory and sentinel effector capacities. (I) On the immunoregulatory hand, this includes regulatory T cells (T_{reg}), which, in particular express the transcription factor, forkhead box protein 3 (Foxp3). (II) On the effector hand, this includes T-Helper (T_H) cells capable of producing pro-inflammatory cytokines, in particular IFN-γ (T_H-1) and IL-17A (T_H-17). **As discussed below, the balance of these opposing elements may exert a crucial influence on the ability of the host to mount an immune response when this site is challenged.** The defining features of these cells, including their induction and their relevance to mucosal immunity will be outlined in the proceeding sections.

I. Foxp3$^+$ regulatory T cells (Foxp3$^+$ T_{reg})

Foxp3$^+$ T_{reg}, which in most secondary lymphoid tissues during steady-state can be distinguished by constitutive expression of the high-affinity IL-2Rα (CD25), are key mediators of the coordination and maintenance of peripheral tissue homeostasis throughout the lifespan of the host (Bennett et al., 2001; Fontenot et al., 2003; Hori et al., 2003; Kim et al., 2007; Wildin et al., 2001). Their absence culminates in fatal inflammatory lesions propagated by the activation and proliferation of self-reactive clones that have escaped negative selection in the thymus (Sakaguchi et al., 2008). Thus, even in the absence of a commensal microbiota, ablation of Foxp3$^+$ T_{reg} results in fatal autoimmunity, albeit with slower kinetics and altered pathogenesis (Chinen et al.). Nevertheless Foxp3$^+$ T_{reg} are still crucial for the prevention of spontaneous reactivity against both the commensal microbiota and food antigen and play an important role in

regulating the intensity of immune responses to foreign pathogens (Izcue et al., 2006; Josefowicz and Rudensky, 2009). They comprise typically 20% of the $CD4^+$ T cell population within the small intestinal Lp and a variable proportion actively produces the potent immunoregulatory cytokine, IL-10, which can also be expressed by Foxp3 cells in this tissue (Maynard et al., 2007). Given their widespread influence, $Foxp3^+$ T_{reg} are not particularly limited in the means by which they can perform their regulatory duties, which include but are not limited to: (**1**) secretion of IL-10 and/or TGF-β(Belkaid, 2002; Li et al., 2007; Rubtsov et al., 2008; Zhang et al., 2009a), (**2**) contact dependent suppression of APC function (Tadokoro et al., 2006; Tang et al., 2006), (**3**) control of DC homeostasis (Liu et al., 2009), (**4**) and abrogation of certain effector functions through an as yet defined mechanism (Mempel et al., 2006). Importantly, none of these mechanisms are mutually exclusive (**Figure 2**).

To date, the precise signals that govern the induction of the $Foxp3^+$ T_{reg} program remain an enigma (Gavin et al., 2007; Lin et al., 2007). Since, seminal thymectomy experiments indicated that thymic development of $Foxp3^+$ T_{reg} was required for the maintenance of self-tolerance (Sakaguchi and Sakaguchi, 1990), many studies have looked to the thymus for clues into the differentiation pathway of these cells. In this regard, several elegant studies have revealed that $Foxp3^+$ T_{reg} can develop and survive negative selection in the presence of strong agonist ligands (Jordan et al., 2001; van Santen et al., 2004). On the other hand, in the absence of a negatively selecting agonist ligand, which can be achieved experimentally upon crossing a naive TCR transgene with a recombination activating gene deficient ($RAG^{-/-}$) mouse, these cells do not develop intrathymically (Walker et al., 2003). Such mice, in turn, have served as ideal tools to assess the extrathymic development of $Foxp3^+$ T_{reg}.

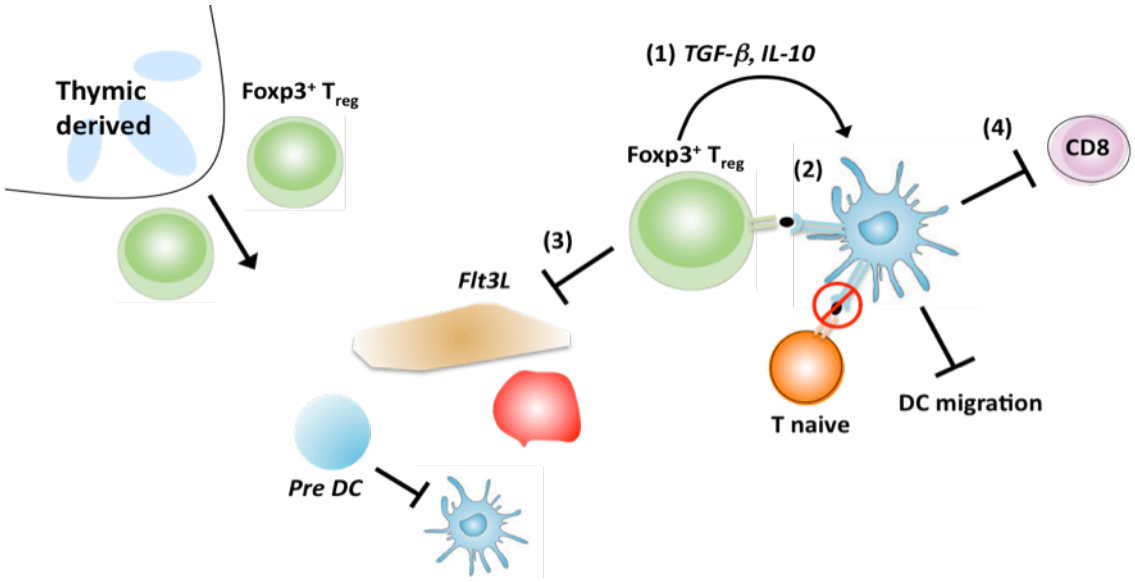


Figure 2. Foxp3$^+$ T$_{reg}$ regulate immune responses through multiple pathways
(**1**) Foxp3$^+$ T$_{reg}$ produce immunoregulatory cytokines, including TGF-β and IL-10. (**2**) Their interactions with dendritic cells (DC) can prevent stable interactions between naïve T cells and antigen presenting DC. They can also inhibit immature DC maturation and/or migration. (**3**) Foxp3$^+$ T$_{reg}$ inhibit the production of FMS-like tyrosine kinase ligand (Flt3L), which promotes DC maturation from precursor Pre DC. (**4**) They also can prohibit certain effector functions, including the capacity of CD8$^+$ cytotoxic T cells to kill. This mechanism of suppression requires TGF-β signaling.

In an effort to determine whether Foxp3$^+$ T_{reg} development could occur extrathymically *in vivo*, Von Boehmer and colleagues administered prolonged sub-cutaneous low-dose infusions of hemagglutinin (HA) peptide to thymectomized, RAG$^{-/-}$ x TCR-HA mice. This technique induced a long-lasting CD25$^+$ population that concomitantly expressed high levels of Foxp3 transcript and possessed suppressive properties similar to Foxp3$^+$ T_{reg} (Apostolou and von Boehmer, 2004). Another study, in which OVA-specific, Foxp3$^-$ CD4$^+$ DO11.10 T cells were transferred into lymphopenic hosts, engineered to secrete OVA, yielded similar findings (Knoechel et al., 2005). Using a more refined strategy, subimmunogenic targeting of peptides to DCs was also shown to induce antigen specific Foxp3$^+$ T_{reg} (Kretschmer et al., 2005). Altogether, these findings suggest that chronic exposure to low doses of antigen and DC presentation in non-inflamed settings can promote the *de novo* acquisition of Foxp3 by peripherally activated cells. These criteria bear remarkable similarity to what one may consider "status quo" in the GI tract, where, in fact, oral feeding of OVA was also shown to induce CD25$^+$ with high transcript levels of Foxp3 (Mucida et al., 2005). However, the immune cells responsible for the coordination of this mechanism were not investigated.

Are DC in the GI tract specially equipped to foster the differentiation of Foxp3$^+$ T_{reg} from naïve precursors?

The cytokine, transforming growth factor-beta (TGF-β) is a heavily regulated cytokine (Annes et al., 2003), which plays a dominant role in immune homeostasis. In addition to promoting thymic Foxp3$^+$ T_{reg} survival and maintaining naïve T cell quiescence, it's been showcased to potently induce Foxp3$^+$ T_{reg} differentiation from naïve CD4$^+$ T cells upon stimulation, *in vitro* (Chen et al., 2003; Li et al., 2006; Marie et al., 2006; Ouyang et al.). TGF-β is also produced in abundance by IEC; this source

potentially serves a non-redundant function in maintaining good relations with the host microbiota and dietary antigen (Barnard et al., 1993; Dignass and Podolsky, 1993). More recent studies have revealed that APC upon phagocytosis of apoptotic cells are also able to produce TGF-β (Perruche et al., 2008; Torchinsky et al., 2009). Assuming that the apoptotic inclusions present in their cytoplasm are a result of phagocytosis, migratory $CD103^+$ LpDC may also be endowed with the capacity to produce this cytokine. Further, this implies that this population may be uniquely poised to induce $Foxp3^+$ T_{reg} under physiological settings. **In Chapter 2, this possibility will be investigated and the role of other mucosal factors in the induction of Foxp3 will be considered.**

II. T-Helper cells in the Lp compartment

Foreign microbial challenge results in the elicitation of an adaptive immune response, which accompanies $CD4^+$ T helper (T_H) cell differentiation. Analogous to this type of challenge, the presence of the commensal microbiota and dietary antigen in the GI tract also elicits an adaptive immune response, resulting in the sustained recruitment of IgA^+ B cells and various T_H subsets into in the Lp (Atarashi et al., 2008; Klaasen et al., 1993; Mazmanian et al., 2005). Until recently, the categories of T_H subsets were cast in essentially binary terms, namely T_H-1 and T_H-2, based on the classic experiments of Coffman and colleagues (Mosmann et al., 1986). However, recent studies have upended this paradigm, leading to the inclusion of several new subsets. While further study is necessary to reconcile these newer subsets, which appear to retain less committed features than their foregoing T_H-1 and T_H-2 counterparts (Locksley, 2009), one subset has emerged as quite an important player in infectious disease and mucosal immunity.

This subset of cells, referred to as T_H-17, will be discussed along with T_H-1 and T_H-2 cells.

T_H-1 cells are defined by production of IFN-γ and canonical expression of the transcription factor, T-bet (Mullen et al., 2001; Szabo et al., 2000). They play instrumental roles in priming and sustaining inflammatory macrophage function (Hu and Ivashkiv, 2009), and as such help mediate adaptive immune responses against a range of intracellular pathogens, including, those which infect the host through the alimentary route, such as microsporidia and *Toxoplasma gondii* (Casciotti et al., 2002; Moretto et al., 2004). T_H-2 cells are defined by production of IL-4, IL-5 and IL-13 and canonical expression of the transcription factor GATA-3 (Loots et al., 2000). They elicit an "alternatively" activated macrophage program that is linked with tissue repair and mediate protective immune responses against helminths (Maizels et al., 2009; Mosser and Edwards, 2008). Further, they are commonly associated with allergic responses (Kim et al.). In contrast to T_H-1 development, previous studies have suggested that normal commensal microbiota derived signals actively constrain steady-state T_H-2 development (Mazmanian et al., 2005; Troy and Kasper). Consistent with these reports, broad-spectrum antibiotic treatment was revealed to trigger oral sensitization to a food allergen (Bashir et al., 2004). However, a more recent study found that cells from the terminal ileum of conventionally reared mice readily secreted T_H-2 cytokines, including IL-4 and IL-13, upon polyclonal T cell restimulation (Gaboriau-Routhiau et al., 2009). Thus, whether the commensal microbiota actively restrains T_H-2 cell development is a matter of debate.

T_H-17 cells are associated with the production of the cytokines: IL-17-A, IL-17F, IL-21 and IL-22 (Khader et al., 2009). While IL-17A and IL-17F are strictly dependent on

the transcription factor, Retinoic acid receptor-related orphan nuclear receptor gamma (RORγ) and, to a much lesser extent, RORα, IL-21 and IL-22 can be generated independently of these transcriptional programs (Ivanov et al., 2006; Korn et al., 2007; Nurieva et al., 2007; Yang et al., 2008; Zhou et al., 2007). Of interest, IL-22 production by T_H-17 cells is strictly dependent on the environmental toxin-activated, transcription factor, aryl hydrocarbon receptor (AHR), which was also demonstrated to amplify production of IL-17A and IL-17F (Veldhoen et al., 2009; Veldhoen et al., 2008a). Shortly after the discovery of IL-17 (IL-17A, specifically) it became associated with human autoimmune syndromes, such as Rheumatoid Arthritis and Lupus (Kotake et al., 1999; Kurasawa et al., 2000; Ziolkowska et al., 2000). In mice, as well, these cells have been pegged as pivotal instigators of autoimmunity and have an extraordinary capacity to both stimulate the release and production of inflammatory mediators from myeloid cells and to recruit innate cells, such as neutrophils to sites of inflammation (Jovanovic et al., 1998; Ye et al., 2001). This has been amply demonstrated in mouse models of collagen induced arthritis and, in particular, experimental autoimmune encephalitis, which has been exploited to great lengths, resulting in an onslaught of molecular insights into the pathways that control the production of IL-17 (Hirota et al., 2007; Ivanov et al., 2006). As such, it is now clear that TGF-β signaling in the context of inflammatory mediators, such as IL-6, IL-1 and IL-21 are critical for T_H-17 differentiation, while IL-23 regulates its effector function *in vivo* (Ahern et al.; Bettelli et al., 2006; Korn et al., 2007; Mangan et al., 2006; McGeachy et al., 2007; McGeachy et al., 2009; Nurieva et al., 2007; Veldhoen et al., 2006; Zhou et al., 2007). Although IL-23 shares an IL-12p40 subunit with IL-12, which drives TH-1 inflammation, this cytokine regulates distinct pathways (Cua et al., 2003; Murphy et al., 2003; Trinchieri et al., 2003). Similar to the reciprocal inhibition of T_H-1 and T_H-2, both IL-4 and IFN-γ potently suppress T_H-17 differentiation (Harrington et al., 2005). However, pathogenic T_H-17 cells, at least, also appear to retain some T_H-1

like features, and can, in fact, morph into IFN-γ producing cells during inflammation (Lee et al., 2009).

Despite the association of this subset with auto-inflammatory disorders, multiple studies have revealed that T_H-17 cells serve as critical effectors against fungal, extracellular bacterial, and in some instances, intracellular bacterial infections (Acosta-Rodriguez et al., 2007; Aujla et al., 2008; Conti et al., 2009; Happel et al., 2005; Ishigame et al., 2009; Khader et al., 2009; LeibundGut-Landmann et al., 2007; Lin et al., 2009; Pitta et al., 2009; Saijo et al.; Zheng et al., 2008). Among their protective functions, IL-17A, IL-17F and IL-22 each have been shown to mediate epithelial cell production of anti-microbial peptides, including β-defensins (Conti et al., 2009; Ishigame et al., 2009; Kao et al., 2004; Wolk et al., 2004) and lectins (Sanos et al., 2009; Zheng et al., 2008). Bearing this in mind, IL-17A secreting T_H-17 cells, a proportion of which also express IL-22 (Ivanov et al., 2009), are typically detected in the small intestinal Lp during steady-state and express the chemokine receptor, CCR6, which favors their proximity to IEC (Ishigame et al., 2009; Ivanov et al., 2008; Nagler-Anderson, 2001). Thus, while clearly in possession of potent inflammatory capacity, in non-inflamed conditions T_H-17 cells seem to be well integrated into the intestinal immune system's microbial containment strategy. Although not the focus of the work to be described, it is worth mentioning that other studies that have examined innate cells, such as γδT cells, lymphoid tissue inducer - like (Lti-like) cells, NK, NKp46$^+$ NK-like cells, and even Paneth cells have noted their capacity to produce IL-17 (γδT, NK*, Lti-like*, Paneth cells*; *upon inflammatory stimulation) and/or IL-22 (γδT*, Lti-like*, NKp46$^+$; *upon inflammatory stimulation) in various contexts, further emphasizing the importance of these cytokines to mucosal barrier regulation (Cua and Tato). Indeed, innate sources of IL-22 were

recently revealed to be absolutely required for the early phase of protection against bacterial pathogens that attach to and efface the IEC barrier (Zheng et al., 2008)

III. Foxp3$^+$ T_{reg}/T_H populations are in a dynamic equilibrium

The preceding description may lead to the misapprehension that the immune system protecting the GALT is strictly designed for coping with the everyday stresses imposed by the commensal microbiota and food antigen; however, this site is also one of the most convenient ports of entry for foreign pathogen, and, therefore, must also be equipped with the capacity to stage a swift and simultaneous immune response. One possibility is that the steady-state immunological landscape in the IEL and Lp compartments has negligible bearing on how the host will respond to foreign microbial challenge. However, such an untethered strategy in this region could prove inefficient and impinge on the capacity of the host to mount a timely and robust immune response. Further, it could potentially disrupt the sensing mechanisms that are in place to maintain and/or restore mucosal homeostasis in the event of a pathogenic challenge. Another consideration, which casts further doubt on this possibility, is that the mucosal immune system is alerted to the presence of a pathogen via the same pattern recognition receptors that are engaged by the commensal microbiota (Sansonetti and Di Santo, 2007). Rather, the alternative hypothesis - that the ongoing steady-state immune response in the GI tract heavily influences the ability of the host to respond to a pathogenic threat – is more likely.

APC within the Lp compartment express a variety of pattern recognition receptors, particularly among the TLR family, suggesting that they can receive

instructive signals directly from the commensal microbiota (Coombes et al., 2007; Uematsu et al., 2008). The precise nature of these signals and their influence on the regulation and distribution of Foxp3$^+$ T_{reg} and T_H subsets within the Lp remain obscure. In view of their sensing, migratory, and priming characteristics, LpDC are potentially strongly equipped for processing and dispatching these cues to CD4$^+$ T cells and, in turn, influencing the Foxp3$^+$ T_{reg} /T_H landscape within the Lp. **In Chapter 3 this possibility will be explored, first, using an *in vitro* assay to identify potential components of the microbiota, which impose natural constraints on the Foxp3$^+$ T_{reg} axis in the GALT.**

One paradigm that has emerged to provide a basic understanding of the homeostatic regulation of T cell subsets in this region is the documented reciprocal nature of Foxp3$^+$ T_{reg} and T_H-17 cells. Both are dependent on TGF-β for their induction and/or survival (Bettelli et al., 2006; Liu et al., 2008; Manel et al., 2008; Mangan et al., 2006; Ouyang et al.; Veldhoen et al., 2006; Volpe et al., 2008) however, where Foxp3$^+$ T_{reg} cells seem inclined to mediate the suppression of inflammatory cascades, T_H-17 cells seem inclined to do just the opposite. Their Janus-like relationship is also apparent in other contexts. In terms of gene regulation, Foxp3 was shown to sequester RORγ and to prevent its binding to DNA, while diversion of cells from the Foxp3$^+$ T_{reg} lineage was shown relieve cells of their inability to produce IL-17 (Gavin et al., 2007; Zhou et al., 2008). In terms of cytokine regulation, *IL-6*$^{-/-}$ mice were shown to lack T_H-17 cells in their Lp, but harbored an enhanced frequency of Foxp3$^+$ T_{reg} (unpublished finding) (Korn et al., 2007). On the other hand, *IL-2*$^{-/-}$ mice were shown to be unable to sustain Foxp3$^+$ T_{reg} cells in their periphery, but harbored an enhanced frequency of T_H-17 cells (D'Cruz and Klein, 2005; Fontenot et al., 2005; Laurence et al., 2007).

Taking into account what is known about the intestinal mucosal environment: abundant TGF-β, a large proportion of Foxp3$^+$ T_{reg}, and the constitutive presence of T_H-17 cells, these findings suggest that the indigenous microbiota may be able to leverage this delicate relationship to shape the inflammatory tone of the tissue. Further, when pathogenic microbial signals converge onto these indigenously derived signals, skewing away from the Foxp3$^+$ T_{reg} axis of control may be amplified. In this regard, Littman and colleagues recently demonstrated that a commensal microbial community, which promotes a steady-state skewing toward the T_H-17 subset, can potentiate immune responses to the T_H-17 eliciting bacterial pathogen, *Citrobacter rodentium* (Ivanov et al., 2009). This study also identified a microbial species known as segmented filamentous bacteria (SFB) that was particularly potent at inducing a prominent T_H-17 subpopulation (Ivanov et al., 2009). However, the precise stimulatory pathways utilized by SFB to promote T_H-17 activity were not completely clarified. **Indeed, the individual contributions of discrete signaling pathways, which are engaged by specific microbial component/pattern recognition receptor interactions, to the regulation of intestinal homeostasis and mucosal immunity are unclear.**

Insofar as this paradigm applies to the Foxp3$^+$ T_{reg} and T_H-17 subsets of T cells, it does not sufficiently encompass the heterogeneity of the microbial communities that are constitutively present in the GI tract, which also should add to T_H diversity and potentially the functional characteristics of Foxp3$^+$ T_{reg} in the Lp compartment. Indeed germ-free mice that were reconstituted with a commensal microbiota from naturally colonized mice displayed dramatic increases in both T_H-1 and T_H-17 subsets within the ileal Lp compartment (Gaboriau-Routhiau et al., 2009). Moreover, commensal derived components, such as the polysaccharide of *Bacteroides fragilis*, have been shown to regulate the T_H-1/T_H-2 balance during steady-state (Mazmanian et al., 2005). Thus the

commensal microbiota was also demonstrated to affect sensitivity to food borne allergies (Bashir et al., 2004). Regarding Foxp3$^+$ T_{reg} cells, specific microbial interactions have been proposed to augment their regulatory activity, including TLR 4 and 5 (Caramalho et al., 2003; Crellin et al., 2005), while others involving TLR 2, 8 and 9 have been proposed to restrain their regulatory activity (Larosa et al., 2007; Liu et al., 2006; Pasare and Medzhitov, 2003; Peng et al., 2005; Sutmuller et al., 2006). **Altogether, these data provide compelling evidence that the composition of the microbiota is able to exert a strong influence on the T cell landscape of intestinal effector sites, which in turn may condition the efficiency of the mucosal response to an invasive pathogen. This concept will be examined in Chapter 3.**

Convergence of nutrient metabolism with the mucosal immune system:

The essential function of the GI tract is to digest food and extract nutrients, some of which are otherwise inaccessible in the absence of the commensal microbiota (Wikoff et al., 2009). Accumulating evidence reveals that the intestinal immune system has adapted to this process through utilization of nutrient metabolites. Similar to microbial driven signals, nutrient metabolite driven signals appear to serve as key moderators of the immunological conversation taking place at mucosal barrier interfaces. In many ways, they are also as well tailored as microbial signals to provide the host with a local immunological advantage. For instance, butyrate, which is a short-chain fatty acid produced by the commensal microbiota upon metabolism of dietary fiber, was shown to inhibit the multiplication of pathogenic enteric bacteria and quelled the inflammatory profile of monocytes, *in vitro* (Bohnhoff et al., 1964; Saemann et al., 2000). Catabolism of the essential amino acid tryptophan, which occurs constitutively in the GALT, was shown to limit basal Ab production to commensal microbiota (Harrington et al., 2008). Recent data also support the hypothesis that tryptophan catabolism positively regulates

the $Foxp3^+$ Treg/T_H-17 ratio (Favre et al.). Another nutrient that especially bares this point is vitamin A and, in particular, its metabolic derivative, retinoic acid.

Vitamin A is a critical nutrient for the GALT immune system:

In the early 20th century E.V. McCollum discovered that certain lipids contained a factor essential for growth, which he termed "fat soluble factor A" or, Vitamin A (Mc, 1952). Mammals and most higher order organisms are dependent on dietary ingestion for acquisition of this vitamin. Its sources include carotenoids, which are present in fruits and vegetables and retinyl esters, which are abundant in meats and poultry. These compounds are broken down into retinol **(1)** for absorption by the small intestine and subsequently esterified for storage in the liver (Harrison, 2005; Mc, 1952).

Retinoic acid can be generated or obtained through multiple pathways:

Retinoic acid (RA) is the end product of a series of enzymatic steps in Vitamin A's metabolism. When required, retinyl esters are hydrolyzed into retinol and deployed into circulation along with its chaperone, retinol-binding protein (RBP) (Wolf, 2007). It's uptake into cells, including IEC and APC, is mediated by the surface receptor, STRA6, which binds with high affinity to RBP (Kawaguchi et al., 2007). Once in the cytosol, the ubiquitously expressed family of alcohol dehydrogenases (ADH) **(2)** reversibly catalyzes the oxidation of retinol to retinal (Mic et al., 2003). Retinal dehydrogenases (RALDH) **(3)** then bind to retinal and catalyze the final and irreversible step in RA synthesis. In the GALT, this predominantly results in all-trans RA, which can eventually isomerize to its 9-cis form. However, this form has yet to be detected *in vivo* (Mic et al., 2003). Notably, RALDH expression is cell and tissue specific, making RA synthesis a controlled process

(Iwata et al., 2004). While the processes regulating RALDH induction are unclear, it was recently demonstrated that lipid mediators, which are widely present in the GALT, induce RALDH2 through the fatty acid binding receptor, PPAR-γ (Szatmari and Nagy, 2008).

As previously mentioned, GALT DC promote lymphocyte homing to intestinal effector sites through coordinate upregulation of $\alpha_4\beta_7$ and CCR9. The first clue that RA controlled the induction of these ligands was the finding that adding it exogenously to APC-free cultures induced their expression on stimulated T cells (Iwata et al., 2004). Then came the seminal observation that adult mice reared on a vitamin A deficient diet had a diminished number of T lymphocytes residing in intestinal effector sites (Iwata et al., 2004). Consistent with their ability to synthesize RA, PpDC and mlnDC expressed messenger RNA for RALDH1 and RALDH2, respectively. Nevertheless, IEC were also revealed to prominently express RALDH1. Therefore, GALT DC may also acquire RA passively from IEC (Iwata et al., 2004). Further, although its physiological significance is unclear, it should be noted that RA is also detectable in serum, albeit at very low levels (Kane et al., 2008a; Kane et al., 2008b).

Retinoic acid contributes to lymphocyte function:

Apart from its immunological influence on homing, there are compelling data that link RA to the functional capacity of lymphocytes, particularly B cells. For example, DC and stromal derived, follicular DC from Pp were revealed to drive naïve B cell differentiation into IgA^+ B cells and/or to preferentially induce IgA^+ class switching in activated B cells (Mora et al., 2006; Suzuki et al.). RA was demonstrated to be essential to these processes, since antagonism of RA signaling inhibited the ability of PpDC to generate IgA^+ B cells, while adding RA in conjunction with IL-6 and/or IL-5 dramatically

enhanced IgA production in peripheral LN DC cocultures (Mora et al., 2006). In concert with these data, Fagarasan and colleagues reported that activating peripheral LN follicular DC with RA synergized with TLR 2 and 4 ligands to induce production of active TGF-β, which is also a critical factor for IgA^+ class switching (Cerutti and Rescigno, 2008; Suzuki et al.). Thus, vitamin A metabolism appears to contribute to the generation of IgA^+ B cells through both direct effects on B cells and indirect effects through DC modulation, although this has not yet been completely resolved.

RA may also exert direct effects on T cells. For instance, RA was shown to enhance T cell proliferation under mitogenic stimulatory conditions, which was correlated with a gross increase in IL-2 production (Ertesvag et al., 2002). However, data have also suggested that RA can inhibit production of IL-2 during naïve T cell activation (Ertesvag et al., 2009). Notably, these findings were obtained in humans and mice, respectively; therefore system differences could account for these discrepancies. RA was also proposed to directly inhibit T_H-1 polarization *in vitro*; however, the effect in these assays was relatively minor and a clear mechanism for the action of RA was not proposed (Iwata et al., 2003). Thus, what role RA plays in T cell activation and T_H polarization, if any, is still ambiguous.

Retinoic acid signaling is mediated through nuclear retinoic acid receptors:

RA signals through several families of nuclear hormone receptors in the nucleus. The best characterized are RA receptors (RAR) which form obligate dimers with retinoid X receptors (RXR) and in turn, transcriptionally activate RA response elements (RARE) in the promoter regions of various genes (Chambon, 1996). Recently, RA was also

shown to be able to signal through PPARβδ; however, the potential importance of this pathway in immune cell populations awaits further scrutiny and was not examined in the studies that will be described in the forthcoming sections (Schug et al., 2007). While RXRs dimerize with several nuclear receptors to transduce signals by other small molecules, RARs bind exclusively with RA (Ziouzenkova and Plutzky, 2008). Three, in particular, have been identified - α, β and γ. Underscoring their influence on a score of genes, as well as their complex regulation, aberrant expression of RARs (positive and negative) has been strongly linked to metabolic disorders and various cancers (de Lera et al., 2007; Hua et al., 2009; Walkley et al., 2007; Yang et al., 2005). RA was shown to mediate TGF-β production in follicular DC via RARβ (Suzuki et al.). However, in contrast to RARα and RARγ, RARβ is not detectable in lymphocytes (Elias et al., 2008). Intriguingly, protein expression of RARα was found to increase dramatically upon $CD4^+$ T cell activation in the presence of TGF-β, and as will be highlighted in the forthcoming chapters, may have a driving influence on $Foxp3^+$ T_{reg}/T_H-17 regulation (Schambach et al., 2007). Furthermore, dysregulation of these receptors has been linked to various cancers, including leukemias. **The prevalence of vitamin A metabolism occurring in the GALT, suggests that RA/RAR interactions may bear significance in the regulation of T cell homeostasis and immunity in this region. Work to be described in Chapter 2 and Chapter 4 will start to address this possibility.**

A role for retinoic acid in infection and immunity

On a typical western diet, retinol absorption exceeds the body's homeostatic requirements. This results in the accrual of retinyl esters in the liver. However, in developing countries, where malnutrition and diarrhea are common, Vitamin A intake and absorption are reduced. When combined with pregnancy, which increases Vitamin A

metabolism, the risk of Vitamin A insufficiency (VAI) both to mother and unborn infant increases dramatically. According to the most recent WHO estimate, there are approximately 250 million children presenting with subclinical and/or clinical signs of VAI at this precise moment (informational website).

VAI has long been linked to increased prevalence of diarrheal diseases and mortality from gastrointestinal infection (Duggan et al., 2002; Villamor and Fawzi, 2000). While numerous studies indicate that Vitamin A supplementation mitigates the incidence and severity of these infections, determining the impact of RA mediated mechanisms on these outcomes has proven difficult (Long et al., 2006a; Long et al., 2007a; Long et al., 2007b; Long et al., 2006b). Complicating this task is the variability in degree of VAI and preexisting disease states within single cohorts. Rodent models of VAI, which prevent these complications, have shed some light on the role of RA in GALT immunity to infection. For example, RA signaling was required for the development of a proper T_H-2 response in mice infected with the helminth, *Trichinella spiralis*, whereas VAI resulted in an aberrant T_H-1 response to this infection (Cantorna et al., 1994; Carman et al., 1992). However, another report demonstrated that RA administered concomitantly during infection with Mycobacterium tuberculosis enhanced T_H-1 responses *in vivo* and facilitated bacterial clearance (Yamada et al., 2007). VAI mice were found to be significantly more susceptible to rotavirus than their WT counterparts. However, while this study compared architectural changes in various tissues, including the gut, immune responses were not assessed in these mice (Ahmed et al., 1990). Thus, the impact of RA mediated signaling on T_H responses is still unclear and may depend on the local site of infection. **To date, there has been a paucity of studies that have considered the role of RA, *in situ*, during intestinal immune responses. Moreover, the precise mechanisms that underlie exacerbated enteric infections during VAI remain**

undefined. Studies in **Chapter 4** will tackle this question and help define how *in situ* Lp responses are impacted by VAI.

Models of infection:

To gain mechanistic insight into the questions that have been raised in this section the following challenge models will be employed:

Encephalitozoon cuniculi (E. cuniculi):

Microsporidia are spore forming obligate intracellular parasites that have recently been classified as fungi (James et al., 2006). They infect a wide range of hosts, primarily through oral transmission (Didier et al., 2004). Species that infect humans, such as *E. cuniculi*, tend to induce a sub-clinical chronic infection accompanied with mild or acute symptoms, most typically diarrhea. Upon ingestion, spores infect the epithelia of the duodenum and replicate in parisotophorous vacuoles (Mathews et al., 2009). Importantly, *E. cuniculi* can infect a variety of other cells, including APC (Orenstein et al., 1992), and can readily disseminate to other tissues in the body. As such, these infections are particularly severe and problematic in immunocompromised individuals (Farthing, 2006; Ferreira et al., 2001; Mertens et al., 1997; Wanachiwanawin et al., 1998). *E. cuniculi* is also a natural mouse pathogen (Keeble, 2001). Studies in laboratory animals have shed light on the course of infection and the immunological requirements for control of this pathogen, which include both $CD4^+$ and $CD8^+$ T cells during the natural route of infection (Moretto et al., 2004; Salat et al., 2006). In order to address how enteric commensal driven signals influence host ability to respond to

infection, mice will be infected with this pathogen. The results obtained from these studies will be described in **Chapter 3**.

Toxoplasma gondii (T. gondii):

Toxoplasma gondii (*T. gondii)* is a protozoan parasite naturally acquired through the oral route that infects up to a third of the world's population (Montoya and Liesenfeld, 2004). While primary infection manifests in a subclinical response, unless treated, *T. gondii* establishes a chronic phase of infection in the host, which is exemplified by the formation of Bradyzoite cysts in the muscle and brain tissue whose pathological consequences are limited by a multitude of host regulatory and inflammatory factors (Stumhofer et al., 2007; Wilson et al., 2005). Extensive mouse studies have established the critical importance of both $CD4^+$ and $CD8^+$ T cells and a controlled T_H-1 response in the development and maintenance of immunity to this parasite (Combe et al., 2005; Gazzinelli et al., 1992; Gazzinelli et al., 1996; Liesenfeld et al., 1996). Consequently, *T. gondii* can have fatal consequences in immunocompromised individuals upon reactivation of the chronic phase (Israelski and Remington, 1988). Based on virulence in mice, *T. gondii* has been classified into 3 strains. Type I are acutely lethal, regardless of the dose of pathogen administered, while Type II and type III strains are generally considered non-lethal, depending on the strain of mice and dose administered (Bradley and Sibley, 2007). For the studies that will be highlighted in **Chapter 4,** a clone of the type II strain, ME-49 will be utilized.

Summary:

The immune system of the gastrointestinal tract has adapted in close apposition with the commensal microbiota, food protein and environmental antigens. As such, it maintains a dynamic presence of both effector and regulatory lymphocyte populations. Yet, the mechanisms in place to orchestrate this balanced dichotomy have not been well defined. To gain insight into this broad question, the studies, which will be discussed herein, were focused particularly on understanding $CD4^+$ T cell regulation within the intestinal Lp compartment as a model of homeostasis. **Chapter 2** explores the convergence of potentially redundant regulatory pathways in this region, specifically, $Foxp3^+$ T_{reg} generation and presents data that identifies retinoic acid as a cofactor in the extrathymic development of $Foxp3^+$ T_{reg}. **Chapter 3** then examines how commensal components intersect with host regulatory strategies to favor the development of mucosal immune responses. Finally, **Chapter 4** inspects more closely the role of vitamin A metabolism in immunity.

CHAPTER 2: GALT Foxp3$^+$ T_{reg} development is critically dependent on vitamin A metabolism

Abstract:

The gastrointestinal tract is a home to a broad reservoir of regulatory T cells, including those which express the transcription factor, Foxp3. Although, most studies and differentiation models mark the origin of these cells as the thymus, recent evidence has showcased their potential to arise from naïve precursors and/or in the absence of a thymically generated pool. In this study, the GI tract and its associated lymphoid tissues (GALT) were studied as potential nucleating sites for the generation of extrathymic Foxp3$^+$ T_{reg}. The following findings emerged:

1. **Antigen introduced through the oral route robustly induced naïve T cell upregulation of Foxp3 protein in the GALT.**
2. **Dendritic cells from the intestinal lamina propria (Lp) and those draining into the mesenteric lymph nodes (mln) uniquely promoted *in vitro* Foxp3$^+$ T_{reg} generation via active TGF-β signaling and the synthesis of retinoic acid (RA).**
3. **RA enhanced TGF-β induced Foxp3$^+$ T_{reg} generation via retinoic acid receptor alpha.**
4. **Vitamin A metabolism was essential for GALT Foxp3$^+$ T_{reg} induction.**

Rationale:

Previous studies have demonstrated that TCR stimulation of naïve $CD4^+$ T cells in the presence of TGF-β could promote the induction of Foxp3 (Chen et al., 2003; Fantini et al., 2004). Further, sub-immunogenic (Kretschmer et al., 2005) or chronic exposure to antigen (Apostolou and von Boehmer, 2004; Knoechel et al., 2005) was also shown to lead to long-lasting adoption of this transcription factor in extra-thymic naïve $CD4^+$ T cells. Therefore, the immune system protecting the GI tract and associated lymphoid tissues (GALT) was hypothesized to serve as a candidate environment for peripheral $Foxp3^+$ T_{reg} development. Indeed, the small intestinal tissue was shown previously to constitutively express large amounts of TGF-β (Barnard et al., 1993) and is continuously exposed to gut commensal microbiota or dietary antigens in what is largely deemed a non-immunogenic manner.

Results:

De novo generation of Foxp3$^+$ T_{reg} cells can occur in the GALT

In order to address if the gastrointestinal tract and its associated lymphoid tissues (GALT) could favor *de novo* production of Foxp3$^+$ T_{reg} from naïve T cells, Ly5.2$^+$ T cells from RAG1$^{-/-}$ OT-II Tg mice, which are specific for the peptide sequence 323-339 of chicken ovalbumin (OVA) protein, were adoptively transferred into Ly5.1$^+$ replete recipients. Because these cells bear a TCR that does not recognize endogenous antigen, they retain a naïve phenotype and are devoid of regulatory features, including CD25 (Jordan et al., 2001) and Foxp3 (Walker et al., 2003). Following transfer, the Ly5.1$^+$ recipients were fed OVA antigen dissolved in drinking water for 5 consecutive days. This feeding regimen was previously demonstrated to suppress airway inflammation if implemented prior to immunization and intra-nasal challenge with OVA antigen, and was associated with upregulated Foxp3 mRNA expression in CD25$^+$ CD4 T cells (Mucida et al., 2005). A recent study by the same group demonstrated that Foxp3 was essential for this effect (Curotto de Lafaille et al., 2008).

After 5 days of OVA administration, Ly5.2$^+$ OVA-specific T cells had expanded and were readily detectable in the GALT, spleen, and even in distal lymph nodes, such as the sub-mandibular (subLN) (**Figure 3A**). However, in spite of the broad dissemination of transferred cells, Foxp3$^+$ expressing T cells were only appreciably detected in the GALT, including the mesenteric lymph nodes (mln), small intestinal lamina propria (Lp) and the Peyer's Patches (Pp) (**Figure 3B**). Although the highest frequency of Foxp3$^+$ converted OVA-specific T cells was consistently found in the Lp,

with a mean frequency of 16.8 ± 6.5%, it is important to note that these mice were sacrificed only 24 hrs post-feeding and that longer resting periods may have led to the redistribution of the cells to other tissues. Nevertheless, these findings argue that in response to oral exposure to antigen, the GALT environment can promote the emergence of Foxp3^{+} T cells from a naïve population in an antigen dependent manner. Importantly, feeding irrelevant antigen, such as bovine serum albumin was shown to not be able to induce Foxp3^{+} upregulation in a similar setting (Coombes et al., 2007).

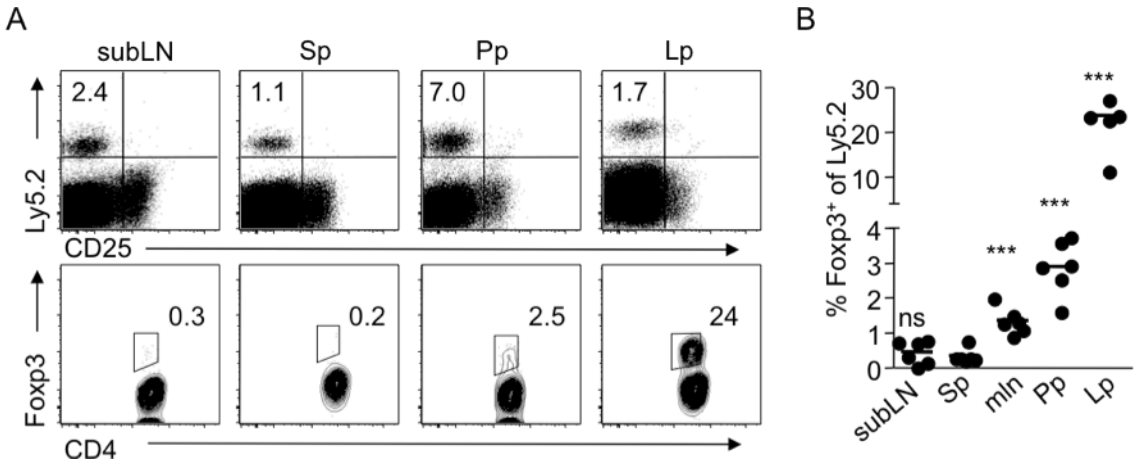


Figure 3. Acquisition of Foxp3 in CD4^{+} T cells responding to orally delivered antigen.
On day 6 after transfer, donor T cells were assessed flow cytometrically for Foxp3 expression via intracellular staining in cell suspensions from the sub-mandibular lymph nodes (subLN), spleen (Sp), mesenteric lymph nodes (mln), Peyer's patches (Pp), and small intestinal lamina propria (Lp). (A) Top dot plots were gated on total CD4^{+} cells. Ly5.2 was used to identify the transferred population. Bottom contour plots show Foxp3 expression by Ly5.2 gated cells (B) Summary of the percentage of Ly5.2^{+} RAG1$^{-/-}$ OT-II T cells expressing Foxp3. Each dot represents a single mouse. Data were combined from 2 individual experiments (3 mice each). Statistical comparisons were performed using the Student's T test, with Sp tissue serving as the baseline comparison for each tissue. ns = not significant,***, $P < 0.001$.

Characterization of lamina propria dendritic (LpDC) cells

The observations above suggest that the GALT microenvironment is particularly poised for the peripheral generation of Foxp3$^+$ T_{reg}. As detailed in **Chapter 1**, tissue resident dendritic cells (DC) constitutively migrate from the Lp into the mesenteric lymph nodes (Huang et al., 2000); this property is essential for the polarization of antigen specific T cells in the draining mln upon antigen feeding (Johansson-Lindbom et al., 2005). It was plausible, therefore, that Lp derived DC may play a dynamic role in the generation of T_{reg}. Examining the dual expression of CD11c and MHC II, which are the two surface antigens typically used to denote DC, in unfractionated Lp tissue suspensions indicated that these cells accounted for typically 25% of the hematopoietic cells within the Lp. As illustrated in **Figure 4A**, CD11c$^+$ MHC II$^+$ cells comprised a heterogeneous population, where two dominant subpopulations emerged: one (I) expressed higher levels of CD11c and slightly less MHC II, while the second (II) stained slightly lower for CD11c, but higher for MHC II. A third subpopulation has also been characterized by another laboratory, which stains much dimmer for CD11c and very bright for MHC II. Recent findings have identified these cells as the serosal macrophages (Bogunovic et al., 2009). As a side note, this population was not sorted in assays in which LpDC were isolated. Consistent with previous reports, when CD11c$^+$MHC II$^+$ cells were assessed for surface integrins, virtually all were CD11b$^+$ (Niess and Reinecker, 2005), while up to 30% expressed αE, also known as CD103 (**Figure 4A**) (Johansson-Lindbom et al., 2005). The majority of CD103$^+$ LpDC co-expressed CD11b. Unlike their counterparts in the Lp, SpDC were almost uniformly CD103$^-$, although in some experiments a portion of the CD11b$^-$ subpopulation

expressed this marker, albeit it dimly (**Figure 4A and data not shown**). Moreover, while these observations were made in C57BL/6 mice, for unclear reasons, the $CD11b^{-}$ $CD103^{+}$ SpDC subpopulation is known to be more prominent in BalbC strains (Annacker et al., 2005). Thus, the basal status of activation within the host may affect the constitutive presence of this population in the spleen. Upon cell sorting, Giemsa staining revealed that LpDC displayed the characteristic features of conventional DC with a stellar shape comparable to freshly sorted SpDC (**Figure 4B**). Analysis of costimulatory molecules on LpDC indicated comparable levels of CD80 and CD86 to SpDC, while CD40 was enhanced (**Figure 4C**). MHC II was also more highly expressed in LpDC. In concert with these findings, LpDC were shown to be at least as efficient as SpDC at inducing T cell proliferation when pulsed with peptide (Johansson-Lindbom et al., 2005).

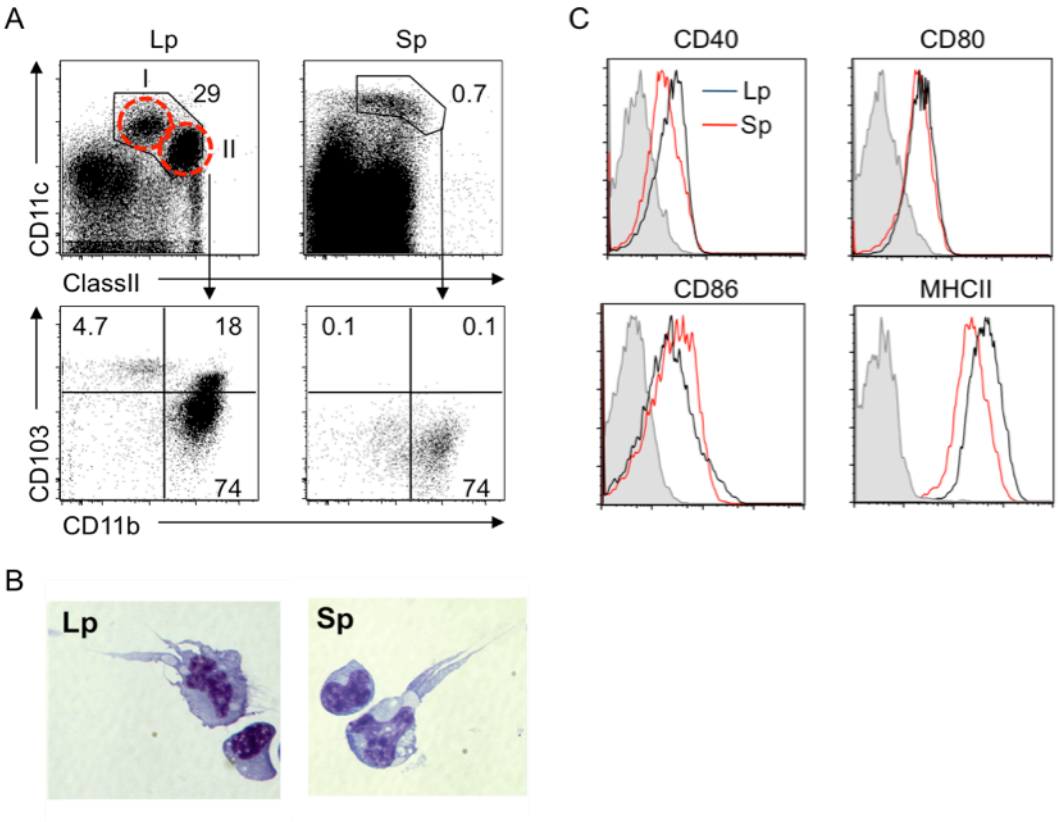


Figure 4. Phenotypic traits of small intestinal lamina propria DC (LpDC).
(**A**) Lp and Sp cell suspensions were labeled with αMHCII, αCD11c, αCD11b and αCD103 mAb. Higher panels indicate the percentages of $CD11c^{+}MHCII^{+}$ cells. Lower panels, gated on CD11c and MHCII, show CD103 versus CD11b expression. Numbers represent the percentage of events in each quadrant. (**B**) * Giemsa staining of sorted Lp (left) and Sp (right) $CD11c^{+}MHCII^{+}$ DC. (Magnification X 1000) (**C**) * Histograms depict the expression levels of CD40, CD80, CD86, and MHC II on LpDC (red) and SpDC (black). Isotype controls are shown in grey (Stained with pooled Lp and Sp samples).

LpDC preferentially induced Foxp3$^-$ T cells to become Foxp3$^+$ T_{reg} cells in T_{reg} polarizing conditions

LpDC were next assayed for their capacity to induce T_{reg} generation. To this end, DC from the Lp or spleen were isolated, co-cultured with purified Foxp3 negative CD4 T cells, and stimulated with polyclonal αCD3 mAb. In order to isolate live Foxp3$^-$ CD4 T cells, we used a transgenic reporter mouse in which an IRES eGFP construct was inserted downstream of the Foxp3 coding sequence (Bettelli et al., 2006). While αCD3 was unable to efficiently induce Foxp3, provision of exogenous TGF-β resulted in the production of Foxp3$^+$ T cells in both cultures, as determined by intracellular cytokine staining (**Figure 5A**). However, under the same conditions and at several T cell to DC ratios, the frequency of Foxp3$^+$CD4$^+$ T cells was typically 3 fold greater in LpDC cultures (**Figure 5B**). Notably, the intensity of Foxp3 expression was higher when induced in the presence of LpDC than with SpDC (**mean fluorescence intensity (MFI): 90.9 vs. 57.4, Figure 5A**). These findings were recapitulated in an antigen specific setting, in which OVA peptide was used to stimulate OT-II transgenic T cells in DC cocultures (**data not shown**). To test the functionality of the induced Foxp3$^+$ T cells, eGFP expressing cells were isolated from cocultures and their suppressive capacity was tested *in vitro* (Thornton and Shevach, 1998). Foxp3$^+$ T cells obtained from both LpDC and SpDC cocultures were as capable as freshly isolated, *ex vivo*, Foxp3$^+$ T_{reg} at curbing the proliferation of Foxp3$^-$ responder T cells upon stimulation, suggesting that these induced cells have regulatory capabilities (**Figure 6**).

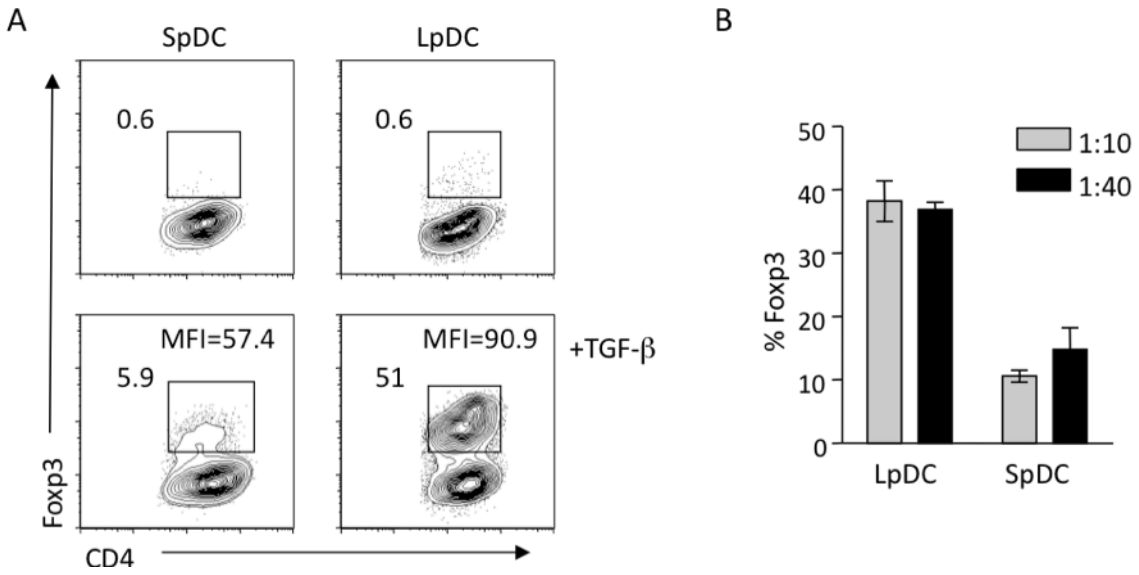


Figure 5. Small intestinal LpDC induce Foxp3$^+$ T_{reg} at a significantly higher rate of efficiency than SpDC.

(**A-B**) 10^5 sorted, Foxp3$^-$ CD4$^+$ T cells were cocultured with 10^4 – 2.5x10^3 Sp or LpDC for 5 days, with αCD3 mAb alone or in concert with TGF-β. Viable cells were then assessed for intracellular Foxp3 expression. (**A**) Contour plots show the percentage of CD4$^+$ T cells that express Foxp3. MFI = mean fluorescence intensity. DC:T cell ratio → 1:10 (**B**) Bar graphs illustrate the frequency of Foxp3$^+$ T cells detected upon termination of culture for each condition. Error bars indicate the high and low values (average of triplicate wells) from two experiments.

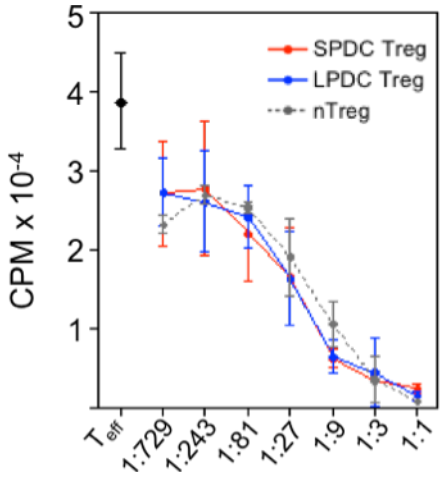


Figure 6. Induced Foxp3$^+$ T cells behave similar to ex vivo isolated Foxp3$^+$ T$_{reg}$.
5 x 10^4 eGFP$^-$CD4$^+$ T (T$_{eff}$) cells were mixed with graded doses of eGFP$^+$ T cells that were sort purified from day 5 SpDC or LpDC cocultures and then stimulated with irradiated splenocytes and αCD3 mAb (0.5 mg/ml). Proliferation was measured by the incorporation of ^{3}H-Thymidine during the last 6 hr of culture. eGFP$^+$ T cells sorted *ex vivo* (nTreg) were used for comparison.

The induced Foxp3$^+$ T_{reg} expressed high levels of CD25 and GITR (**data not shown**) regardless of the origin of the stimulating DC, while CD103 expression was consistently enhanced when induced by LpDC (**MFI: 594 vs. 275, Figure 7A**). Since CD103 expression was previously shown to be upregulated by TGF-β signaling (El-Asady et al., 2005), the influence of the dose of TGF-β on Foxp3 T cell generation was examined in DC cocultures. Significantly, the ability of SpDC to induce Foxp3 was much more sensitive to the dose of TBF-β, where lowering the dose led to a precipitous reduction in the frequency of this induced population. Conversely, the ability of LpDC to induce a sizable Foxp3$^+$ population remained robust across various TBF-β concentrations (**Figure 7B**). These findings raised the possibility that TGF-β signal transduction is augmented in T cells stimulated by LpDC. Though not part of these studies, it would be intriguing to investigate the kinetics and degree of smad phosphorylation, which is downstream of TGF-β receptor signaling (Li and Flavell, 2008), in T cells during stimulation with Lp vs. SpDC.

CD103 expression by DC is required for suppression of T cell mediated colitis in the adoptive transfer model, suggesting that this subset possesses regulatory function *in vivo* (Annacker et al., 2005). Therefore, it was important to test whether the CD103$^+$ subset was the subpopulation of LpDC that was promoting preferential Foxp3$^+$ T_{reg} induction. To address this, LpDC were further sort purified based on CD103 expression. Strikingly, CD103$^+$ LpDC, were uniquely able to induce Foxp3 expression in the absence of exogenous TGF-β (**Figure 8**). Moreover, in the presence of TGF-β they consistently induced a higher frequency of Foxp3$^+$ cells, though this was not very dramatic, especially when compared with SpDC. Purification of the CD103$^+$ DC subpopulation from the intestinal draining mln indicated that these cells shared the same competency

to induce Foxp3$^+$ T_{reg} as their counterparts in the Lp. This held true both in absentia and in the presence of exogenous TGF-β (**Figure 8**). On the other hand, even with exogenous TGF-β, the CD103$^-$ subpopulation still induced less than half of the Foxp3 frequency when compared to the CD103 expressing subset (**Figure 8**). These findings suggest that the CD103$^+$ DC subset in the mln originates from the intestinal Lp, while the CD103$^-$ subset likely does not. Consistent with this idea, an elegant study demonstrated that, although DC were readily detected, the CD103$^-$ subset was selectively excluded from lymph vessels draining the intestine (Schulz et al., 2009).

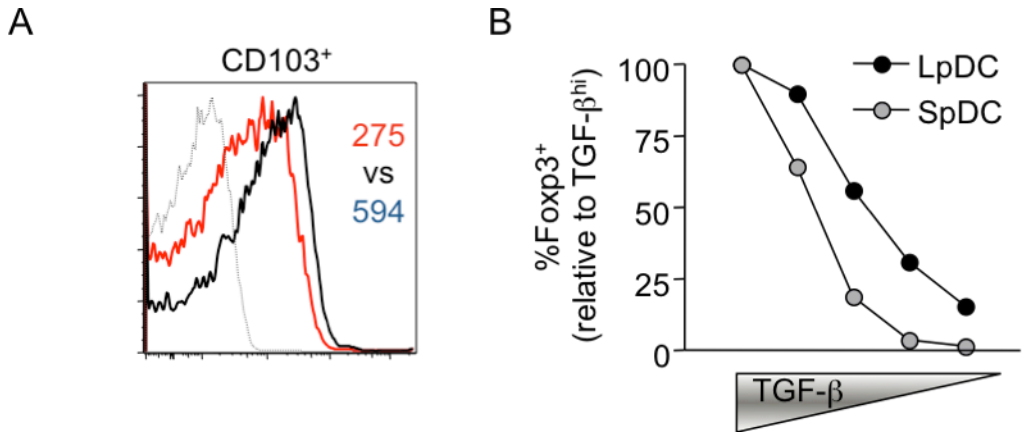


Figure 7. TGF-β responsiveness is enhanced in the presence of LpDC
(**A**) eGFP$^-$CD4$^+$ T cells were cocultured at a 10:1 ratio with SpDC or LpDC in T_{reg} polarizing conditions. On day 5, eGFP$^+$ T cells were assessed for CD103 expression via flow cytometry. Red = SpDC, Black = LpDC. (**B**) As in A; however, the amount of TGF-β in coculture was tested at several concentrations.

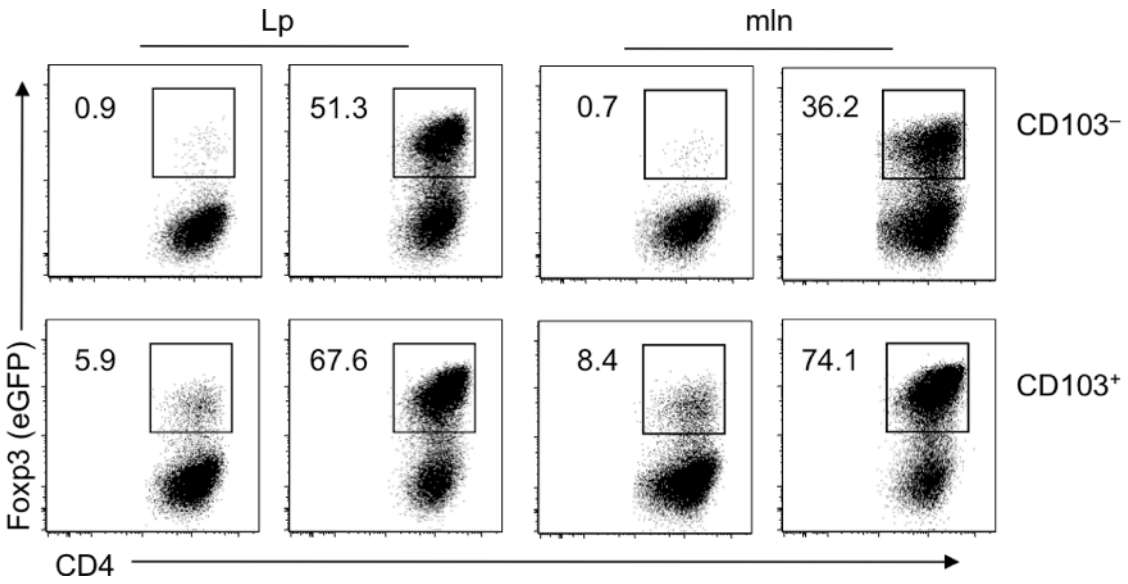


Figure 8. CD103$^+$ Lp and mln DC are specialized to induce Foxp3$^+$ T$_{reg}$ in the absence of exogenous factors.
eGFP$^-$CD4$^+$ T cells were cocultured at a 10:1 ratio with CD103$^+$ or CD103$^-$ DC purified from Lp or mln tissues and stimulated with αCD3, in the presence or absence of TGF-β. Cells were harvested 5 days later, and eGFP expression was assessed on viable CD4$^+$ T cells as a surrogate for Foxp3.

LpDC induce sustained Foxp3 expression

The relative paucity of converted T_{reg} in SpDC cocultures may have been due to a lower viability or a reduced proliferative potential of cells that acquired Foxp3. To determine the potential contribution of these elements, the survival of *in vitro* activated T cells was measured. In both SpDC and LpDC cocultures, $Foxp3^+$ T cells remained significantly more viable than the Foxp3 null population. Thus, the lower yield of T_{reg} cells in SpDC cocultures was not the result of increased cell mortality (**Figure 9A**). T cell proliferation in cocultures was explored next. Since eGFP and CFSE share overlapping emission spectra, naïve $CD4^+$ T cells (Foxp3 contamination was < 0.5%) were isolated from non-transgenic mice (However, in subsequent experiments it became clear that eGFP is not retained when fix/permeabilized with the Foxp3 staining buffer kit offered by eBioscience). Consistent with findings using $CD4^+$ T cells from reporter mice, the proportion of converted T_{reg} continued to dominate in LpDC co-cultures (**54.5% vs. 14.9%, Figure 9B**). CFSE dilution profiles indicated that $CD4^+$ T cells proliferated vigorously regardless of the origin of DC during the length of incubation. While the $Foxp3^+$ T_{reg} lineage is typically distinguished by its hypoproliferative characteristics (Ref), this observation may in part be explained by the polarizing system used, in which IL-2, a potent growth factor for T_{reg} in these stimulating conditions, was added at 24 hrs and 72 hrs post-culture. Importantly, even at late division cycles the proportion of $Foxp3^+$ T_{reg} remained high in LpDC cocultures (**Figure 9C**). These data suggest an initiation of a more stable $Foxp3^+$ T_{reg} program in the presence of LpDC.

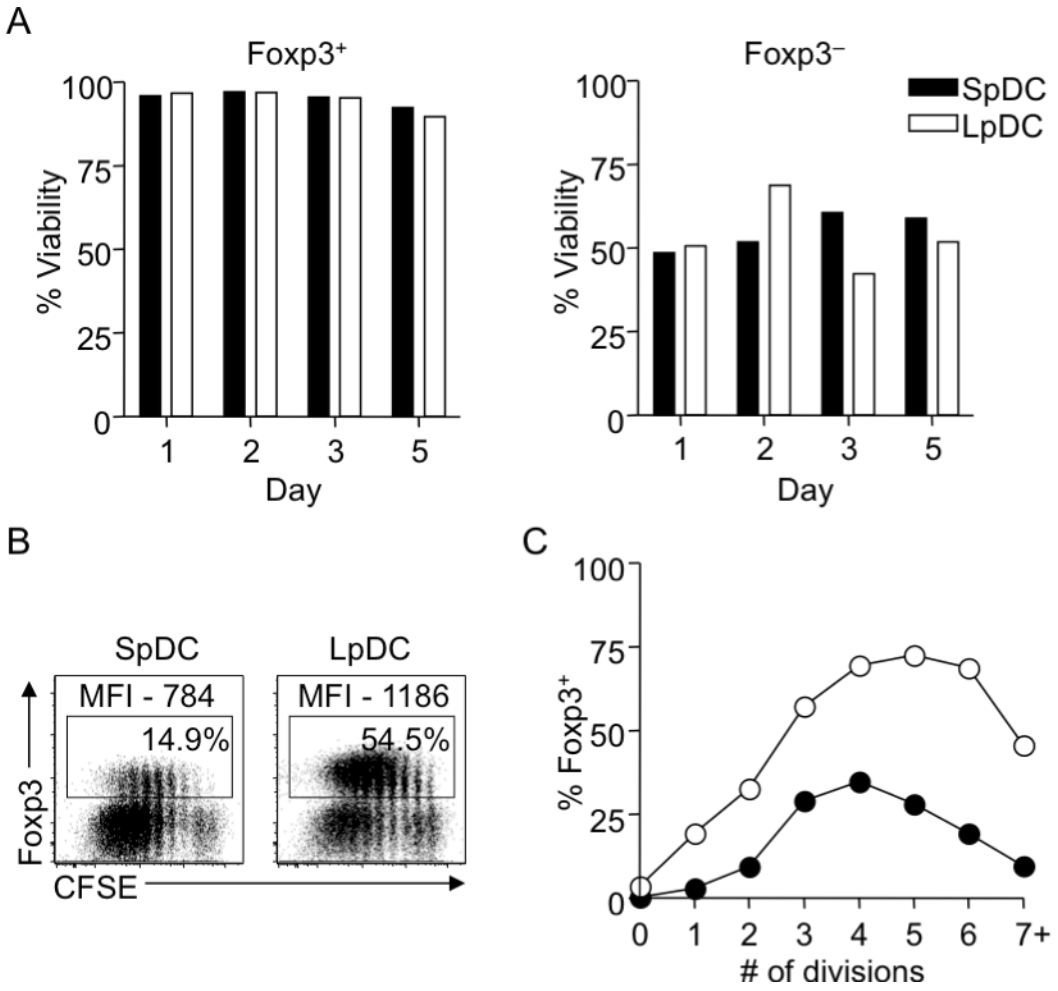


Figure 9. Maintenance of Foxp3 contributes to higher frequency of Foxp3$^+$CD4$^+$ T cells in the presence of LpDC.

(**A**) eGFP$^–$CD4$^+$ T cells were co-cultured with purified DC at a 5:1 ratio in T_{reg} polarizing conditions. At indicated time points, cells were harvested, and stained with 7AAD (dead) and annexinV (apoptotic). Foxp3 expression was determined based on eGFP fluorescence. The percentage of 7AAD$^–$ annexinV$^–$ cells is depicted by white bars for LpDC and black bars for SpDC. Data are from one of 2 independent experiments. (**B**) CFSE labeled naive CD4$^+$ T cells were co-cultured with purified DC as described above. On day 5, cells were harvested and stained for CD4 and intracellular Foxp3. Dot plots of Foxp3 versus CFSE are illustrated. The percentage of Foxp3$^+$ cells and the MFI was defined as the bordered population. (**C**) The proportion of Foxp3$^+$ cells among CD4 T cells is plotted as a function of the number of cell divisions. LpDC (○) of SpDC (●).

LpDC are equipped to metabolize vitamin A derivatives in retinoic acid

Prior to this work, a number of studies have attributed several unique functional properties to GALT DC. Specifically, these studies found that lymphocytes activated in the presence of GALT DC, including those from the Lp, selectively up-regulate the gut homing surface molecules CCR9 and $\alpha_4\beta_7$, which as mentioned in **Chapter 1** results in their preferential migration into intestinal tissue (Johansson-Lindbom et al., 2005; Johansson-Lindbom et al., 2003; Mora et al., 2003; Mora et al., 2006). This upregulation was recently shown to depend on retinoic acid (RA) (Iwata et al., 2004; Mora et al., 2006). Corresponding to this feature, GALT DC from Pp and mln were found to uniquely express high levels of messenger RNA for certain retinaldehydrogenases (RALDH), which are the enzymes that irreversibly catalyze the metabolism of the vitamin A derivative, retinal, into RA (Mic et al., 2003). In order to assess for the functional expression of this family of enzymes in LpDC, a cell-membrane-penetrating probe that fluoresces upon binding to active RALDH was incubated with Sp and Lp tissue cell suspensions, and then examined flow cytometrically. Strikingly, only LpDC displayed high levels of functional RALDH; more specifically, these high levels were restricted to the $CD103^+$ LpDC population (**Figure 10A**). Nevertheless, inhibition of RALDH activity using the alcohol dehydrogenase inhibitor, diethylaminobenzaldehyde (DEAB), during incubation with the RALDH probe indicated that both $CD103^-$ LpDC and SpDC have marginal, albeit detectable RALDH activity. Consistent with more RALDH activity in LpDC, upregulated $\alpha_4\beta_7$ expression was consistently observed on induced $Foxp3^+$ T cells in LpDC but not in SpDC cocultures (**Figure 10B**).

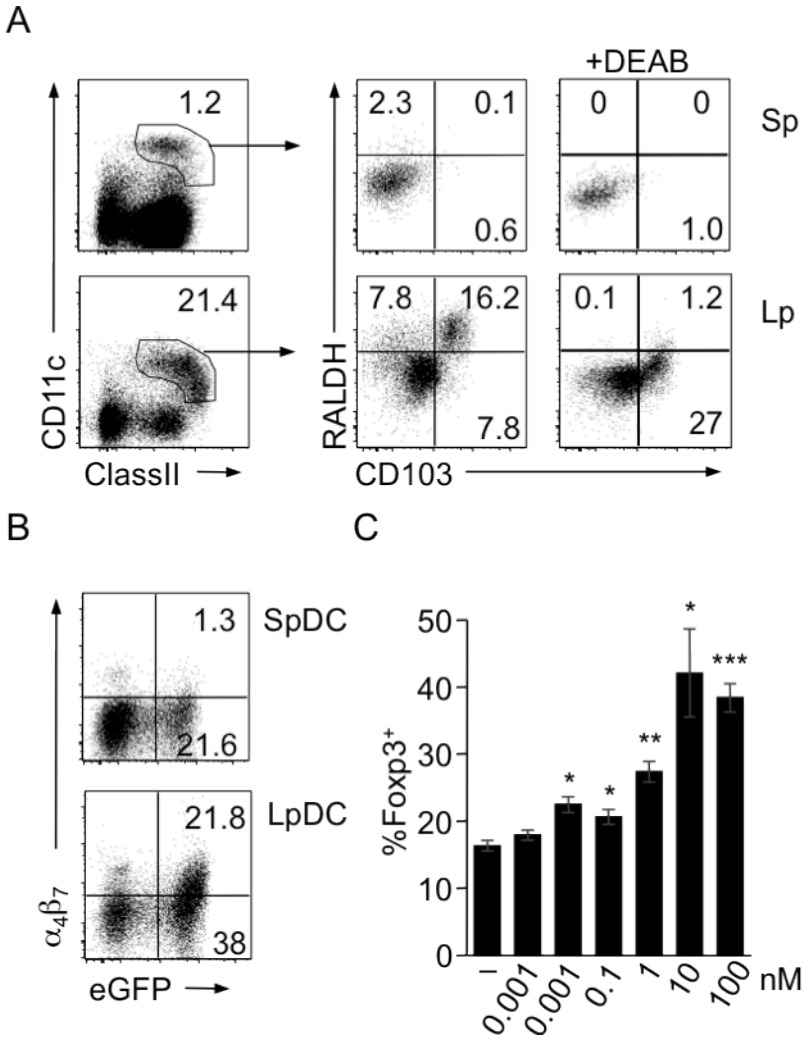


Figure 10. LpDC synthesize retinoic acid, which can enhance Foxp3$^+$ T_{reg} generation in cooperation with TGF-β.

(**A**) Sp and Lp cell suspensions were incubated with a RALDH substrate possessing a fluorescent indicator. Where indicated, cell suspensions were concomitantly treated with a RALDH inhibitor, DEAB. RALDH activity was assessed by flow cytometry, with gating strategy showed. (**B**) *In vitro* cocultured cells were stained for $\alpha 4\beta 7$ and assessed for eGFP fluorescence (Foxp3) by flow cytometry. (**C**) The dose responsiveness of eGFP (Foxp3) expression by CD4 T cells cocultured with SpDC was determined by flow cytometry. Error bars represent the standard error of the mean (s.e.m.) of 3 individual samples from one experiment. Statistical significance was determined using the Student's T test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Retinoic Acid enhances Foxp3$^+$ T_{reg} generation in SpDC cocultures via retinoic acid receptor alpha (RARα)

To proceed with the previous findings, the capacity of RA to enhance the generation of Foxp3$^+$ T cells in T_{reg} polarizing conditions was tested. To this end, SpDC/T cell cocultures were prepared with αCD3 and TGF-β in the presence of increasing doses of the synthetic RA, all-trans RA, which is the predominant physiologically detectable form of retinoic acid (Mic et al., 2003). While RA alone did not induce Foxp3 expression, RA in the presence of TGF-β enhanced the frequency of Foxp3$^+$ T_{reg} recovered in culture in a dose dependent manner, also leading to upregulated $\alpha_4\beta_7$ on these cells (**Figure 10C and data not shown**). The enhanced generation of Foxp3$^+$ T cells was markedly significant at RA concentrations of 1nM and above, and plateaued at 10nM, with the percentage of Foxp3$^+$ cells reaching 40-55% (**Figure 10C**). Since exogenous RA in the absence of TGF-β was unable to induce T_{reg} generation *in vitro*, the effects of this molecule seem to require TGF-β. In this regard, RA was recently shown to enhance smad3 phosphorylation and its nuclear translocation in T_{reg} polarizing conditions (Nolting et al., 2009; Xiao et al., 2008). Nevertheless, RA was capable of augmenting the frequency of Foxp3$^+$ cells in the absence of smad3 (Nolting et al., 2009). Therefore, although RA may influence other aspects of TGF-β signaling in the absence of smad3, another plausible explanation is that RA signals complement the effect of TGF-β signals. Regardless of these interpretations, TGF-β appears requisite for the establishment of a baseline level of Foxp3 differentiation, *in vitro*, at least.

To determine the signaling pathway utilized by RA to induce this outcome, naïve CD4$^+$ T cells deficient in retinoic acid receptor alpha (*Rara*), beta (*Rarb*), or gamma (*Rarg*), respectively, were incubated with WT SpDC in T_{reg} polarizing conditions, with or

without RA. Strikingly, only in the absence of RARα did RA fail to enhance the frequency of Foxp3$^+$ T cells recovered in cocultures (**Figure 11A**). Indicative that RARα was an important transcriptional effector of RA driven pathways, $\alpha_4\beta_7$ upregulation was also attenuated in the absence of this gene (**Figure 11B**). Not examined in these experiments was whether the effect of other isoforms, such as 9-cis RA, was also diminished in the absence of RARα. This form of RA, although not readily detected *in vivo*, was also shown to enhance Foxp3 generation (Mucida et al., 2007).

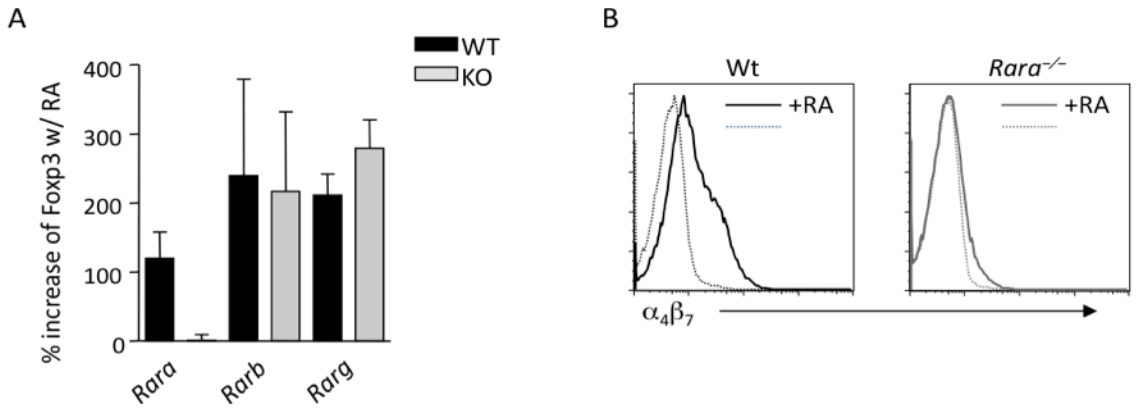


Figure 11. RA signaling through RAR-alpha enhances Foxp3$^+$ T_{reg} generation.
(**A**) Naïve CD4$^+$ T cells from *Rara*, *Rarb*, or *Rarg* deficient mice and their WT littermates were cocultured at a 10:1 ratio with WT SpDC in T_{reg} polarizing conditions, ± RA (10nM). Intracellular Foxp3 expression was assessed on day 5. Bars depict the average percent increase (of duplicate samples) in the proportion of Foxp3$^+$ T cells recovered when cultured with RA. Percent increase was calculated based on the average frequency of Foxp3$^+$ T cells recovered from duplicate wells cultured w/o RA. Error bars mark the high and low values. (**B**) $\alpha_4\beta_7$ expression was measured on total CD4$^+$ T cells after coculture.

LpDC facilitate Foxp3 induction via retinoic acid mediated signaling

Exogenous RA in the presence of TGF-β in LpDC cocultures also increased the proportion of Foxp3$^+$ T cells generated and diminished the intrinsic differences between CD103$^+$ and CD103$^-$ subsets' abilities to induce T_{reg} (**Figure 12A**). Notably, in LpDC cultures containing the CD103$^+$ subset, in which the frequency of Foxp3$^+$ cells was already high, RA exerted a more dramatic effect on $\alpha_4\beta_7$ than on Foxp3 expression. This suggested that the signaling pathways evoked by RA for the generation of Foxp3 and upregulation of $\alpha_4\beta_7$ may differ, independent of their dependency on RARα, and that the effects of RA on the regulatory pathway is saturated at smaller concentrations of RA. Interestingly, addition of RA to CD103$^+$ LpDC cocultures stimulated without TGF-β, in which a small frequency of Foxp3$^+$ T cells was already induced, did not lead to the expansion of this population (**data not shown**), indicating that the endogenous level of RA is not the limiting component in these conditions.

The accumulating data suggested that the capacity of LpDC to synthesize RA was, in part, responsible for Foxp3$^+$ T_{reg} induction observed in these cocultures. To address this hypothesis the synthetic RA receptor antagonists LE540 and LE135 (RAi) were added to LpDC cocultures in an attempt to block RA mediated signaling. Remarkably, the addition of 1μM each of these inhibitors inhibited T_{reg} conversion by 67% and 57% in CD103$^+$ and CD103$^-$ LpDC cocultures, respectively (**Figure 12A**). Drawing from these data, we hypothesized that RA signals conveyed by LpDC synergized with TFG-β to favor T_{reg} induction. In support of this, either blockade of TGF-β or RA receptor signaling resulted in a significant decline of spontaneous αCD3 induced T_{reg} induction by CD103$^+$ LpDC, (87%, $P < 0.0005$ and 73%, $P < 0.001$, respectively) (**Figure 12B**).

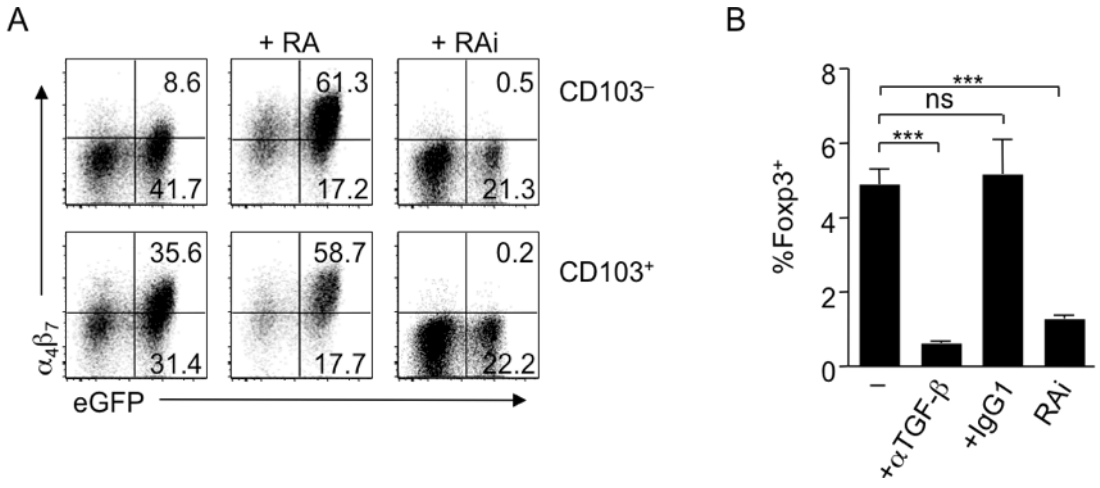


Figure 12. RA production by LpDC is requisite for optimal Foxp3$^+$ T_{reg} induction. (**A**) Foxp3$^-$ CD4$^+$ T cells were cocultured with both CD103$^\pm$ LpDC at a 10:1 ratio in T_{reg} polarizing conditions. However, in some wells, RA (100nM), or the RA receptor antagonists (RAi), LE540 and LE135 were added. Cells were subsequently stained and assessed for $\alpha4\beta7$ and eGFP fluorescence (Foxp3) by flow cytometry. (**B**) Foxp3$^-$ CD4$^+$ T cells were cocultured with CD103$^+$ DC and stimulated with αCD3 ± TGF-β neutralizing antibody (isotype IgG1) or LE540 and LE135. Error bars depict the s.e.m. of three independent experiments. Statistical significance was determined using the Student's T test. ns = non significant; ***, $P < 0.001$.

Vitamin A metabolism is essential for GALT generation of Foxp3$^+$ T_{reg} cells in response to antigen feeding

Although RA receptor blockade prevented T_{reg} generation *in vitro*, it remained unclear whether vitamin A metabolism was an essential mediator of T_{reg} induction in the GALT upon antigen feeding. To begin to address this, a natural model of diet-induced, vitamin A insufficiency (VAI) was employed, in which mice no longer received vitamin A in their diet starting at day 14.5 *in utero* (Smith et al., 1987). At 10 weeks of age, these mice, which displayed normal gut histology and thymic T cell development (**data not shown**), were deficient in vitamin A and its derivative metabolites as evidenced by diminished $\alpha_4\beta_7$ expression on CD44^{hi} CD4$^+$ T cells (**Figure 13A**). Moreover, the capacity of LpDC to synthesize RA, based on assessment of functional RALDH, was abrogated during VAI (**Figure 13B**). These data suggested that the metabolism of vitamin A in LpDC is controlled by a positive feedback loop. Further, they indicated that even in the event that residual vitamin A/metabolites lingered in the VAI host, LpDC would be unable to induce RA mediated signals during polarization.

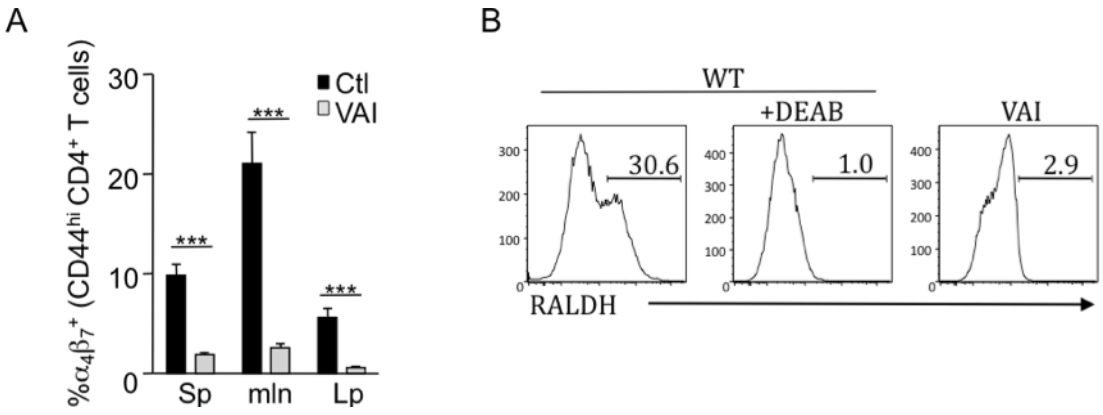


Figure 13. RA synthesis is abolished in vitamin A deficient diet fed mice at 10 wks of age.

(**A**) Cell suspensions from Sp, mln and Lp of Ctl and VAI mice were stained for T cell markers, $\alpha 4\beta 7$, and CD44 and analyzed flow cytometrically. $\alpha_4\beta_7$ expression was then assessed on gated $CD44^{hi}$ $CD4^+$ T cells. Bars depict the average values (n = 4 per group). Error bars depict the standard deviation (s.d.). ***, $P < 0.001$. (**B**) Lp cell suspensions from Ctl and vitamin A insuffient (VAI) mice were pooled (2 per group) and incubated with the functional RALDH probe. RALDH activity on LpDC was assessed by flow cytometry. The RALDH inhibitor, DEAB, shows the specificity of the probe.

To determine whether VAI mice retained the capacity to promote GALT T_{reg} generation upon antigen feeding, $Foxp3^{-}Ly5.2^{+}$ T cells from $Foxp3^{eGFP}$ X OT-II Tg mice were adoptively transferred into $Ly5.1^{+}$ VAI recipients. As expected, after 5 days of OVA feeding, an appreciable frequency of transferred cells adopted Foxp3 in the Lp of mice fed a vitamin A sufficient diet (control – Ctl) (**Figure 14A**). In contrast, transferred cells failed to upregulate Foxp3 in any of the tissues analyzed in VAI hosts, including the Lp, Sp and mln (**Figure 14A-C)**. This outcome was unlikely due to a defect in antigen presentation, as CFSE labeling indicated that OVA specific cells readily divided in response to OVA feeding. Nevertheless, despite the breakdown of this pathway, thymic $Foxp3^{+}$ T_{reg} development appeared grossly unimpaired in VAI animals (**Figure 15A-B).** Taken together, these results unequivocally reveal a requirement for vitamin A in GALT Treg induction *in vivo*.

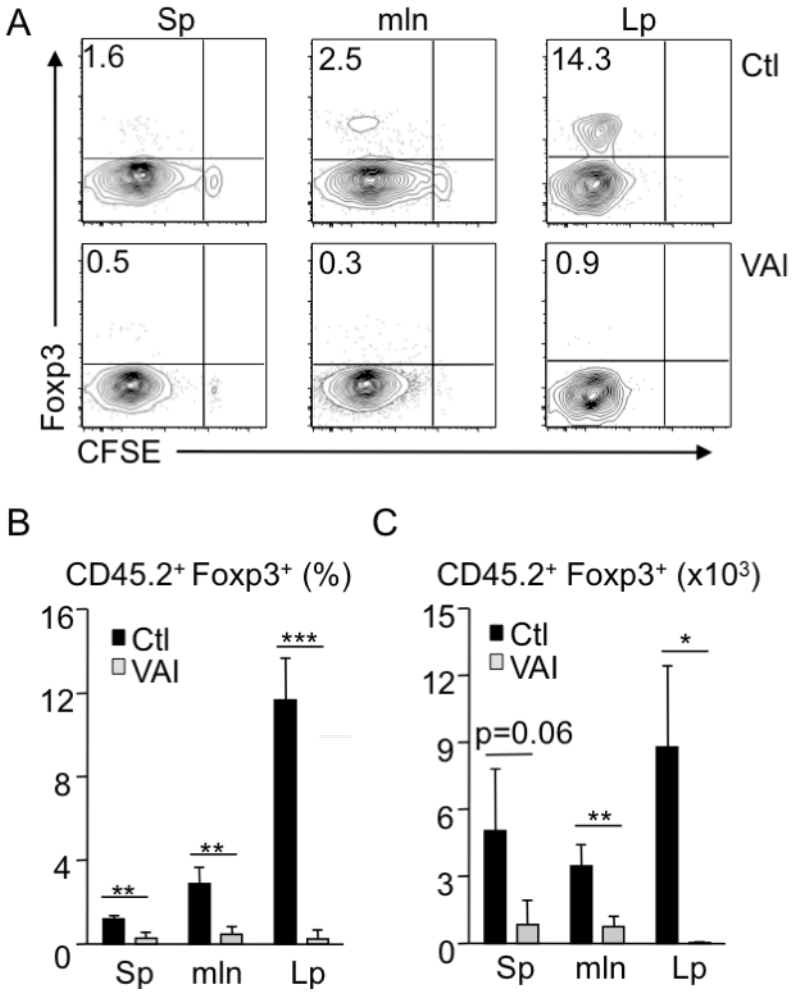


Figure 14. Vitamin A insufficiency results in diminished GALT Foxp3$^+$ T_{reg} induction.

(**A**) CD45.1$^+$ WT and VAI mice were intravenously injected with CFSE labeled, sorted CD45.2$^+$ Foxp3$^-$ OTII transgenic T cells and fed OVA antigen in their drinking water for 5 consecutive days. Transferred cells were assessed in the Sp, mln, and Lp for *de novo* expression of intracellular Foxp3 via flow cytometry. Representative dot plots gated on CD45.2$^+$ CD4$^+$ T cells show Foxp3 as a function of CFSE. (**B**) Summary of the frequency Foxp3 expression in transferred cells. (**C**) Summary of the absolute number of induced Foxp3$^+$ T_{reg}. Error bars illustrate the s.d. Statistical comparisons were performed using the unpaired Student's *t* test *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ns = not significant. n = 3-4 mice per group.

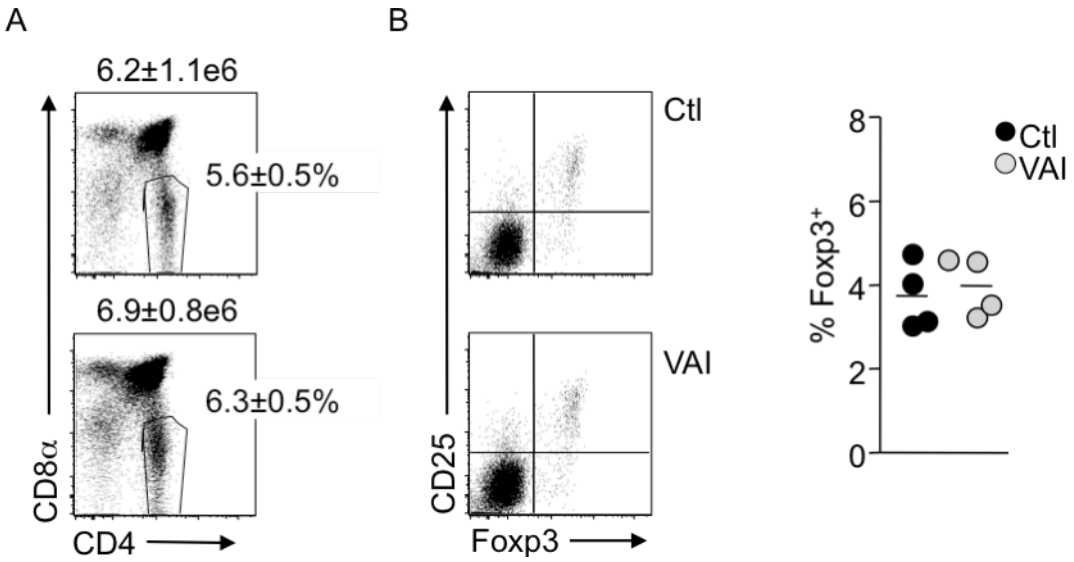


Figure 15. CD4$^+$ T cell development appears normal in VAI mice.
(**A**) Thymic CD4 and CD8 profile of Ctl and VAI mice at 10 wks of age. Absolute number of CD4 single positive cells is displayed above each dot plot. n = 4 mice per group. (**B**) Cells were fixed and intracellularly stained for Foxp3. Dot plots illustrate CD25 versus Foxp3 expression upon gating on viable CD4$^+$ cells. The frequency of Foxp3$^+$ cells is summarized on the right-hand side. Each dot represents and individual mouse.

Discussion

The data herein demonstrate that the GALT environment is able to promote the *de novo* generation of Foxp3$^+$ T_{reg}. Specifically, the findings described reveal that DC from the small intestinal lamina propria are uniquely endowed with the capacity to induce Foxp3$^+$ T_{reg} *in vitro* via a mechanism that, in addition to TGF-β, is dependent on the vitamin A metabolite, retinoic acid. Underscoring the relevance of this pathway for the development of peripheral Treg *in vivo*, mice devoid of RA are unable to induce T_{reg} in the GALT upon antigen feeding.

The induction of oral tolerance has been linked to a number of immunoregulatory processes, including: the polarization of TGF-β (T_H-3) and IL-10 (T_R-1) producing T cells, clonal deletion in the periphery, and more recently, the upregulation of Foxp3$^+$ mRNA, which is essential for the programming of Foxp3$^+$ T_{reg} cell function (Chen et al., 1995; Chen et al., 1994; Gavin et al., 2007; Lin et al., 2007; Mucida et al., 2005). It is now clear that Foxp3 protein expression is acquired, as well, during this process (Coombes et al., 2007; Sun et al., 2007). The high frequency of Foxp3$^+$ T_{reg}, sometimes exceeding 20%, detected within the small bowel Lp suggests that this site may serve as a niche for this population of cells. Lending support to this hypothesis, this lab has demonstrated using a lymphopenic transfer model that induced Foxp3$^+$ T_{reg} preferentially accumulate in the Lp (Sun et al., 2007).

Based on the capacity of LpDC to induce Foxp3$^+$ T_{reg}, compounded with the high frequency of these cells that was recovered from the Lp, one interpretation is that *de novo* Foxp3 production occurs *in situ*. However, this possibility is unlikely for several reasons:

1. Naïve cells are rarely present in the intestinal tissue (<5% based on CD44 and CD62L staining, **data not shown**).
2. Though not tested on naïve T cells per se, Foxp3 could not be induced in $CD4^+$ T purified from the Lp. Regardless, it would be worthwhile to repeat this experiment with Lp T cells that were isolated based on expression of naïve surface markers.
3. From a kinetic standpoint, impressive Foxp3 induction initially occurred in the mln and Pp. One caveat of these findings, however, is that the adoptive transfer system that was used for these experiments involved the injection of $1x10^6$ OT-II T cells or less. As such, it was difficult to identify transferred OTII cells within the Lp shortly after transfer (i.e. within 36 hrs).
4. Removal of the mesenteric lymph nodes or genetic disruption of the ability of LpDC to migrate into them was previously demonstrated to abrogate oral tolerance induction (Worbs et al., 2006).

It was recently revealed that the $CD103^+$ LpDC population is the predominant migratory subset of $CD11c^+MHCII^+$ cells within the intestinal Lp and is much more efficient than the $CD103^-$ subset at polarizing T cells (Bogunovic et al., 2009; Schulz et al., 2009). Thus, whether the $CD103^-$ subset plays a physiological role in GALT $Foxp3^+$ T_{reg} generation and maintenance of oral tolerance is unclear. Based on their capacity to promote T_{reg} *in vitro* in the presence of exogenous TGF-β, as well as their capacity to efficiently take-up antigen (Schulz et al., 2009), it's plausible that these cells could act to maintain or propagate *de novo* generated $Foxp3^+$ cells. Arguing in favor for such a role, $F4/80^+CD11c^+CD11b^+$ cells, i.e. surface markers, which correspond to the $CD103^-$ subset, were shown to prevent T cell mediated transfer colitis via IL-10 production and

the maintenance of the Foxp3 T_{reg} (Murai et al., 2009). Intriguingly, another study described a macrophage-like subset, which expressed low/negative levels of CD11c, and potently induced $Foxp3^+$ T_{reg} generation in vitro via IL-10 (Denning et al., 2007). These cells were also found to express message for RALDH2 enzyme.

Although the antigen-feeding model employed in these assays favors the emergence of T_{reg} in the GALT, this protocol in other studies has been shown to limit airway inflammation in a Foxp3 dependent manner (Curotto de Lafaille et al., 2008), underscoring the circulatory capacity of these induced regulatory cells. Moreover, suggestive that a similar tolerogenic process occurs in tissues that are in contact with other natural sites of opportunistic entry, such as the nasal cavities, intranasal administration of antigen also was revealed to curb airway inflammation. It would be interesting to examine whether $Foxp3^+$ T_{reg} induction in this system also depends on vitamin A metabolism. Interestingly, RA was recently demonstrated to limit airway responses in a model of allergic asthma; however, the cellular-source of RA responsible for these effects was not clear, nor was the dependency on $Foxp3^+$ T_{reg} induction (Goswami et al., 2009).

Indeed, the capacity to synthesize RA is not restricted to GALT DC. For example, epithelial cells are also endowed with this feature (Iwata et al., 2004). More recently, it was revealed that the stroma of the mesenteric lymph nodes also share this capability (Hammerschmidt et al., 2008). In fact, in elegant surgical experiments, this capacity was demonstrably essential for the effective upregulation of $\alpha 4\beta 7$ and CCR9 on newly activated T cells upon antigen feeding. This finding indicates that, rather than merely serving as a collecting reservoir for migrating LpDC, the mln may actively participate in the fates of activated T cells. In this regard, it would be of interest to test how selective

ablation of RA synthesizing capacity in the mln stroma would effect Foxp3^{+} T_{reg} induction and development of oral tolerance.

RA and TGF-β: partners in oral tolerance

While the potential participation of other sources of RA in the induction and regulation of GALT Foxp3$^+$ Treg generation remains enigmatic, the importance of vitamin A metabolism in this process is unequivocal. Thus, VAI mice that are devoid of vitamin A and its metabolites were unable to support the differentiation of Foxp3$^+$ T_{reg} in response to antigen feeding. Based on this finding, a reasonable question to test is whether oral tolerance is also hindered in these animals. Nevertheless, the manifold problems that accompany VAI may confound the interpretation of such experiments. Rather, with consideration to the importance of RA/RARα driven signals in Foxp3$^+$ Treg generation *in vitro*, it may be more straightforward to test the requirement of RARα by T cells in this process *in vivo* and its significance in oral tolerance induction. However, this experiment would first require crossing *Rara*$^{-/-}$ mice to OTII transgenic mice.

Although vitamin A metabolism is essential for GALT Foxp3$^+$ T_{reg} generation, it is not sufficient. TGF-β signaling was also critical for the capacity of LpDC to induce Foxp3 in CD4$^+$ T cells *in vitro*. This point was illustrated in experiments using αTGF-β mAb. This finding, however, was not altogether surprising, as virtually every report detailing the peripheral requirements for the generation of CD25$^+$ or Foxp3$^+$ T_{reg}, both *in vitro* and *in vivo*, has noted the critical involvement of TGF-β (Chen et al., 2003; Fantini et al., 2004; Kretschmer et al., 2005; Wan and Flavell, 2005). Nevertheless, this is the first demonstration that LpDC and their mln counterparts are capable to produce and/or process active TGF-β (Coombes et al., 2007; Sun et al., 2007). Indeed, in comparing DC subsets in the mln, Powrie and colleagues found that the CD103$^+$ mlnDC subset was uniquely capable of expressing TGF-β related genes (Coombes et al., 2007). Further

highlighting the potential importance of this finding, it was recently demonstrated that the ability of DC to activate TGF-β on their surface via integrin alpha(v) is vital to the maintenance of intestinal homeostasis (Lacy-Hulbert et al., 2007; Travis et al., 2007). Assimilating these findings and putting them into context with the data reported above tempts speculation that the $CD103^+$ LpDC population is selectively armed with this integrin. Importantly, CD103 expression on DC is important for protection against T cell transfer mediated colitis(Annacker et al., 2005), yet LpDC from animals genetically deficient in this marker are still capable of synthesizing RA (Jaensson et al., 2008). Thus, one possibility is that CD103 expression regulates the expression of alpha(v) integrins, potentially via cellular crosstalk, as CD103's chief binding partner is E-cadherin, which is broadly expressed throughout the GI tract.

It is still unclear why RA acts merely as a cofactor with TGF-β to enhance Foxp3 generation *in vitro*, yet seems absolutely required for T_{reg} generation in the GALT. Perhaps this discrepancy could derive from alterations to the endogenous LpDC population in VAI mice that transcend a deficit in RA production. Related to this possibility, deficiency in vitamin A has been shown to alter the intestinal epithelial landscape, as well as mucous production (Cha et al.). Additionally, TGF-β activity may also be disrupted during VAI. Consistent with this possibility, a recent study showed that TGF-β production was impaired in follicular DC - which do not share their origin with hematopoietic DC - from Pp of VAI mice (Suzuki et al.).

Influence of environmental cues on Foxp3 promoting capacity of LpDC

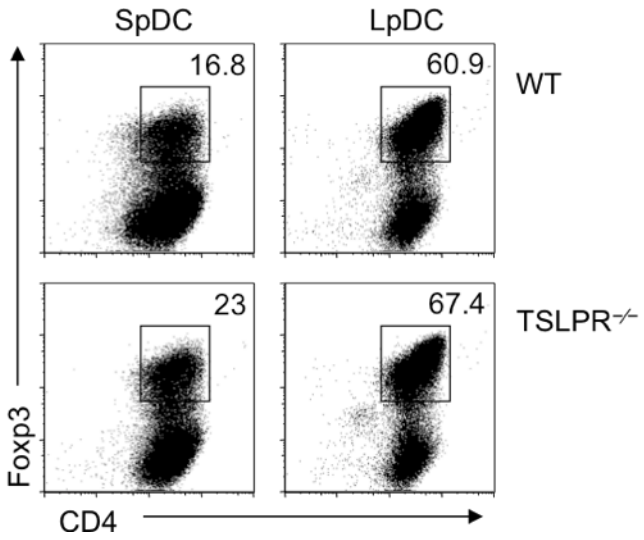


Figure 16. TSLP signals are not required for the ability of LpDC to induce Foxp3$^+$ T_{reg}.
Foxp3$^-$ cells were cultured with Sp or LpDC and stimulated in T_{reg} polarizing conditions at a 10:1 ratio for 5 days. Dot plots are gated on CD4$^+$ cells and the percentages of Foxp3$^+$ cells are shown.

Importantly, changes in epithelial homeostasis may have broad implications on the conditioning of DC within the Lp. For instance, intestinal epithelial production of the cytokine, thymic stromal lymphopoietin (TSLP), was revealed to promote IL-10 secretion by DC and diminish their capacity to polarize a T_H-1 response (Rimoldi et al., 2005) (Zaph et al., 2007). TSLP was also found to promote T_{reg} induction in human thymic T cells via effects on DC maturation (Watanabe et al., 2005). In light of these discoveries, a potential role for TSLP in controlling the ability of LpDC to preferentially induce Foxp3$^+$ T_{reg} was evaluated. However, LpDC from TSLP receptor deficient (TSLPR$^{-/-}$) mice were as competent as their WT counterparts at promoting high frequencies of Foxp3$^+$ T cells in coculture (**Figure 16**). Moreover, these animals displayed no alterations in the frequency and absolute number of Foxp3$^+$ T_{reg} across several lymphoid and non-lymphoid tissues (**data not shown**). Yet, it is still possible that other conditioning factors produced by epithelia contribute to the capacity of LpDC to drive a Foxp3 program. Highlighting this possibility, RA and TGF-β released from an intestinal epithelial line were

shown to confer RA synthesizing capacity, CD103 and active TGF-β in conditioned bone marrow DC (Iliev et al., 2009). Alternatively, LpDC may receive environmental cues from other cells within the lamina propria. For example, GM-CSF secreted from an F4/80 (macrophage like) population was proposed to potentiate RA synthesizing capacity in LpDC (Yokota et al., 2009).

In summary, the data provided here suggest that the intestinal immune system, through the simultaneous production of TGF-β and RA, has evolved a self-contained strategy to promote Foxp3$^+$ T_{reg} induction. The dual necessity for these factors would likely restrict this process to lymphatic sites draining tissues that reside in close contact with opportunistic points of entry, such as the gastrointestinal, nasopharyngeal, and possibly the urogenital tract. Notably, in response to antigen feeding, Foxp3$^+$ induced cells were found to preferentially accumulate in the Lp tissue and were detected less commonly in the draining mln. This may be a reflection of the sentinel role played by these cells, which could facilitate their rapid deployment into tissue. In this regard, it deserves mention that a recent and elegantly conducted study, which compared TCRα sequences across various CD4$^+$ T cell subsets in an effort to correlate TCR specificity with peripherally versus thymically generated Foxp3$^+$ T_{reg}, concluded that the frequency of peripherally generated Foxp3$^+$ T_{reg} was quite low (Lathrop et al., 2008). However, several caveats in this study may have led to an underestimation of the contribution of peripherally induced Foxp3$^+$ T cells to the Foxp3$^+$ T_{reg} repertoire. First, the mice used for analysis possessed a fixed TCRβ chain, so only alpha rearrangements could occur. Second, the authors did not evaluate Foxp3$^+$ T_{reg} and non-T_{reg} repertoires in tissue sites, where the data described here would predict most of the induced Treg reside. Thus, based on a high probability that the thymus and gut, as well as other tissues exposed to

non-sterile environments maintain a degree of non-overlapping antigen repertoires, it is plausible that peripheral Foxp3$^+$ T_{reg} generation potentially expands the Foxp3$^+$ T_{reg} repertoire.

CHAPTER 3: Commensal DNA limits peripheral Foxp3$^+$ T_{reg} generation and is an adjuvant of intestinal immune responses

Abstract:

The intestinal tract is in intimate contact with the commensal microbiota. Yet, how commensal signals communicate with cells in this tissue to ensure immune homeostasis is still unclear. In addition, the importance of these signals in the regulation and generation of mucosal immune responses has not been amply investigated. Continuing from findings described in **Chapter 2**, this study begins with an assessment of the influence of commensal components on the Foxp3$^+$ T_{reg} polarizing capacity of DC from the intestinal lamina propria. Remarkably, only TLR9 engagement was found to have a significant impingement on the induction of Foxp3$^+$ T_{reg}. Pairing these findings with work initiated by Dr. Nicolas Bouladoux, resulted in a collaborative effort (each figure that was a collaboration is indicated by an (*)), which produced the following data:

1. **TLR9 stimulation of LpDC limited Foxp3$^+$ T_{reg} generation in favor of the generation of T_{eff} equipped with mucosal homing capacity.**
2. **Commensal microbiota derived DNA (gfDNA) was a natural TLR9 ligand that impaired Foxp3$^+$ T_{reg} generation**
3. ***TLR9*$^{-/-}$ mice experienced dysregulated T_{reg}/T_H homeostasis in the GI tract, and displayed an expansion of Foxp3$^+$ T_{reg} that contributed to impaired mucosal T cell responses following oral infection with *Encephalitozoon cuniculi*.**
4. **gfDNA was a natural adjuvant of mucosal T_H responses.**

Rationale:

The ability of the gastrointestinal tract and its associated lymphoid tissues to support the induction Foxp3$^+$ T_{reg} may help curb untoward responses to dietary, floral and environmental antigens, which otherwise put the host at risk for allergic hyperresponsiveness and tissue damage (Curotto de Lafaille et al., 2008). Yet this mode of regulation, as well as others that are constitutively supported in this tissue must be tempered in order to avoid compromising effective immunity. Commensal and pathogenic microbes routinely reveal and radio their presence to the host immune system through conserved ligands that are cardinal features of microorganisms, which commonly signal through the toll like family of receptors (TLR) and subsequently through the adaptor protein, Myd88 (Sansonetti and Di Santo, 2007) (Medzhitov et al., 1998). These signals positively regulate the sampling of luminal content by DC in the underlying Lp tissue (Chieppa et al., 2006; Niess and Reinecker, 2005), indicating that LpDC constitutively "sense" commensals and dietary antigen. Indeed, the constant exposure of the intestinal immune system to flora and its constituent ligands provides a rationale to consider the physiological impact of TLR signaling not just on GALT T_{reg} generation, maintenance and regulation, but more broadly in the control of T cell homeostasis in this region. Such an understanding will be critical to unraveling how regulatory thresholds are set and overcome in the control of host tissue immunity.

Results:

TLR9 signaling limits LpDC mediated Foxp3$^+$ T_{reg} induction *in vitro.*

To begin to address how the commensal flora may influence peripheral Foxp3$^+$ T_{reg} induction in the GALT, LpDC were cocultured with naïve CD4$^+$ T cells in T_{reg} polarizing conditions and exposed to various purified and synthesized TLR ligands. With intent to avoid direct responses of the T cells to TLR stimulation, T cells from *Myd88* deficient mice were used for these experiments. LpDC were revealed to express weak to undetectable levels of TLRs 2 and 4, yet readily expressed message for TLRs 5 and 9 (Uematsu et al., 2008). Thus, the addition of peptidoglycan (PGN), or lipopolysaccharide (LPS), which engage TLRs 2 and 4, respectively, only marginally affected the outcome of Foxp3$^+$ T cell generation in coculture (**Figure 17A**). The addition of flagellin (Flgn), which engages TLR5 (Uematsu et al., 2006), also had no obvious effect on Foxp3$^+$ T_{reg} induction even at a high input concentration, raising the possibility that TLR5 expression may vary depending on the animal facility in which the mouse is reared. Conversely, the addition of ODN1826 (CpG), which stimulates cells through TLR9, resulted in a potent reduction in the frequency of Foxp3$^+$ T_{reg} recovered at the end of coculture (**Figure 17A-B).** This inhibition was observed at CpG doses equal to or exceeding 100ng/ml (**Figure 17A-B)**. Analysis of $\alpha_4\beta_7$ revealed that rather than being inhibited, the expression of this retinoic acid signaling dependent integrin was increased, most notably on the Foxp3 negative population (**Figure 17C)**. Consequently, the tolerogenic and homing effects of RA are decoupled in the presence of a strong adjuvant, such as CpG.

Cells that failed to up-regulate Foxp3 had potentially outgrown T_{reg} cells in CpG stimulated cocultures. To assess this, naïve *TLR9*$^{-/-}$ T cells were CFSE labeled prior to

culture. However, gating on the Foxp3^{-} cells indicated that they proliferated comparably in both CpG stimulated and non-stimulated cocultures (**Figure 17D**). The next question was whether TLR9 signaling could inhibit naïve T cell up-regulation of Foxp3 during differentiation. In fact, even before Foxp3^{+} cells had undergone a single division, their frequency was reduced 50% in cocultures containing CpG.

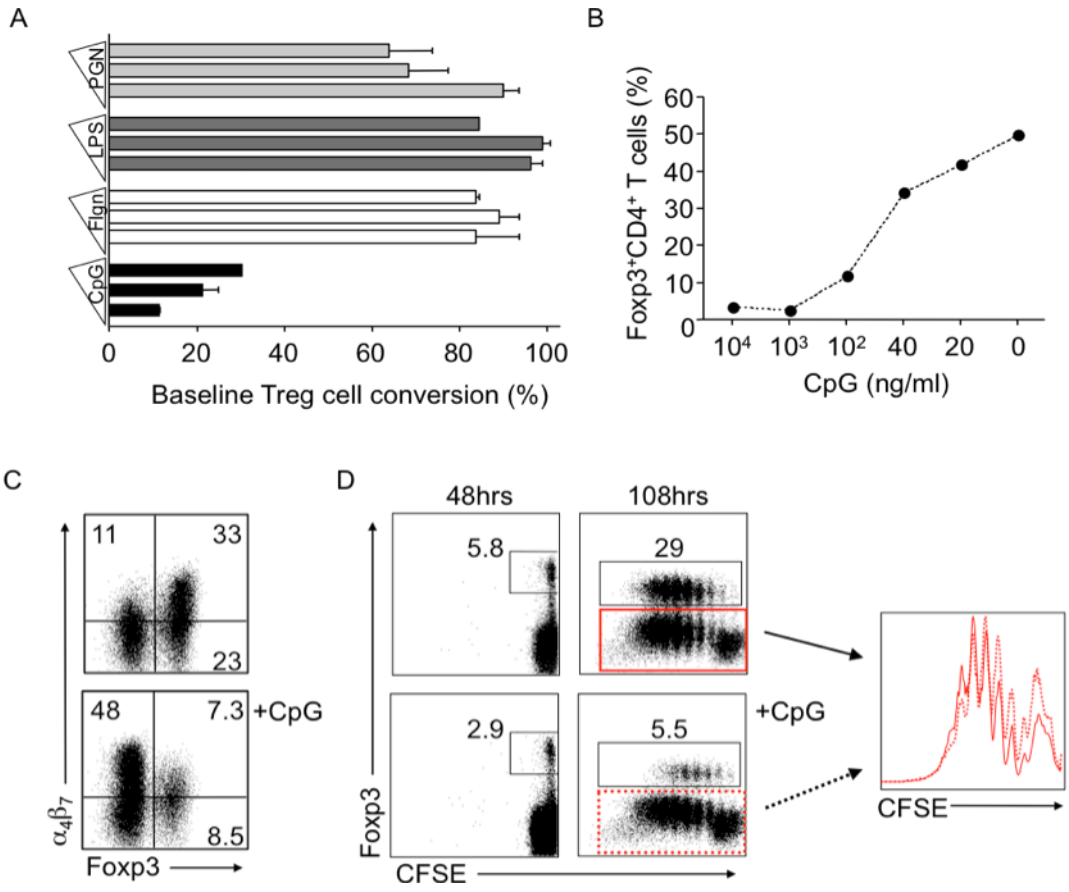


Figure 17. TLR9 signaling inhibits LpDC induced Foxp3$^+$ T_{reg} generation.
(A) FACS-sorted naïve CD4$^+$CD25$^-$CD44^{lo} T cells isolated from myd88$^{-/-}$ mice were cultured in T_{reg} polarizing conditions with WT LpDC at a 10:1 ratio in the presence of the indicated TLR ligand for 5 days. PGN (TLR2), LPS (TLR4), Flgn (TLR5) or CpG (TLR9) was added at a starting concentration of 2 μg/ml, 10 μg/ml, 1 μg/ml or 10 μg/ml, respectively. Two subsequent five-fold dilutions of each ligand were tested (wedge). Results were then normalized to the frequency of Foxp3$^+$ T cells generated in T_{reg} polarizing conditions without TLR ligands Cross bars indicate the high and lows of duplicate cultures. (**B**) Dosage effect of CpG on LpDC induced Foxp3$^+$ T_{reg} generation. **(C)** Same as in **A**. Dot plots are gated on viable CD4$^+$7-AAD$^-$ T cells and illustrate $\alpha_4\beta_7$ versus Foxp3 expression following culture with or without CpG (10 μg/ml). **(D)** Naïve CD4$^+$CD25$^-$CD44^{lo} T cells were isolated from *TLR9*$^{-/-}$ mice, labeled with CFSE, and cultured as in **A**. CFSE dilution was assessed as a function of Foxp3 at the indicated time points. Histogram overlay of CFSE dilution profiles for CD4$^+$Foxp3$^-$ T cells, indicated in the boxed in regions (solid line: control; dotted line: in presence of CpG).

TLR9 signaling promotes T_H cell differentiation in lieu of Foxp3$^+$ T_{reg}.

CpG stimulation of LpDC was found to induce their production of proinflammatory cytokines, including: IL-6, IL-12p40, and TNF-α (**Figure 18A**). The IL-12p40 subunit can form a heterodimer with IL-12p35 to comprise IL-12 or with IL-23p19 to comprise IL-23; however, these cytokines were not examined in these experiments. IL-27 was also not examined, although it deserves mention that LpDC have been shown to express both of the subunits comprising this cytokine, EBI3 and IL27p28 at steady-state (Oldenhove et al., 2009). Nevertheless, in comparison to other TLR stimuli, CpG was the only one that induced a strong proinflammatory cytokine profile by LpDC (**Figure 18B**). Consequently, in place of Foxp3, TLR9 activated LpDC differentiated naïve T cells into T_H capable of IL-17A, IFN-γ as well as IL-4 secretion (**Figure 19**). Thus, coupled with its ability to induce $\alpha_4\beta_7$, TLR9 stimulation of LpDC may help shape T_{reg}/T_H composition throughout the GALT.

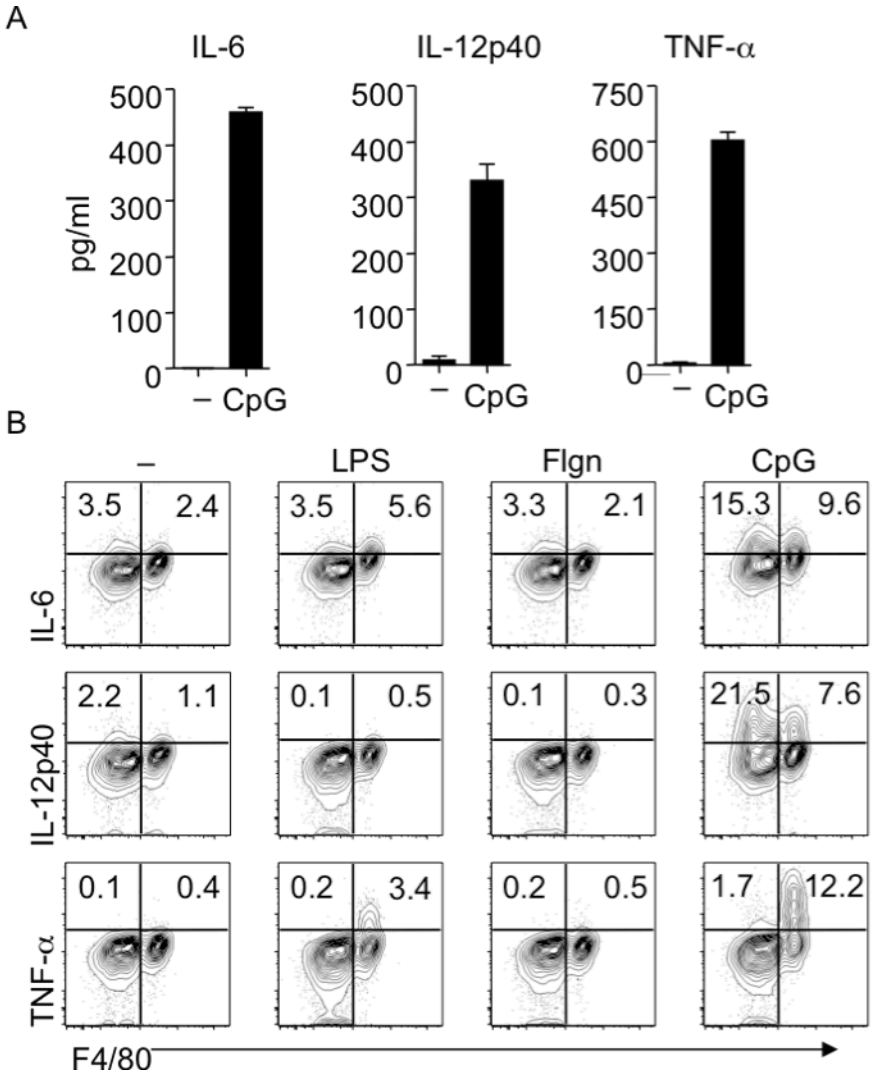


Figure 18. TLR9 stimulation of LpDC promotes robust pro-inflammatory cytokine production.

(A) 5x10^4 FACS purified LpDCs were stimulated over night in the presence or absence of CpG (1 μg/ml) in complete media supplemented with 40 ng/ml of GM-CSF. Supernatants were assessed for cytokines by ELISA. **(B)** Lp cell suspensions were stimulated with the indicated TLR ligands (1μg/ml) for 4 hrs and subsequently surface stained with CD11b, CD11c, Class II, F4/80, and fixable live/dead dye. Upon fixation/permeabilization, cells were stained intracellularly for IL-6, IL-12p40 and TNF-α. Counter plots are gated on CD11c^{hi} and CD11c^{hi} CD11b$^+$ cells.

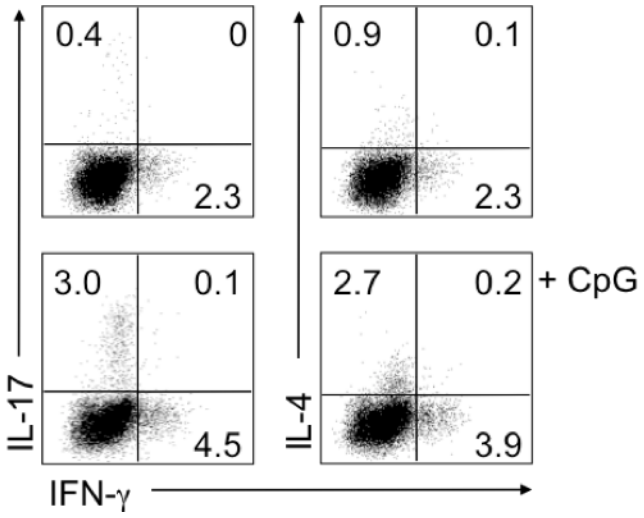


Figure 19. TLR9 stimulated LpDC induce T_{eff} programs in Foxp3$^+$ T_{reg} polarizing conditions. CD4$^+$ CD25$^-$CD44^{lo}Foxp3$^-$ T cells from *Tlr9*$^{-/-}$ Foxp3^{eGFP} mice were cultured in T_{reg} polarizing conditions in the presence or absence of CpG for 5 days and then restimulated with PMA + ionomycin for assessment of intracellular cytokine production. Cytokine percentages in bordered regions are expressed as a percentage of viable CD4 cells.

TLR9 signaling abrogates Foxp3$^+$ T_{reg} generation through undefined innate pathways

Among the innate cytokines, IL-6 and IL-27 were previously reported to potently inhibit Foxp3$^+$ T_{reg} generation *in vitro* (Bettelli et al., 2006; Korn et al., 2007). Depending on the conditions and source of costimulation, IL-12 was shown to have a marginal to moderate negative impact on T_{reg} polarization (Bettelli et al., 2006; Wei et al., 2007), while IL-23, had a minimal influence (Izcue et al., 2008). To test the relative significance of TLR9-induced IL-6 production on the subversion of Foxp3$^+$ T_{reg} induction, IL-6 signaling was blocked with anti-receptor and neutralizing Abs (αIL-6) or bypassed with LpDC genetically deficient for IL-6 (*Il-6*$^{-/-}$). While both of these measures curbed IL-17A production, neither was sufficient to reverse the effect of TLR9 stimulation on T_{reg} inhibition (**data not shown and Figure 20A**). Additional blockade with antibody against IL-27p28 also failed to induce any appreciable rebound; nevertheless it is important to note that the efficacy of this mAb was not confirmed for these assays (**Figure 20A**). Assuming that αIL-27 reduced IL-27 signaling in the assays described, these data indicated that TLR9 signaling hindered the capacity of LpDC to induce Foxp3$^+$ T_{reg} through complex innate pathways. In this regard, the group that showed that IL-23 was unable to inhibit Foxp3$^+$ T_{reg} induction *in vitro* more recently showed that the IL-23R was a cell intrinsic regulator of the differentiation/accumulation of Foxp3$^+$ T_{reg} *in vivo* (Ahern et al.). As such, IL-23 signaling may negatively regulate Foxp3$^+$ differentiation/accumulation in concert with other cytokines. Although further addition of an Ab directed against IL-12p40, which has been validated to block both free IL-12p40 and the IL-12 heterodimer, in cocultures containing *Il-6*$^{-/-}$ LpDC also failed to relieve Foxp3 inhibition, the efficacy of this Ab against IL-23 has not yet been tested (**Figure 20A**). In light of the effectiveness of Abs directed against αIL-6/αIL-6R, it may be

worthwhile in the future to perform these experiments with LpDC obtained from *IL12p40*$^{-/-}$ mice.

Intriguingly, blockade of IL-6 treatment consistently enhanced IFN-γ, IL-4, and IL-10 production (**Figure 20B**). While IL-10 was recently demonstrated to only slightly reduce the frequency of Foxp3^{+} T cells generated in T_{reg} polarizing conditions (Bettelli et al., 2006), IFN-γ and especially IL-4, were revealed to antagonize Foxp3^{+} T_{reg} generation in a Stat1 and Stat6 dependent manner, respectively (Wei et al., 2007). Moreover, enforced expression of T_H-1 and T_H-2 transcription factors was sufficient to effect this inhibition in the absence of these cytokines. These findings coupled with the cytokine modulation that occurred in the face of IL-6 blockade indicated that deviation towards T_H-1 and T_H-2 programs, in addition to T_H-17, was potentially complicit in TLR9-induced Foxp3 inhibition. In accord with this hypothesis, the addition of neutralizing Abs against both IL-4 and IFN-γ, in addition to αIL-6, resulted in a ~70% rebound in the frequency of Foxp3^{+} T_{reg} cells (**Figure 21A and B**). IL-4 neutralization drove a greater rebound in the frequency of Foxp3^{+} T cells at the end of culture than IFN-γ neutralization. Complementing these data, addition of each of these cytokines blocked LpDC-induced Foxp3 development in a dose dependent fashion, where IL-6 exerted the most potent inhibition at all concentrations tested. Notably, suppression was further enhanced when the cytokines were combined (**Figure 21C**). These data demonstrated that TLR9 activation overrode preferential T_{reg} induction via non-specific T_H programming.

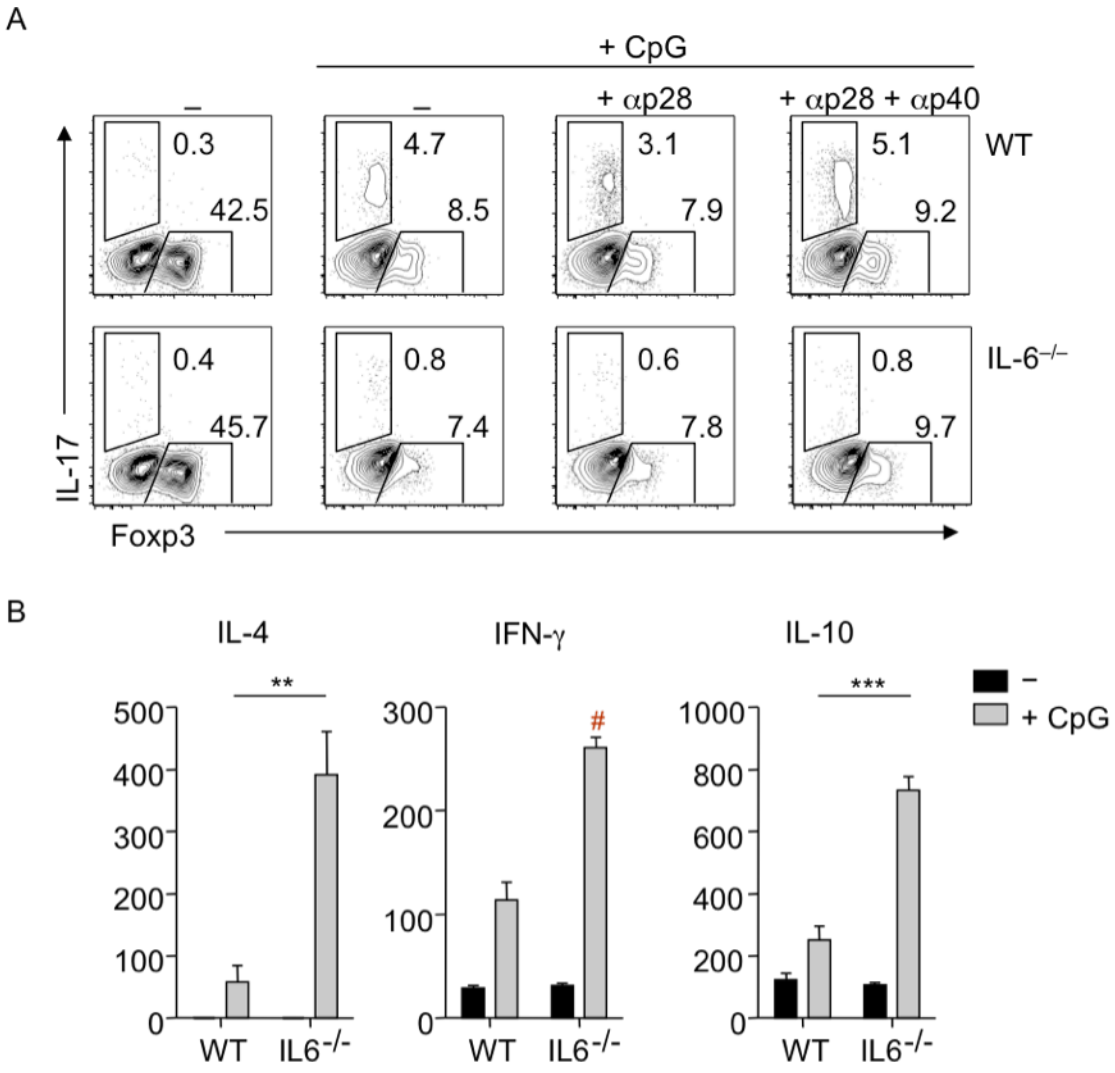


Figure 20. TLR9 stimulated LpDC redirect polarization of T cells in Foxp3^{+} T_{reg} polarizing conditions via multiple innate pathways.
(A) CD4^{+}CD25^{-}CD44^{lo}Foxp3^{-} T cells from *Tlr9*$^{-/-}$ Foxp3^{eGFP} mice were cocultured with LpDCs purified from WT or *IL-6*$^{-/-}$ mice for 5 days. In wells where CpG (10μg/ml) was added, the effect of various cytokine blocking Abs was assessed, including: αIL-27p28 and αIL-12p40/p70. Each condition was tested in triplicate. **(B)** Supernatants from cocultures in **A** were assayed for IL-4, IFN-γ and IL-10 by ELISA. Error bars depict the s.e.m. (#, sample was tested in duplicate; **, *P* <0.01; ***, *P* <0.001).

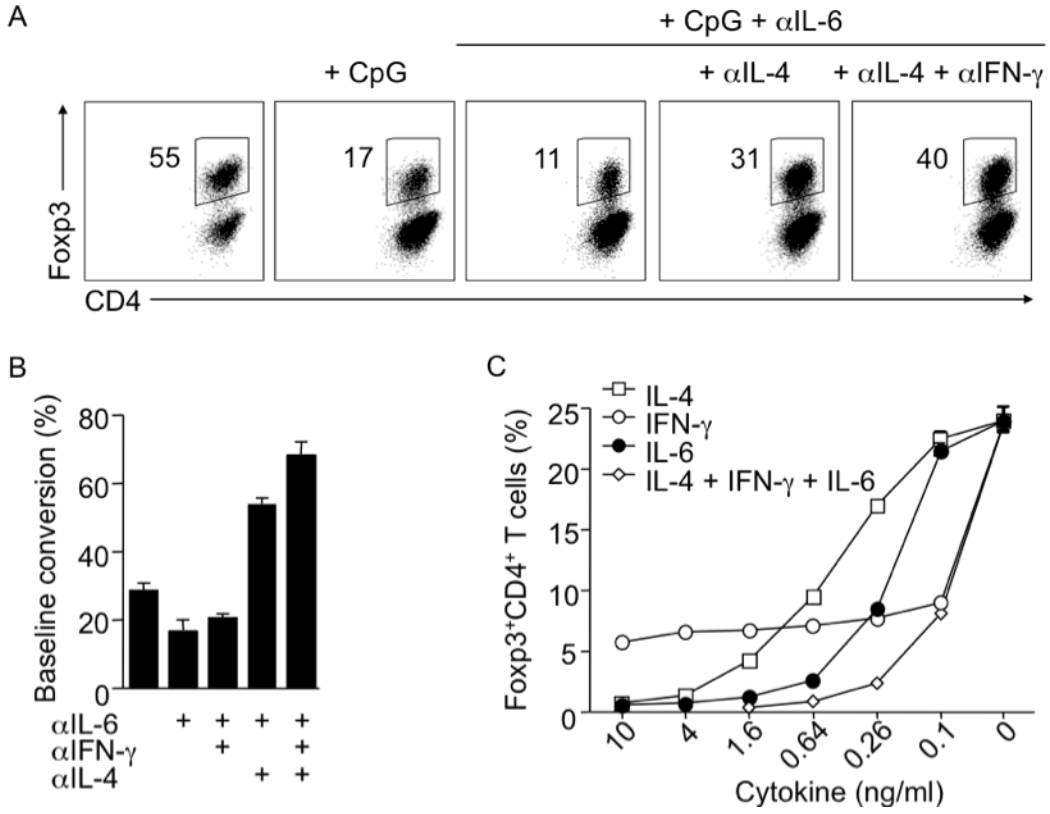


Figure 21. Partial rescue of Foxp3 development in the face of TLR9 activated LpDCs.

(A) Naïve $CD4^+CD25^-CD44^{lo}$ T cells from *Myd88*$^{-/-}$ mice were cultured in T_{reg} polarizing conditions for 5 days in the presence or absence of CpG (10μg/ml). In wells containing CpG, antibodies to IL-6 and IL-6 receptor α (αIL-6), IL-4 (αIL-4) and IFN-γ (αIFN-γ) were added at the start of culture as indicated. Bracketed areas indicate the percentage of $Foxp3^+$ cells. **(B)** Summary of results in **A** normalized to baseline T_{reg} induction. Crossbars represent the high and lows of conditions cultured in duplicate. **(C)** Blockade of LpDC induced $Foxp3^+$ T_{reg} generation by IL-6, IL-4 and IFN-γ. Naïve $CD4^+CD25^-CD44^{lo}$ T cells were cocultured with LpDCs in T_{reg} polarizing conditions. Various doses of IL-6, IL-4, IFN-γ or a combination of all 3 cytokines were added at the start of culture. Cytokine concentration is shown on the x-axis. In wells containing cytokine combinations, the same concentration of each cytokine was used.

Engagement of TLR9 by commensally derived gut flora DNA inhibits Foxp3$^+$ T_{reg} induction *in vitro*

TLR9 recognizes unmethylated CpG dinucleotides, which are abundant in the DNA of various prokaryotic species populating the GI tract (Dalpke et al., 2006). To test whether physiological ligation of TLR9 influenced LpDC in a manner similar to CpG, DNA was extracted from the gut content of caeca (gfDNA) and colons in naïve C57BL/6 mice and added to LpDC cocultures in Foxp3$^+$ T_{reg} polarizing conditions. In the absence of a defined sequence length for gfDNA, a control condition to guarantee cellular uptake was also included. In this condition, gfDNA was complexed to the monocationic lipid transfection reagent, DOTAP, prior to stimulation (Bucci et al., 1992). Notably, cathelicidins, which are a family of cationic antimicrobial peptides secreted by intestinal epithelial cells, and innate cells, including neutrophils and macrophages, may serve a physiologically analogous role to DOTAP, by virtue of their ability to package DNA (Lande et al., 2007), Regardless of whether DOTAP was present, gfDNA consistently and significantly reduced the frequency of Foxp3$^+$ T_{reg} recovered from culture, and upregulated $\alpha_4\beta_7$ on Foxp3$^-$ cells (**Figure 22A-B**). This effect was abolished when purified LpDC from *Tlr9*$^{-/-}$ mice were used, demonstrating that gfDNA targeted TLR9.

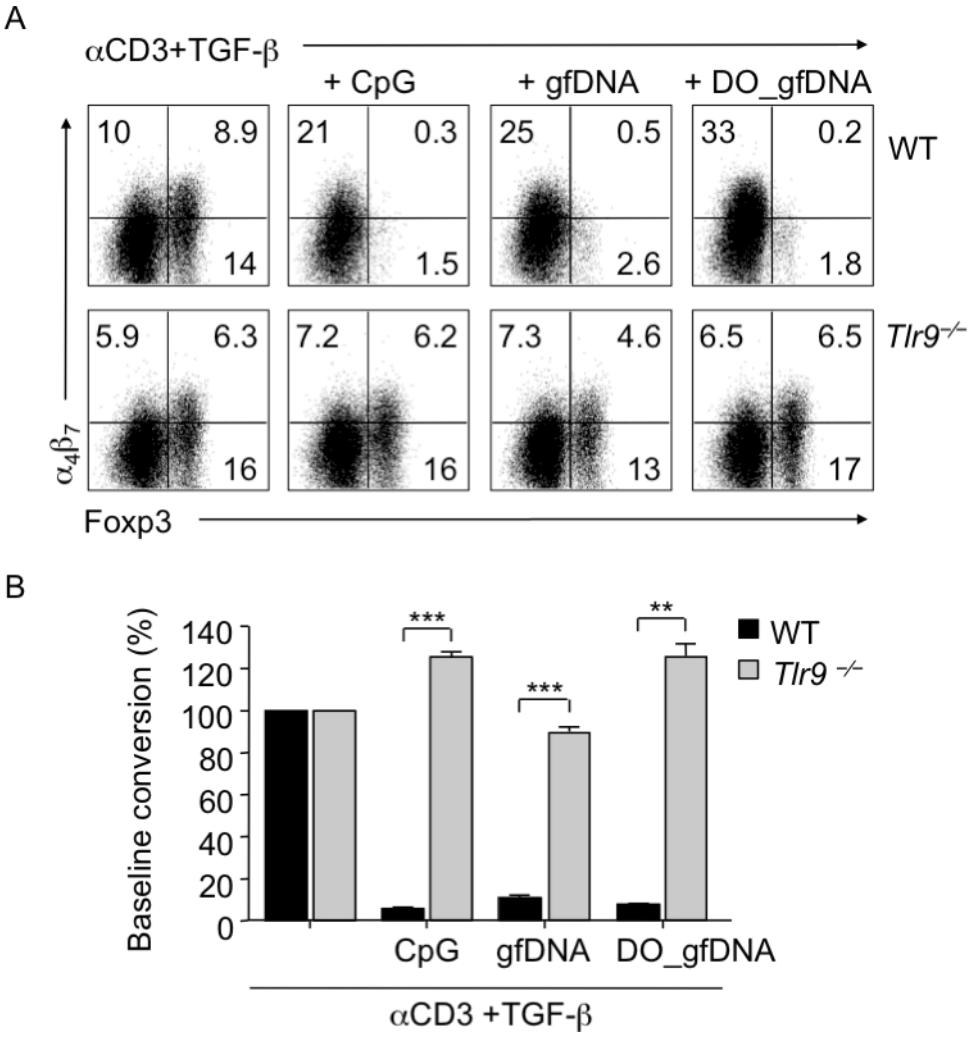
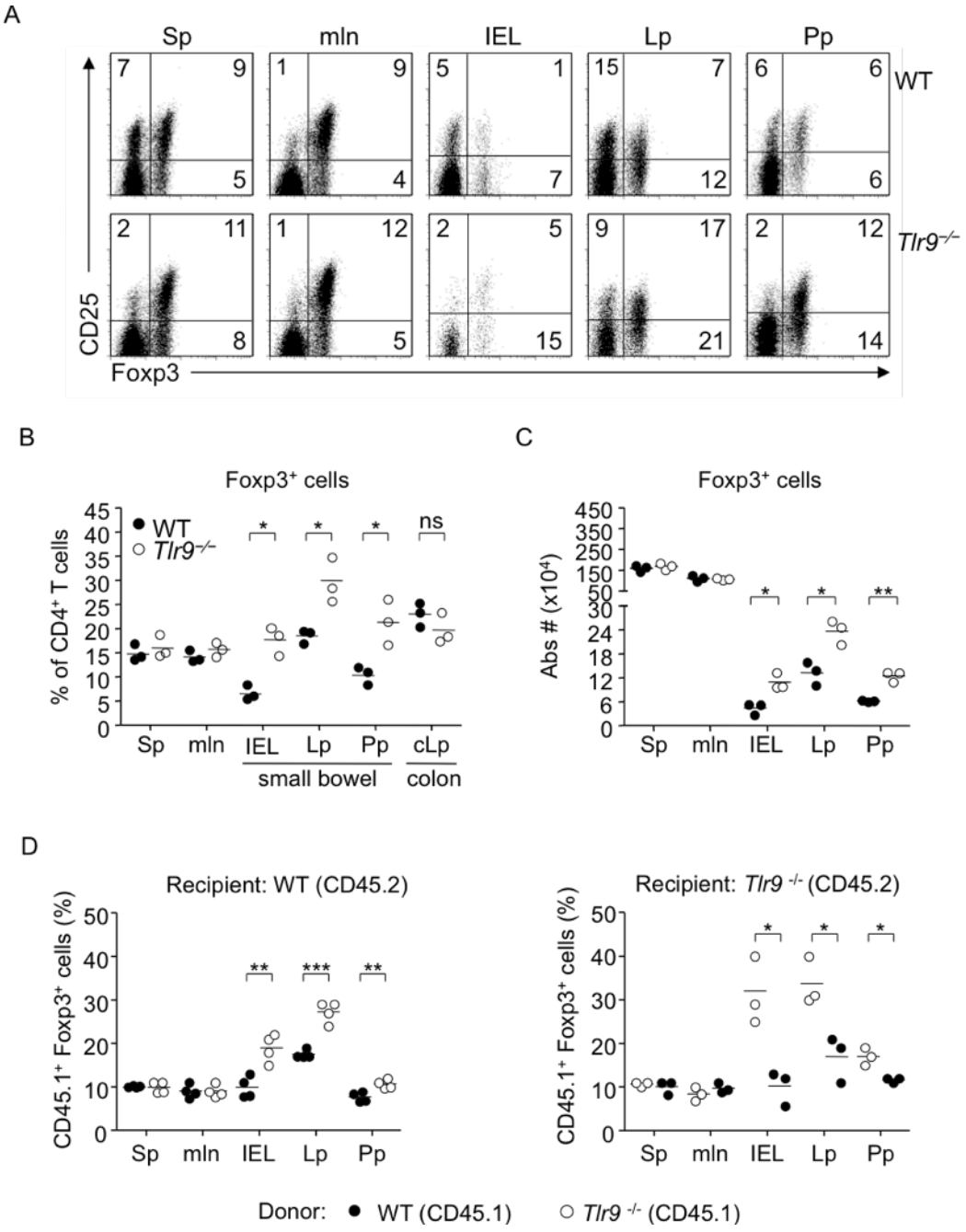


Figure 22. DNA enriched from the gut flora prevents Foxp3$^+$ T_{reg} production in a TLR9 dependent manner.

(A) Naïve CD4$^+$CD25$^-$CD44^{lo} T cells isolated from WT or *Tlr9*$^{-/-}$ mice were cultured in T_{reg} polarizing conditions with LpDC from WT or *Tlr9*$^{-/-}$ mice, respectively. In some culture wells, CpG (10μg/ml), DNA enriched from murine gut flora (gfDNA) or gfDNA formulated with the cationic liposome, DOTAP, was added (DO_gfDNA). (**A**) Foxp3 and $\alpha_4\beta_7$ expression was analyzed on day 5. Dot plots are gated on viable CD4$^+$ cells. **(B)** Summary of results from **A** (normalized to the baseline T_{reg} frequency. Error bars represent the s.e.m. of triplicate cultures (**, P <0.01; ***, P <0.001).

TLR9 signaling regulates Foxp3$^+$ T_{reg}/T_H cell ratio in intestinal tissues

The preceding data suggested that gfDNA/TLR9 signaling contributed to T cell homeostasis in the GALT. To test this hypothesis, Foxp3$^+$ T_{reg} populations were first assessed in various tissues of naïve *Tlr9*$^{-/-}$ and WT mice. Strikingly, *Tlr9*$^{-/-}$ mice displayed greater percentages and absolute numbers of Foxp3$^+$ T_{reg} cells than their WT counterparts throughout the GALT, including the intestinal epithelial lymphocyte (IEL), small intestinal Lp, and Peyer's patch (Pp) compartments (**Figure 23A-C**). This trend did not extend to the mesenteric lymph nodes (mln), nor was it observed outside of the small intestinal compartment. To exclude the possibility that these findings arose due to distinct commensal populations residing within these animal strains, these differences were assayed and reconfirmed in animals that were co-housed for several weeks (**data not shown**). Importantly, the selective expansion of Foxp3$^+$ T_{reg} in the GALT was observed in chimeric WT mice reconstituted with *Tlr9*$^{-/-}$ bone marrow, while it was reversed in chimeric *Tlr9*$^{-/-}$ mice reconstituted with WT bone marrow (**Figure 23D**). These data demonstrated that the hematopoietic compartment controlled Foxp3$^+$ T_{reg} homeostasis in the GALT. Further, based on the previous *in vitro* experiments, it was plausible that this likely occurred, at least in part, via constitutive gfDNA/TLR9 interactions in LpDC.


A
Sp
mln
IEL
Lp
Pp
WT
Tlr9−/−
CD25
Foxp3
B
Foxp3+ cells
% of CD4+ T cells
WT
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ns
Sp
mln
IEL
Lp
Pp
cLp
small bowel
colon
C
Foxp3+ cells
Abs # (x10⁴)
D
Recipient: WT (CD45.2)
Recipient: Tlr9 -/- (CD45.2)
CD45.1+ Foxp3+ cells (%)
Donor: WT (CD45.1) Tlr9 -/- (CD45.1)

Figure 23. (*) Hematopoietic derived TLR9 signals regulate Foxp3$^+$ T_{reg} homeostasis in the gastrointestinal tract during steady-state.
(A) Comparative assessment of CD4$^+$Foxp3$^+$ T_{reg} cells in the spleen (Sp), mesenteric lymph node (mln), intestinal epithelium lymphocyte (IEL), intestinal lamina propria (Lp) and Peyer's patch (Pp) compartments of age-matched naïve WT and *Tlr9*$^{-/-}$ mice. Cells gated on CD4 and TCR-β^+ were analyzed for expression of Foxp3 and CD25 by flow cytometry. Numbers in quadrants refer to the percentage of each subset. **(B)** Summary of T_{reg} frequencies in naïve WT (closed circle) and *Tlr9*$^{-/-}$ (open circle) mice. cLp = colon lamina propria. Each dot represents the results from one experiment (three mice pooled per group). Crossbars depict the mean of three independent experiments. **(C)** Same as **B**, but a summary of the absolute number of T_{reg}. **(D)** Frequency of Foxp3$^+$ T_{reg} was analyzed in various tissues of BM chimeric mice, in which the hematopoietic or the non-hematopoietic compartment lacked TLR9 expression. CD45.1+ cells were gated on CD4 and TCR-β^+, and then analyzed for Foxp3 expression. (*, P <0.05; **, P <0.01; ***, P <0.001)

To further probe how constitutive TLR9 signaling shaped the T cell landscape in the GI tract, Lp tissue cell suspensions, in which most conventional $\alpha\beta$ T cells are of the CD4$^+$ lineage, and IEL tissue cell suspensions, in which a majority are of the CD8$^+$ lineage, from both WT and *Tlr9*$^{-/-}$ mice were restimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and then analyzed for cytokine production. In *Tlr9*$^{-/-}$ mice, a significant reduction in both the frequency and absolute number of IL-17A and IFN-γ producing cells was observed in the Lp compartment, while IL-10 was less affected (**Figure 24A-B**). A similar reduction in the frequency and absolute number of IFN-γ producing cells was also observed in the IEL of *Tlr9*$^{-/-}$ mice (**Figure 24A-B**). Thus, the loss of TLR9 signaling in the GI tract resulted in disequilibrium of T cell subset distribution within this tissue, culminating in an expansion of Foxp3$^+$ T_{reg} cells and a concomitant reduction in T_H cells.

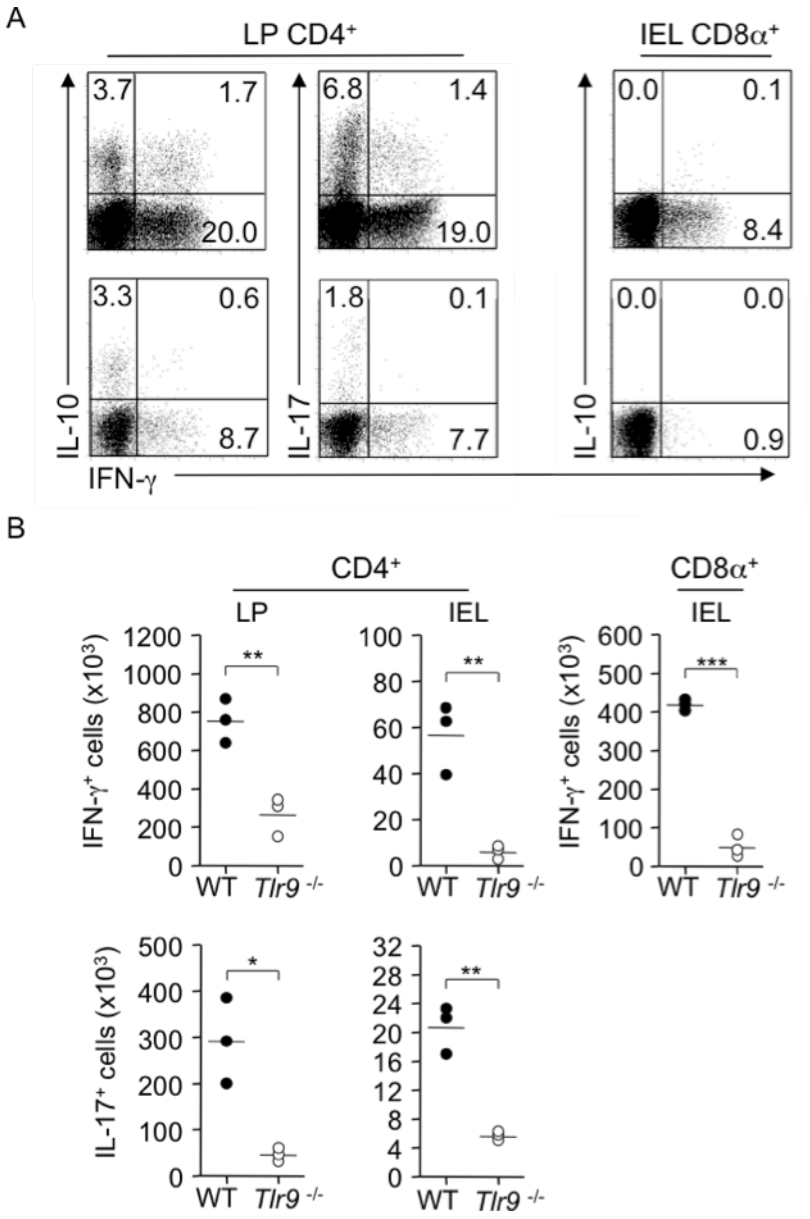

Figure 24. (*) TLR9 signaling contributes to the basal T_H tone of the GI tract.
(A) Thy 1.2 enriched cells from the Lp and IEL compartments were stimulated with PMA+ionomycin, and then stained intracellularly for IL-10, IL-17 and IFN-γ. Cytokine percentages in bordered regions are expressed as a percentage of CD4 cells. **(B)** A summary of the absolute numbers of $CD4^+$ and/or $CD8\alpha^+$ T lymphocytes expressing IFN-γ and IL-17 in naïve WT (closed circle) and *Tlr9*$^{-/-}$ (open circle) mice. Each dot represents a single mouse and each bar delineates the mean. (*, *P* <0.05; **, *P* <0.01; ***, *P* <0.001).

TLR9 signaling is requisite for optimal responses to oral infection

The profound influence of constitutive TLR9 signaling on the activation status of T cells within the GI tract suggested that gfDNA/TLR9 interactions played a potential role in establishing and amplifying protective immunity to pathogenic encounters in the GALT. To test this, *Tlr9*$^{-/-}$ and WT mice were infected with the obligate intracellular fungal parasite, *Encephalitozoon cuniculi*, (*E. cuniculi*), which naturally infects its host through the alimentary tract and belongs to the microsporidia phylum (Didier, 2005). In addition to the oral route, *E. cuniculi* induces a productive T_H-1 response following intraperitoneal (IP) challenge (Khan and Moretto, 1999; Moretto et al., 2004). To assess whether any systemic priming defects were present in *Tlr9*$^{-/-}$ mice, animals were first inoculated with *E. cuniculi* IP Measurement of the cardinal T_H-1 cytokine, IFN-γ, following *in vitro* antigen recall on day 11 of infection demonstrated that *Tlr9*$^{-/-}$ mice mounted immune responses that were comparable to their WT counterparts (**Figure 25A**). Parasite burdens were also similar between knockout and WT animals (**Figure 25B**). Together, these data indicated that systemic priming did not require TLR9 signaling. They further implied that TLR9 recognition of *E. cuniculi* was dispensable for triggering immunity to this parasite.

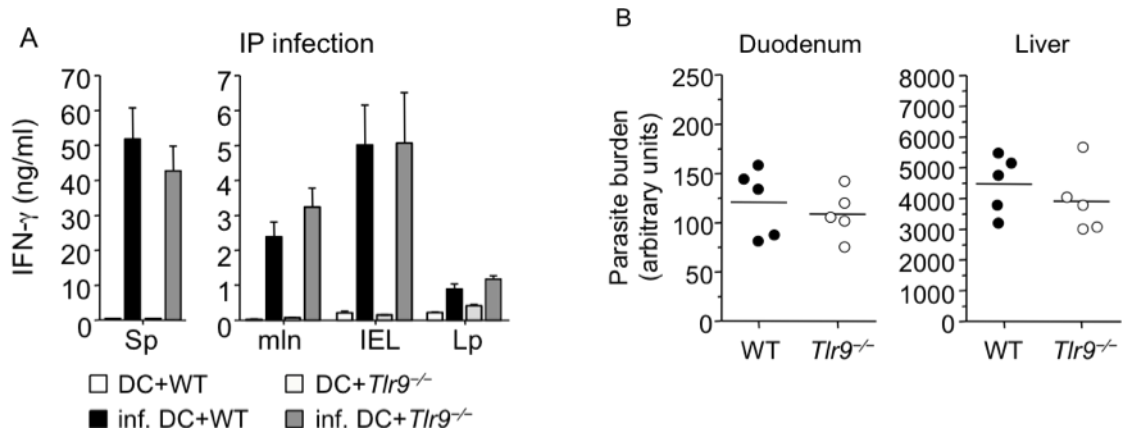

Figure 25. (*) Systemic priming and immunity to *E. cuniculi* is independent of TLR9 signaling.

WT and *Tlr9*$^{-/-}$ mice were infected with 5×10^6 freshly isolated *E. cuniculi* spores i.p. **(A)** On day 11 of infection, pooled, single cell suspensions from various tissues were incubated in triplicate with uninfected (DC) or *E. cuniculi*-infected BMDC (inf. DC). ELISA was performed on supernatants 72 hrs later. Histograms depict the mean cytokine concentration ± s.d. (**B**) Measurement of parasite load in the duodenum and liver using quantitative real-time pcr. Each dot represents an individual mouse. n = 5 per group. Bars indicate the means.

In contrast to IP inoculation, oral inoculation with *E. cuniculi* produced a significantly different picture in *Tlr9*$^{-/-}$ and WT mice. Specifically, antigen specific IFN-γ and IL-17A production were severely impaired in the IEL and LP of *Tlr9*$^{-/-}$ mice, manifesting in defective parasite clearance both in the primary (duodenum) and dissemination (liver) sites of infection (**Figure 26A-B**). Consequently, systemic immune impairments, determined by splenocyte recall of IFN-γ and IL-17A production, were also observed (**Figure 26A**). There are numerous instances illustrating that inefficient control of parasitic infection can be a consequence of improper T cell polarization (Else et al., 1994; Scott, 1991; Zaph et al., 2007). Nevertheless, although impaired T_H-1 and T_H-17 responses were accompanied with higher systemic concentrations of IL-10 and IL-4, deviation towards a (T_H-2) response was not observed in the GALT of *Tlr9*$^{-/-}$ mice (**Figure 26C and data not shown**).

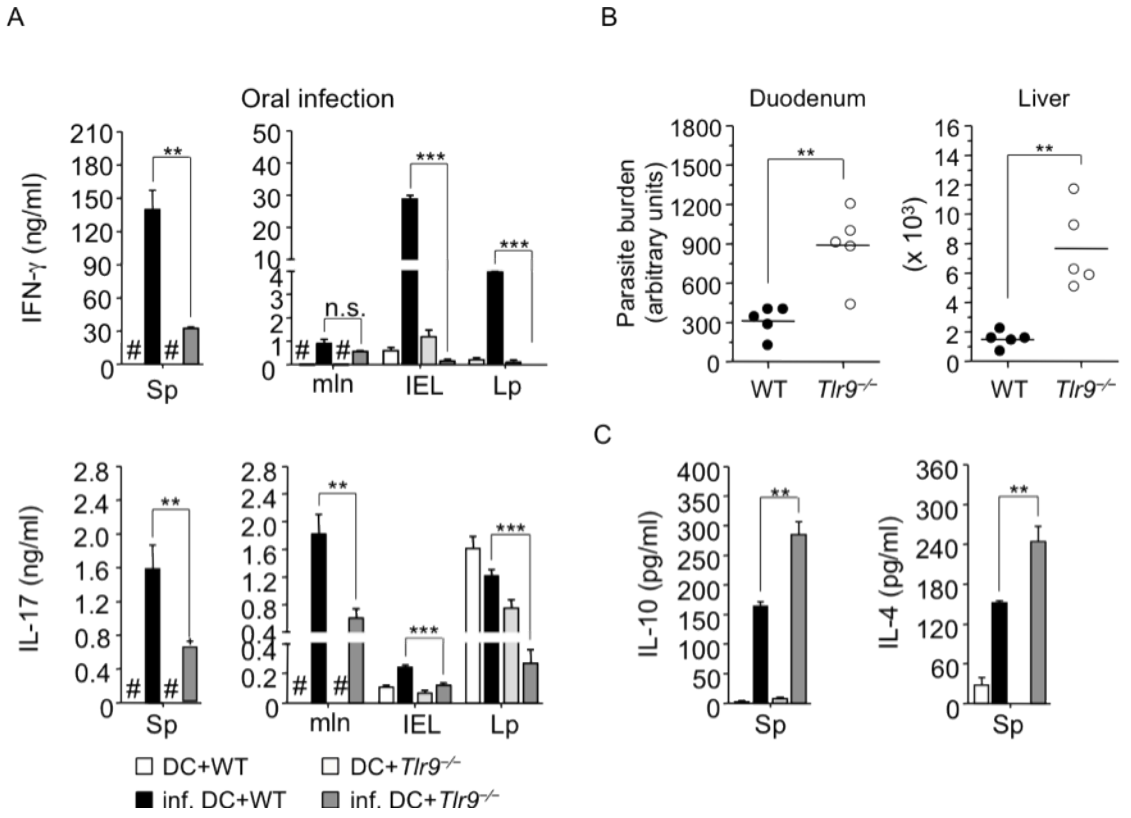

Figure 26. (*) Immunity to oral infection with *E. cuniculi* is dependent on TLR9 signaling.

WT and *Tlr9*$^{-/-}$ mice were infected by gavage with 5×10^6 freshly isolated *E. cuniculi* spores. **(A)** On day 11 of infection, pooled, single cell suspensions from various tissues were incubated in triplicate with uninfected (DC) or *E. cuniculi*-infected BMDC (inf. DC). ELISAs for IFN-γ and IL-17 were performed on supernatants 72 hrs later. Histograms depict the mean cytokine concentration ± s.d. (**B**) Measurement of parasite load in the duodenum and liver using quantitative real-time pcr. Bars indicate the means. (**C**) Same as in **A** measuring IL-10 and IL-4. (# = not detected; n.s. = not significant; **, P <0.01; ***, P <0.001). Each dot represents an individual mouse. n = 5 per group.

The expansion of GALT Foxp3$^+$ T_{reg} in the absence of TLR9 signaling contributes to deficient mucosal immune responses

Increased Foxp3$^+$ T_{reg} cell frequencies within the intestinal tissue persisted in orally infected *Tlr9*$^{-/-}$ mice (**Figure 27A**). These cells have the capacity to negatively modulate the effector stages of infectious immunity through both antigen specific and non-specific means (Belkaid and Tarbell, 2009; Oldenhove et al., 2009; Shafiani et al.). Thus, it was possible that the enhanced number and frequency of Foxp3$^+$ T_{reg} in the GI tract contributed to the impaired T_H response that ensued following oral inoculation with *E. cuniculi*. To address this hypothesis, WT and *Tlr9*$^{-/-}$ animals were treated with an antibody against CD25 (clone PC-61), an activation marker mainly restricted to Foxp3$^+$ T_{reg} during steady-state conditions (Fontenot et al., 2003; Khattri et al., 2003). This antibody was previously shown to reduce the absolute number of Foxp3$^+$ T_{reg} by as much as 50% *in vivo* and inhibit CD25 expression (Couper et al., 2007). Although CD25 is also a hallmark of activated T cells, the depletion protocol employed for these assays consistently enhanced pro-inflammatory T_H-1 cytokine production in both WT and *Tlr9*$^{-/-}$ mice, suggesting that bystander loss of T_H cells did not pose a significant confounding element to consider in the interpretation of infectious outcomes in treated animals (**Figure 27B**). Strikingly, α-CD25 treatment markedly improved parasite clearance in the duodenum and liver of infected *Tlr9*$^{-/-}$ mice, while having no discernible effect in WT mice, in which parasite burdens were already relatively low (**Figure 27C**). While these data argue that the elevated proportion of Foxp3$^+$ T_{reg} (in the GI tract of *Tlr9*$^{-/-}$ mice) constituted a barrier to efficacious mucosal immunity upon oral challenge, Lp IFN-γ production was still reduced in α-CD25 treated *Tlr9*$^{-/-}$ animals in comparison to isotype Ab treated WT animals post-infection. Based on the low expression of CD25 on T_{reg} in the Lp, one explanation was that α-CD25 failed to trigger sufficient depletion of these

cells in this tissue. The implication of this type of scenario could be that Foxp3$^+$ T_{reg} negatively regulate the amplitude and/or maintenance of T_H responses *in situ*. However, a previous report demonstrated that TLR9 signaling in DC made T_H resistant to T_{reg} regulation (Pasare and Medzhitov, 2003), indicating that gfDNA/TLR9 interactions may also promote T_H responses independent of T_{reg}.

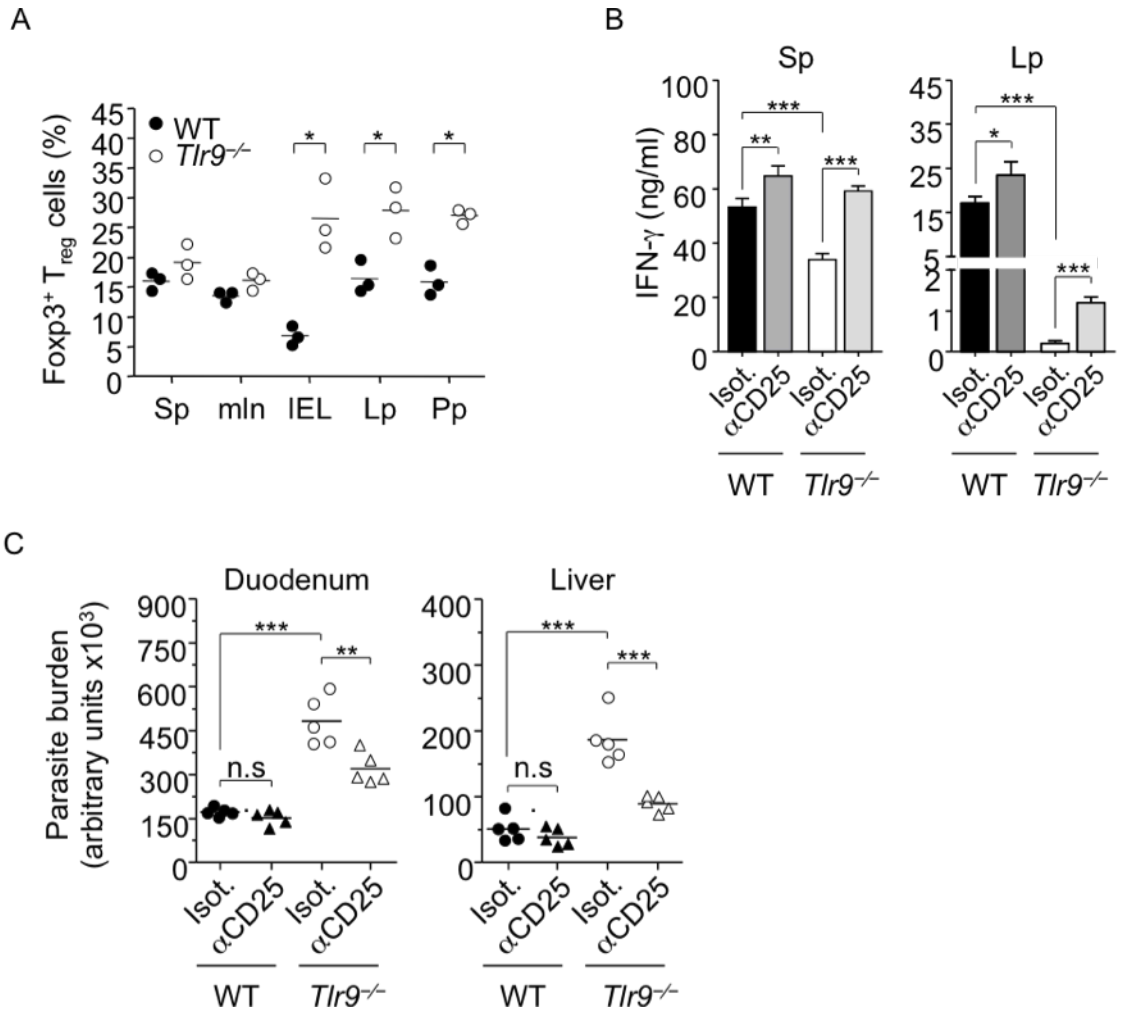

Figure 27. (*) The expansion of T_{reg} in the GI tract contributes to impaired mucosal immunity in the absence of TLR9.
WT and *Tlr9*$^{-/-}$ mice were infected by gavage with 5×10^6 freshly isolated *E. cuniculi* spores. **(A)** On day 11 of infection, the percentage of CD4$^+$Foxp3$^+$ T_{reg} cells in various tissues was assessed via flow cytometry. **(B-C)** Mice 3 days preceding and on day 0 and day 7 of infection were injected i.p. with αCD25 or isotype control Ab (Isot). (**B**) *In vitro* recall was performed as described in **Figure 26A**. Histograms depict the mean cytokine concentration ± s.d. (**C**) Measurement of parasite load in the duodenum and liver using quantitative real-time pcr. Each dot represents an individual mouse. n = 5 per group. (*, P <0.05; **, P <0.01; ***, P <0.001).

Gut flora DNA from conventional gut flora is a natural adjuvant of intestinal immune responses

Taken together, the preceding *in vitro* and *in vivo* data suggested that commensal gfDNA engagement of TLR9 provided pro-inflammatory cues during both steady-state and pathogenic challenge, thereby promoting mucosal immunity. To test this, the impact of other gut floral signals in the GI tract were minimized by placing mice on a cocktail of antibiotics (ATB) (Rakoff-Nahoum et al., 2004), while LPS, CpG, or gfDNA was provided orally once per week. 6 wk into treatment, mice were orally infected with *E. cuniculi*. While the frequency and absolute number of $CD4^+$ and $CD8^+$ T cells remained comparable between all different treatment groups (**data not shown**), ATB treated mice mounted noticeably impaired IFN-γ and IL-17A responses in both the IEL and Lp compartments (**Figure 28A**). Paralleling these impairments, parasite burden was consistently elevated in ATB treated animals (**Figure 28B**). These data demonstrated that the commensal flora were required to promote and sustain mucosal immune responses. Providing back LPS (a TLR4 ligand) led to a restoration of the systemic immune response to *E. cuniculi* in ATB treated animals, based on the IFN-γ recall response in the spleen. However, this commensal product failed to boost the immune response in the small intestinal Lp (**Figure 28C**). As such, the parasite burden in LPS treated mice remained elevated and comparable to mice receiving ATB alone (**Figure 28B**). In contrast, the addition of CpG induced a significant rebound in systemic cytokine production and propelled mucosal IFN-γ and IL-17A cytokine production to levels observed in control (Ctl) mice, which did not receive any ATB (**Figure 28A and C**).

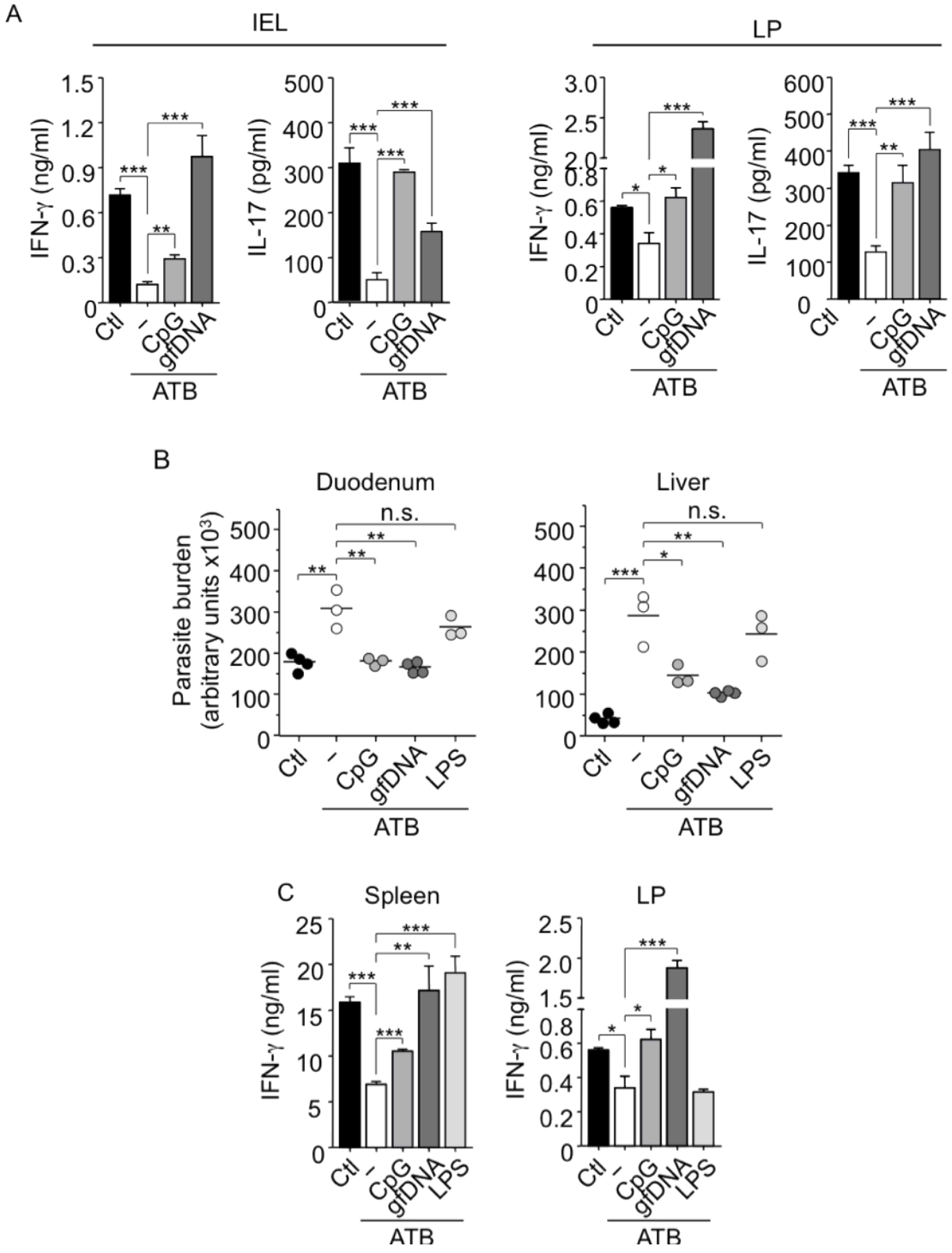
A
IEL
LP
IFN-γ (ng/ml)
IL-17 (pg/ml)
Ctl
–
CpG
gfDNA
ATB
B
Duodenum
Liver
Parasite burden (arbitrary units x10³)
n.s.
LPS
C
Spleen
LP

Figure 28. (*) Gut floral DNA restores immune responses in commensally depleted mice orally infected with *E. cuniculi*.
3 wk old mice received a commensal depleting antibiotic cocktail (ATB) in their drinking water for 6 weeks and, in tandem, received oral weekly treatments of PBS vehicle or 100 μg of CpG, 500 μg gfDNA, or 25 mg/kg of lipopolysaccharide from *Escherichia coli* (LPS). Control mice received no ATB treatment. Mice were infected orally with *E. cuniculi* after 6 weeks of treatment. **(A)** On day 11 of infection, pooled IEL and Lp suspensions from each group were restimulated as previously described. Supernatants were assessed for IFN-γ and IL-17 by ELISA. Histograms depict the mean cytokine concentration ± s.d. **(B)** 8 wk-old mice were treated and infected as described in **A**. Parasite burden was evaluated in the duodenum and liver on day 11 of infection by quantitative real-time PCR. Each dot represents one mouse and each bar the mean of three or four mice analyzed **(C)** Cell suspensions from **B** were *in vitro* restimulated and IFN-γ production was measured by ELISA. n.s. = not significant; *, P <0.05; **, P <0.01; ***, P <0.001.

This recovery of the mucosal response in CpG treated animals was also associated with a reduced parasite burden (**Figure 28B**). These data suggest that flora derived TLR9 signaling is a significant driver of mucosal immunity. Nevertheless, ATB treating *Tlr9*$^{-/-}$ mice also resulted in a reduction in the mucosal immune response, confirming that other commensally derived components can amplify mucosal immune responses, either normally or through compensatory mechanisms when TLR9 signaling is deprived or disrupted (**data not shown**). Strikingly, gfDNA restored the mucosal response to *E. cuniculi* in WT ATB treated mice, but not in *Tlr9*$^{-/-}$ mice (**Figure 28-B and data not shown**). Thus, gfDNA functions as a natural TLR9 specific adjuvant of mucosal immune responses.

Discussion

The tissues of the GI tract are constantly exposed to TLR ligands harbored by the commensal gut flora (Pamer, 2007); yet how these interactions factor into the regulation and development of immunity in this region have not been investigated in depth. The data revealed in this study, demonstrated that TLR9 stimulation, via gfDNA, antagonizes the T_{reg} polarizing capacity of LpDC. As such, in the absence of natural TLR9 signals *in vivo*, the GI tract harbored an expanded frequency of $Foxp3^+$ T_{reg} and a concomitant reduction in T_H, which in turn subdued the development of an efficient protective response upon mucosal challenge. Loss of TLR9 signaling in the hematopoietic compartment recapitulated this homeostatic dysregulation. Strikingly, orally introduced gfDNA potently adjuvanted the mucosal response to infection with *E. cuniculi*, when signals from other gut floral compents were diminished. Altogether, these data highlight the importance of *in situ* signaling from gut floral components in the generation of protective immune responses at sites of mucosal challenge.

Stromal vs hematopoeitic TLR9 engagement - controlling injury vs immunity

In contrast to hematopoeitic cells, which recognize TLR9 intracellularly (Ahmad-Nejad et al., 2002; Latz et al., 2004), epithelia in the GI tract basally express TLR9 on their surface (Ewaschuk et al., 2007; Lee et al., 2006). Although intracellular compartmentalization was revealed as a prerequisite for TLR9 activation in antigen presenting cells (Ewald et al., 2008), physiologically acidic environments may confer functional activity to TLR9 expressed on epithelial surfaces. In this regard, epithelial driven TLR9 signals were shown to promote the expression of proteins that drive Paneth cell maturation and have the potential to regulate flora composition, thereby linking lumenal signals from gfDNA to intestinal homeostasis (Lee et al., 2006). Accordingly, the

absence of TLR9 led to exacerbated colitis induced by oral administration of the polysaccharride, DSS, which disrupts epithelial integrity and facilitates bacterial translocation (Lee et al., 2006; Okayasu et al., 1990). These findings were reminiscent of an earlier study, which revealed that commensal sensing by the intestinal epithelial compartment was essential for recovery from DSS induced colitis (Rakoff-Nahoum et al., 2004). Coupled with a study demonstrating the MyD88 dependece of Paneth cell mediated barrier protection (Vaishnava et al., 2008), these findings suggest that gfDNA/TLR9 interactions on the intestinal epithelia promote barrier protection and facilitate recovery from acute intestinal epithelial injury.

While intestinal epithileal sensing of gfDNA appears to drive mucosal homeostasis through barrier repair and maintenance of tissue integrity, this function is distinct from gfDNA's impact on steady-state T cell equilibrium within the GI tissue. Thus, the absence of TLR9 expression in the hematopoeitic compartment was sufficient to promote $Foxp3^+$ T_{reg} expansion within the GI tract. Further, although the data were not presented here, this absence resulted in an attenuated immune response to oral infection with *E. Cuniculi* (Hall et al., 2008). The question then to arise from these findings is which hematopoeitic cells does gfDNA signal through to drive robust mucosal immune responses. In this study, LpDC were singled out: first, for their known function in initiating primary immune responses (Banchereau and Steinman, 1998); second, for their capacity to sense luminal bacterial components (Chieppa et al., 2006). Indeed, engaging LpDC with TLR9 ligands, including gfDNA, hindered their capacity to promote a $Foxp3^+$ T_{reg} program in T_{reg} polarizing conditions, and instead could promote T cell differentiation into T_H-17, T_H-2 or T_H-1 cells. This *in vitro* finding remarkably paralleled the differences in *ex vivo* activation statuses that was observed between $CD4^+$ T cells isolated from the Lp of WT versus *Tlr9*$^{-/-}$ mice. Consistent with a role for LpDC in the

effector status of T cells in the GI tract, temporary ablation of LpDC via oral administration of diptheria toxin (DT) to mice expressing a Diptheria toxin receptor transgene under the control of the CD11c promotor (CD11c-DTR) was shown to reduce constitutive T_H-17 production in the Lp (Denning et al., 2007).

TLR9 signaling on other immune cells

Nevertheless, gfDNA signaling may act on other immune cell populations besides DC within the GI tract, which in turn could contribute to the promotion and maitenance of mucosal immune responses. In this regard, DT administration to CD11c-DTR mice would likely ablate resident macrophages in addition to DC in the Lp, since they also express CD11c. Indeed, based on the current controversy and difficulty associated with discerning Lp macrophages from classical LpDC (Varol et al., 2009); Schulz, 2009 #1360}, it is possible that the LpDC preperations used in the experiments described above contained some proportion of contaminating macrophages. As such, TLR9 ligands may also act directly on macrophages, which themselves or indirectly via effects on LpDC could provide inflammatory cues to naive T cells in culture or to already activated cells *in situ*. Plasmacytoid DC also reside within the intestinal Lp (Yrlid et al., 2006a) and their activation *in situ* can direct the migration and maturation of $CD103^+$ LpDC (Yrlid et al., 2006b). Thus, it would be interesting to assess whether gfDNA can induce pDC activation, which may facilitate antigen transport into the draining mln. Based on their potent capacity to produce type I IFNs upon stimulation, secretion of these cytokines upon stimulation *in vitro* could first be examined to address this possibility (Theofilopoulos et al., 2005).

Recent studies have indicated that MyD88 intrinsic signals promote sustained T cell responses in various infection models (LaRosa et al., 2008; Rahman et al., 2008). Along these lines, TLR9 expression has been reported in naive and activated $CD4^+$ T cells (Gelman et al., 2004). Further, TLR9 activation via CpG was shown to directly enhance T cell proliferation and T_{eff} survival (Gelman et al., 2006). Based on these findings, one could speculate that gfDNA also provides survival/activation cues directly to T cells in the GI tract. This possibility could be investigated with the generation of 50:50 (*Tlr9*$^{-/-}$/WT) mixed bone marrow chimeras.

TLR9 signaling – polarization versus amplification

The implication that other immune cell populations are directly affected by the sensing of gfDNA and that these TLR9 interactions contribute to the efficacy of mucosal immune responses raises another important question, at what step of the immune response is gfDNA sensing the mucosal response? The evidence that the ability of LpDC to polarize a regulatory respnse is abrogated upon TLR9 engagement would suggest that gfDNA sensing plays a role in polarization. However, this has not been confirmed *in vivo*, and is difficult to do in an infection for which immunodominant peptides are not known, such as *E. cuniculi*.

Although not described in the data section, the importance of TLR9 stimulation in the GI tract was also verified using the mucosal adjuvant, LT(129G) (a non-toxic mutant of the *E. coli* labile toxin) (Chong et al., 1998), which was administered in conjuntion with OVA as a model antigen. This vaccination protocol is typically administered several times prior to assessment of the immune response; however, if the vaccine were introduced into WT or *Tlr9*$^{-/-}$ mice that were adoptively transferred with $CD4^+$ T cells

expressing the OT-II transgene, then a potential contributing role of gfDNA/TLR9 interactions to T_{eff} polarization could be better investigated. A role for gfDNA/TLR9 interactions in GALT T_{eff} polarization notwithstanding, the sensing of gfDNA may also amplify mucosal immune responses directly *in situ*. While this possibility could still implicate LpDC, gfDNA interactions with resident macrophages, resident plasmacytoid DC, T_H, as well as Foxp3$^+$ T_{reg}, would need to be considered.

In situ regulation of immune responses via Foxp3$^+$ T_{reg}

The selective expansion of Foxp3$^+$ T_{reg} in the GI tract of *Tlr9*$^{-/-}$ mice strongly correlated with the specificity of the immune impairments in response to the mucosal route of challenge. Moreover, partial T_{reg} depletion with αCD25 during infection led to a resurgence both in mucosal cytokine production and parasite clearance. Together, these data argue that the expansion of these cells was complicit in the suboptimal development/maitenance of mucosal immune responses in the absence of gfDNA sensing. In addition to augmented numbers, there is also recent evidence that the absence of TLR9 increases Foxp3$^+$ T_{reg} suppressive function in a cell intrinsic manner. This is based on unpublished data from Dr. Nicolas Bouladoux of the lab as well as a recent report showing that CpG hindered T_{reg} suppressive capacity *in vitro* (**data not shown**) (Larosa et al., 2007). As there have been multiple recent demonstrations of the ability of Foxp3$^+$ T_{reg} to inhibit a variety of innate processes, including: DC differentiation from immediate precursors (Liu et al., 2009), DC migration from tissues (Zhang et al., 2009a), not to mention inflammatory chemokine production from multiple cell sources, such as DC, NK cells and stroma (Lund et al., 2008), the impact of augmented T_{reg} proportions in the GI tract of *Tlr9*$^{-/-}$ mice could be two-fold.

gfDNA/TLR9 intreactions and T cell homeostasis in the GI milieu

Although their steady-state increase is likely one of the root causes of impaired mucosal immunity, the mechanisms that drive Foxp3$^+$ T_{reg} expansion in the absence of TLR9 signals within the GI tract are still unclear. Based on the *in vitro* data presented here, one potential explanation is that gfDNA/TLR9 interactions place natural constraints on extrathymic GALT T_{reg} generation. Indeed TLR9 engagement of LpDC drove multiple proinflammatory signals, including: IL-6, IL-12/23p40, and likely IL-27 (although not investigated in these studies), which alone or in concert have been shown to impede the development of Foxp3$^+$ T_{reg} *in vitro* and/or *in vivo* (Bettelli et al., 2006; Korn et al., 2007) (Ahern et al.; Wei et al., 2007). *IL-6*$^{-/-}$ mice, in particular, display several features that are reminiscent of *Tlr9*$^{-/-}$ mice. First, they lack T_H-17 cells in the intestinal Lp during steady-state (Ivanov et al., 2006). Second, they fail to mount robust immune responses, on account of an inability to overcome T_{reg} suppression (Korn et al., 2007; Pasare and Medzhitov, 2003). Thus, the effect of IL-6 blockade on LpDC induction of Foxp3$^+$ T_{reg} was tested during TLR9 stimulation *in vitro*. Rather than restoring Foxp3$^+$ cell generation, both antibody blockade and coculture with *IL-6*$^{-/-}$ LpDC resulted in enhanced T_H-1 and T_H-2 cytokine production, while impeding T_H-17. These data emphasize that TLR9 sensing induces an inflammatory cytokine profile with the potency to induce a diversity of T_{eff} programs, which are indeed observed in the GI tracts of WT mice during steady-state. While further attempts at innate cytokine inhibition also failed to rescue Foxp3$^+$ T_{reg} generation (suggesting that a microarray screen of TLR9 engaged LpDC versus an unstimulated control may be useful, to acquire a full sense of the inflammatory signature that results), antibodies against IL-4 and IFN-γ in addition to IL-6 led to a significant restoration. Both IL-4 and IFN-γ were recently revealed to inhibit T_{reg} development *in vitro* (Wei et al., 2007). Further, IL-4 in conditions containing TGF-β was

shown to induce IL-9 secretion by T cells (T_H-9) (Dardalhon et al., 2008; Veldhoen et al., 2008b), a cytokine whose role in intestinal homeostasis is not clear and which was not examined in these experiments. The transcription factor, interferon regulatory factor -4 (IRF-4) is essential for T_H-2, T_H-17, and T_H-9 development (Brustle et al., 2007; Staudt et al.). In light of these findings, it would be interesting to assess how steady-state IRF-4 expression in T cells within the GI tract is affected by gfDNA sensing.

Controll of extrathymic Foxp3$^+$ T_{reg} generation

If extrathymic GALT Foxp3$^+$ T_{reg} generation is controlled by gfDNA sensing, an important consideration is the stage at which this inhibition is occurring. The two scenarios that are known to induce Foxp3 in T cells within the GALT are lymphopenia and oral exposure to antigen in the absence of pathogenic signals (Coombes et al., 2007; Lathrop et al., 2008; Sun et al., 2007; Zheng et al.). Mice are obviously in a state of lymphopenia immediately after birth and the time after weaning might also represent a time of dynamic change within the GI tract upon loss of maternal antibodies. Thus, it would be worthwhile to measure the number and frequency of Foxp3$^+$ T_{reg} within the GI tract of WT and *TLR9*$^{-/-}$ mice as a function of age, which would offer insight into when gfDNA sensing limits the *de novo* generation and/or expansion of these cells. Foxp3$^+$ T_{reg} induction in response to exposure to antigen traversing the gut is also likely a dynamic process. As such, these cells may turn over very fast and their acquisition of a Foxp3 program may at early time points be subject to destabilizing factors, such as gfDNA, both at sites of priming and in Lp tissue itself. Therefore, the absense of TLR9 signals may promote an induced Foxp3$^+$ T_{reg} that is longer lived. An alternative possibility with a similar outcome is that in the absence of TLR9 signaling, signaling by other TLRs can gain precedence. TLR2 signaling, for example favors T_{reg} cell expansion through

both cell intrinsic (Liu et al., 2006; Sutmuller et al., 2006) and cell extrinsic means. The latter being regulated via the induction of retinoic acid synthesizing capacity in DC (Manicassamy et al., 2009). Accordingly, Foxp3^{+} T_{reg} cell frequencies are decreased in *TLR2*$^{-/-}$ mice (Liu et al., 2006; Sutmuller et al., 2006). Altogether, these findings suggest that TLR ligands can discretely influece T cell homeostasis in the GI tract, which may explain why *MyD88*$^{-/-}$ and *MyD88*$^{-/-}$ *Trif*$^{-/-}$ mice have no apparent gross changes in the frequency of Foxp3^{+} T_{reg} or T_{H}-17 cells in this tissue (Atarashi et al., 2008; Cha et al.; Ivanov et al., 2008; Min et al., 2007).

Not all TLR ligands are created equal

Minimization of gut floral signals with broad-spectrum antibiotic treatment (ATB) also impaired the mucosal immune response to infection with *E. Cuniculi* and resulted in a concomitant increase in parasite burden. TLR9 but not TLR4 activation was sufficient to rescue immune responses in ATB treated animals, illustrating both the discrete functions of individual TLR ligands in the promotion and regulation of immunity and the potency of TLR9 engagement as an adjuvant. Intriguingly, TLR4 activation with LPS, while failing to improve parasite control and immune responses within the GI tract, elicited a complete rebound in the systemic cytokine response to oral *E cuniculi* infection. This finding is consistent with recent reports illustrating that peripheral TLR4 signals, which occur upon floral translocation, can enhance the activation status of T cells (Brenchley et al., 2006; Paulos et al., 2007). Despite these findings, however, TLR4 signals may also operate in other ways, specifically within the GI tract. In this regard, exposure of the intestinal epithelia to LPS during birth was found to result in tolerance of this tissue to subsequent LPS stimulation (Lotz et al., 2006). More recently, LPS engagement of TLR4 was also revealed to temper T cell activation via enhancement of

phosphatase activity in a cell intrinsic manner (Gonzalez-Navajas et al.). This may be especially relevant in sites where T cell interactions with LPS are likely, such as the GALT.

The experiments in this study illustrated that DNA purified from the entire gut flora exerted an adjuvant effect on mucosal immune responses; yet it is important to bear in mind that gut flora bacteria possess differential capapacities to stimulate TLR9 (Dalpke et al., 2006). Moreover, some DNA motifs may, in fact, exert inhibitory effects on TLR9 activation or actively promote regulatory features, which are still dependent on TLR9 (Trieu et al., 2006). As such, experiments are currently underway by other members of the laboratory to address whether gfDNA derived from different commensal species imparts specific effects on the development, control and maintenance of mucosal immune responses and whether these effects can be extrapolated to the types DNA motiffs present in these species.

TLR9 in other infections

In contrast to sterile sites, mucosal sites in the body are in ritual contact with commensals and their byproducts. The finding that *Tlr9*$^{-/-}$ mice exhibited a specific mucosal defect in response to infection, in this instance the fungal parasite, *E. cuniculi,* suggested that gfDNA/TLR9 interactions in the GI tract shape mucosal immune responses through release of immunoregulatory elements. One in particular that was showcased in these findings was immunosuppression via Foxp3^{+} T_{reg}. Previous immunological studies of infections that are initiated at mucosal sites, such as *Toxoplasma gondii* and *Herpes simplex* virus, have also defined a role for TLR9 in the regulation of mucosal inflammation and viremia, respectively (Lund et al., 2006; Minns et

al., 2006). Although TLR9 is clearly important in the recognition of these microbes, it is also plausible that the absence of gfDNA/TLR9 interactions at these sites of challenge both during steady-state and infection could have additional bearing on the phenotype observed.

CHAPTER 4: Essential role for retinoic acid in the promotion of $CD4^+$ T cell effector responses via retinoic acid receptor alpha

Abstract:

The data presented in **Chapter 2** along with numerous recent reports indicate that the vitamin A metabolite, retinoic acid (RA), promotes $Foxp3^+$ T_{reg} induction *in vitro* and *in vivo*. However, incorporation of the data from **Chapter 3** into these findings also suggested that RA signaling, rather than suppressing the differentiation of T_{eff} cells, could also promote their migration into the intestinal mucosa. While an initial interpretation of these findings could suggest context dependent roles of RA during tolerance and infection, they also could imply that RA possesses a more fundamental function in the regulation of T cell activation/differentiation. To begin to address this hypothesis, immune responses to the parasite, *T. gondii*, and the mucosal adjuvant, LT(R129G), were examined in mice reared on a vitamin A deficient diet. Unexpectedly, these studies led to the following insights:

1. **T_H-1 and T_H-17 immunity were abrogated in the absence of vitamin A metabolites.**
2. **Retinoic acid rescued T_H-1 and T_H-17 immune responses in the absence of vitamin A.**
3. **RA receptor alpha expression was necessary for $CD4^+$ T cell immunity and homeostasis.**
4. **RA receptor driven signals maintained the ability of T cells to properly respond to TCR/CD3 stimulation in a cell intrinsic manner.**

Rationale:

In **Chapter 2**, LpDC and their migratory counterparts in the mesenteric lymph nodes were revealed to be specially equipped with the capacity to induce Foxp3$^+$ T_{reg} (Coombes et al., 2007; Sun et al., 2007). This feature was dependent on their ability to convey both TGF-β and retinoic acid mediated signals to T cells. At approximately the same time that these findings emerged, other groups demonstrated that exogenous RA was able to: reduce T_H-17 polarization via inhibition of IL-6 and IL-23 receptors (Elias et al., 2008; Mucida et al., 2007; Xiao et al., 2008); facilitate Foxp3 induction in the face of high levels of costimulation (Benson et al., 2007), and negatively modulate cytokine production by effector/memory T cells during restimulation (Hill et al., 2008). Prior to these reports, RA was also shown to promote IgA-secreting plasma B cell differentiation (Mora et al., 2006). Altogether, these findings reinforced an emerging perception that RA drove anti-inflammatory programs and synchronized with the paradigm of mucosal tissues as hypo-responsive environments during steady-state conditions. Nevertheless, in the event of pathogenic exposure, overcoming these regulatory hurdles is essential and how RA is integrated into this framework is unclear. In **Chapter 3**, adjuvant activated LpDC were revealed to lose their capacity to induce Foxp3$^+$ T_{reg}; however, this effect was likely not a consequence of impaired RA signaling, as the RA-inducible integrin heterodimer, alpha4beta7 ($\alpha_4\beta_7$) was strongly upregulated on Foxp3$^-$ T cells. Indeed, $\alpha_4\beta_7$ upregulation is a well-appreciated hallmark of T cells activated in the GALT, which in turn drives their migration into the GI tract (Johansson-Lindbom et al., 2003).

Deficiency in vitamin A, which is the precursor of RA, is known to manifest in susceptibility to mucosal infections (Sommer, 2008; Underwood, 2004). Although this susceptibility, in part, may be driven by impairments in epithelial barrier protection

(Biesalski and Nohr, 2004; Stephensen, 2001), to date, the *in situ* regulation of mucosal $CD4^+$ T cell responses via RA and/or other retinoids has not been investigated. Gaining an understanding of these pathways will help unravel how vitamin A metabolism contributes to host protective immunity.

Results:

Retinoic acid mediated signaling occurs during systemic inflammation

To address the role of vitamin A metabolism in immunity, the induction of mucosal homing markers was evaluated during oral infection with *Toxoplasma gondii* (*T. gondii*). This parasite induces a strong inflammation and a robust systemic T_H-1 response (Gazzinelli et al., 1992; Suzuki et al., 1988). Mice were inoculated with 10 cysts of the type II avirulent *T. gondii* strain, ME-49, and $CD4^+$ T cells were examined during the acute stage of infection, at which time they are the predominant responding T cell subset. Accordingly, a large proportion of activated cells within the small intestinal lamina propria, the draining mesenteric lymph nodes, and the spleen expressed the transcription factor, T-bet, which mediates interferon gamma (IFN-γ) production (**Figure 29A**). Expression of, $\alpha_4\beta_7$ and CCR9, which are indicative of retinoic acid signaling (Iwata et al., 2004; Svensson et al., 2008), were also observed on a proportion of activated $CD4^+$ T cells in each of these tissues (**Figure 29A and data not shown**). Notably, expression of these markers was mainly confined to T-bet^+ cells (**Figure 29A**). To determine whether $\alpha_4\beta_7$ induction was unique to the oral route of infection with *T. gondii* animals were next inoculated intraperitoneally (IP). Strikingly, a large proportion of activated $CD4^+$ T cells expressed $\alpha_4\beta_7$, and, again, most of these coexpressed T-bet (**Figure 29B**). These results suggested that RA signaling was sustained and occurred systemically during inflammatory responses.

An alternate scenario compatible with these findings was that signaling molecules, other than RA, contributed to $\alpha_4\beta_7$ expression in response to infection. To

address this possibility, vitamin A insufficient (VAI) mice at 10 wks of age were inoculated with *T. gondii*.

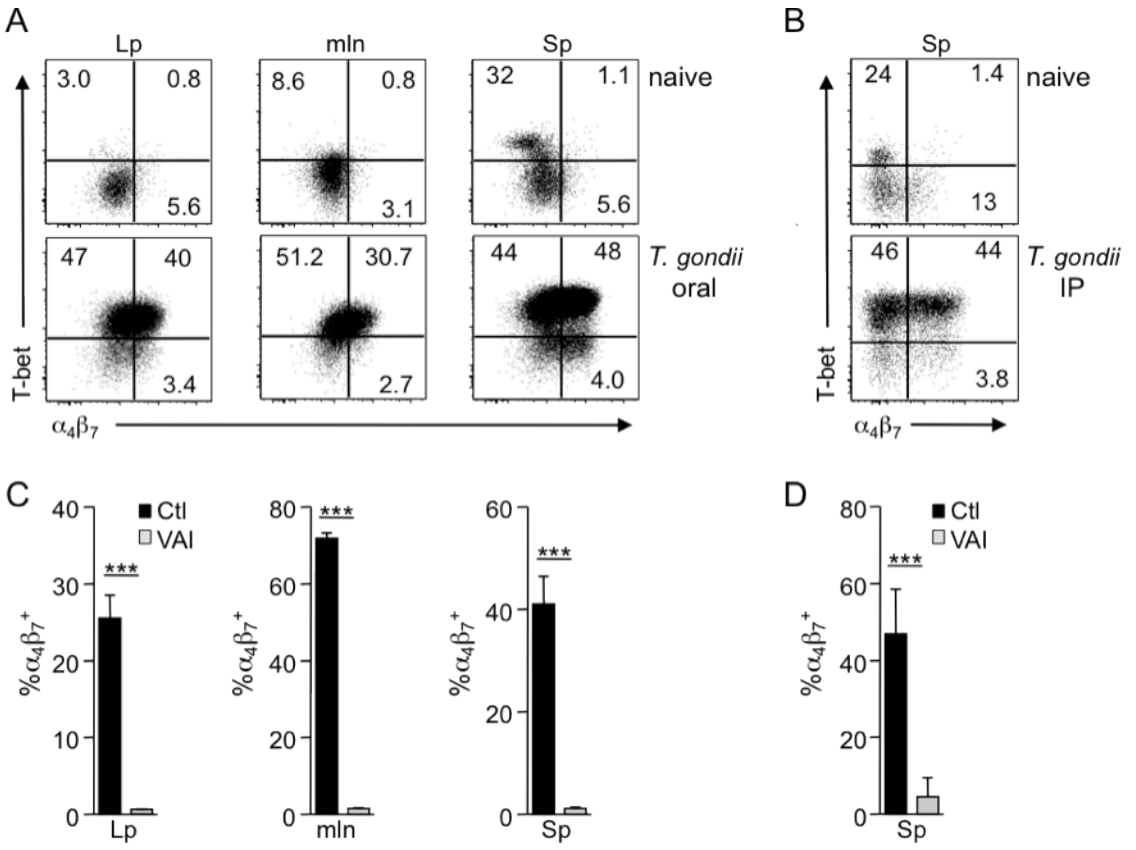

Figure 29. Vitamin A metabolite dependent signaling is sustained and systemic during *T. gondii* infection

(**A**) C57BL/6 mice were infected per-orally with 10 bradyzoite cysts of ME-49 clone C1. On day 8 post-infection (p.i.), single cell suspensions prepared from the spleen (Sp), mesenteric lymph nodes (mln) and small intestinal lamina propria (Lp), were stained with fluorochrome labeled antibodies and assessed for a_4b_7 and T-bet expression by flow cytometry. Dot plots are gated on Foxp3$^-$ CD44^{hi} CD62L^{lo} CD4$^+$ T cells and representative of 3 mice per group. (**B**) C57BL/6 mice were infected intraperitoneally with 10 bradyzoite cysts of ME-49 clone C1. On day 8 p.i., single cell suspensions prepared from the Sp were stained and assessed as described in A (**C**) Ctl and VAI mice were infected per-orally with *T. gondii*. On day 8 single cell suspensions were stained for $\alpha_4\beta_7$. Bar graphs summarize the average frequency of Foxp3$^-$ CD44^{hi} CD4$^+$ T cells expressing $\alpha_4\beta_7$, n = 3-4 mice per group. (**D**) Ctl and VAI mice were infected intraperitoneally with *T. gondii* and assessed as described in part c. n = 6-8 mice. For C and D, error bars illustrate the s.d. Statistical comparisons were performed using the unpaired Student's *t* test ***, $P < 0.001$, ns = not significant.

Significantly, 8 days post infection, $\alpha_4\beta_7$ remained virtually absent on activated $CD4^+$ T cells in VAI mice infected either by the oral or IP route with *T. gondii*, strongly suggesting that its upregulation was strictly dependent on RA during both steady-state and inflammatory conditions (**Figure 29C and D**). Thus, RA signaling was sustained during infection and exerted a systemic influence on $CD4^+$ T cells regardless of the route of infection, an indication that vitamin A metabolism may imprint features that exceed homing potential during infection.

Mucosal and systemic $CD4^+$ T cell immunity is impaired in the absence of vitamin A metabolites

To explore the functional consequence of vitamin A insufficiency during infection, the acute-phase T_H-1 response in VAI mice was evaluated following oral inoculation with *T. gondii*. To this end, magnetically enriched T cells from mice on day 8 post-infection were restimulated with soluble *T. gondii* antigen (STAg) in the presence of BMDC *in vitro*. 48hrs later, IFN-γ in supernatants was measured. Strikingly, T cells enriched from the mln, Lp, as well as the Sp of infected VAI mice produced significantly smaller amounts of IFN-γ than their control counterparts upon recall (**Figure 30A**). The reduction in T_H-1 responsiveness was reflected in the enhanced parasite burden observed both systemically (Sp) and in the Lp of VAI mice (**Figure 30B**). IP infection with *T. gondii* yielded similar findings, in which, $CD4^+$ T cells removed from VAI animals and rechallenged with STAg produced lower levels of IFN-γ on a per cell basis (MFI = 1800±380 in VAI versus 5300±870 for Ctl, $p < 0.003$). Enhanced parasite burdens in the peritoneal exudates (Pecs) and Sp of these animals were also observed (**Figure 30C**), emphasizing that impaired T_H-1 immunity to *T. gondii* during vitamin A insufficiency is not solely a consequence of defective responsiveness in the GALT. These data suggested a

direct or indirect role for vitamin A metabolites in the capacity of $CD4^+$ T cells to acquire effector function during infection.

T. gondii infects all nucleated cells and triggers multiple innate pathways that synergize to induce a strong adaptive T_H-1 response. Therefore, the influence of vitamin A metabolism on mucosal immunity was also tested in a non-infectious system with a model antigen. To this end, mice were orally vaccinated with a mixture of OVA and the mucosal adjuvant, LT (R129G), a non-toxic mutant of the heat-labile enterotoxin of *Eschericia coli*. This regimen previously was demonstrated to induce a T_H-1 and robust T_H-17 response (Hall et al., 2008); the latter distinguished by expression of the transcription factor, ROR(γ)t, in T cells (Ivanov et al., 2006). After two rounds of vaccination one week apart, abundant IFN-γ and IL-17A were detected in the supernatants of *in vitro* restimulated Lp and Peyer's patch (Pp) tissues from control mice, while less was detected in the mln (**Figure 30D**). Coincident with IL-17A protein, flow cytometric analysis revealed substantial expression of ROR(γ)t in $Foxp3^-$ $CD4^+$ T cells from the Lp (44.8±6.4%) and Pp (9.3±0.4%) (**Figure 30E-F**). Yet, GALT cells from VAI mice secreted only marginal amounts of IFN-γ and IL-17A. This reduction in IL-17A corresponded with diminished ROR(γ)t expression in both the Lp (13.7±5.9%) and Pp (1.7±0.8%). Moreover, the frequency of $CD4^+$ T cells expressing the nuclear proliferation antigen, Ki-67 (Gerdes et al., 1983), was significantly reduced, indicating decreased activation/proliferation in response to vaccination in VAI mice (**Figure 30E-F**). Collectively, these results suggest that vitamin A is critical for optimal T cell responses.

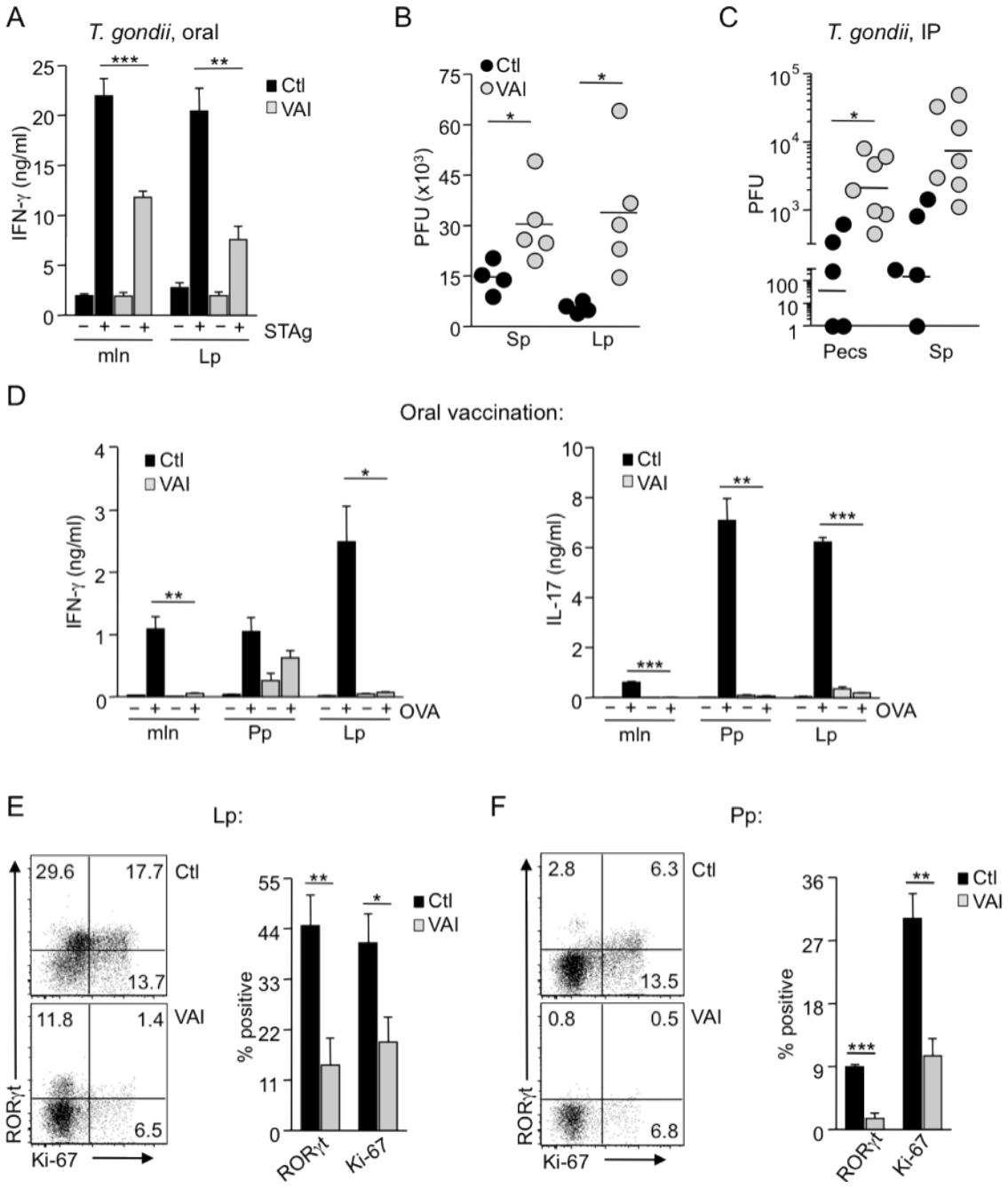
A
T. gondii, oral
IFN-γ (ng/ml)
Ctl
VAI
STAg
mln
Lp
B
PFU (x10³)
Sp
Lp
C
T. gondii, IP
PFU
Pecs
Sp
D
Oral vaccination:
IFN-γ (ng/ml)
IL-17 (ng/ml)
OVA
mln
Pp
Lp
E
Lp:
29.6
17.7
13.7
11.8
1.4
6.5
RORγt
Ki-67
% positive
F
Pp:
2.8
6.3
13.5
0.8
0.5
6.8

Figure 30. **T_H-1 and T_H-17 immune responses are impaired in the absence of vitamin A metabolites**
(**A-B**) Ctl and VAI mice were infected per-orally with *T. gondii* (**A**) At day 8 p.i., pooled tissue suspensions were enriched for T cells and cultured with irradiated BMDC +/- soluble *T. gondii* antigen (STAg) for 48 hr. IFN-γ was measured in triplicate supernatants by ELISA. n = 4-5 mice per group. (**B**) Parasite burdens in Sp and Lp of individual mice from A were determined by plaque assay. Results are expressed as plaque forming units (PFU). Each dot represents an individual mouse (**C**) Ctl and VAI mice were infected intraperitoneally with *T. gondii*. Parasite burden was assessed day 8 p.i. as described in **B**. n = 6-8 mice per group. (D-F) Ctl and VAI mice were immunized orally with a mixture of OVA and the mutant *E. coli* labile toxin, LT(R129G), once per week. On day 14, pooled cell suspensions from mln, Pp and Lp were cultured with BMDC infected with recombinant vaccinia virus expressing OVA (iDC) for 72 hours. Triplicate supernatants were assayed for IFN-γ and IL-17A by ELISA. n = 3-4 mice per group. (**E-F**) Lp (**E**) and Pp (**F**) suspensions from individual mice were assessed for intracellular RORγ(t) and Ki-67 by flow cytometry. Representative dot plots from Lp of WT and VAI mice gated on viable $Foxp3^-$ $CD4^+$ T cells are shown. Bar graphs summarize the frequency of $Ki\text{-}67^+$ and $ROR\gamma(t)^+$. Error bars in **A** and D depict the s.e.m.; error bars in **E** and **F** depict the s.d. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

RA restores $CD4^+$ T cell immunity in the absence of vitamin A

Impaired T_H-1 and T_H-17 responses in VAI mice could also emerge from a developmental defect in these animals, and not reflect a genuine role for vitamin A metabolites on T_H-1 and T_H-17 development (Mora et al., 2008; Ziouzenkova et al., 2007). Vitamin A metabolism produces several derivatives with signaling capacity in the host. RA, in particular, has been shown previously to induce strong effects on a variety of immune cell populations *in vitro* (Geissmann et al., 2003; Mora et al., 2008) and in vitamin A-replete settings *in vivo* (Mucida et al., 2007; Xiao et al., 2008). However, the effect of RA on T cell immunity in the absence of other vitamin A metabolites, which may exert confounding influences, has never been interrogated. To test if RA could restore protective immunity *in vivo*, VAI mice were prophylactically treated with RA every other day. On day 5 of treatment, they were innoculated orally with *T. gondii*. Administration of RA or the vehicle control continued every other day until day 8 of infection, when the animals were analyzed. In contrast to vehicle treated animals, *T. gondii* infected VAI

mice that received short-term RA displayed a striking recovery in *ex vivo* T cell IFN-γ responses to STAg, both in the Sp and Lp (**Figure 31A**). Assessment of intracelular IFN-γ further revealed that Lp $CD4^+$ T cells responded as potently as their control counterparts to antigen restimulation (**Figure 31B**). Importantly, this rebound culminated in functional immunity to toxoplasmosis, enhancing parasite clearance so that parasite burdens mirrored those observed in control infected animals (**Figure 31C-D**). The effect of RA was also examined on T_H-17 responses via oral vaccination with LT(R129G) and OVA. For these experiments, mice were treated with the same drug regimen. To accommodate this, the vaccination schedule was performed four days apart, rather than one week. Significantly, Lp $Foxp3^- CD4^+$ T cells from RA treated animals regained their capacity to produce IL-17A, in some instances to a degree that exceeded what was observed in control animals (**Figure 31E**). This capacity was coupled to a rebound in RORγ(t) expression and markedly enhanced T cell proliferation, as measured by intracellular Ki-67 (**Figures 31E-F**). The efficacy of short-term treatment with RA, argued that rather than a developmental deficit causing impaired $CD4^+$ T cell immunity in VAI mice, this metabolite provided essential signals to mediate T_H-1 and T_H-17 responses.

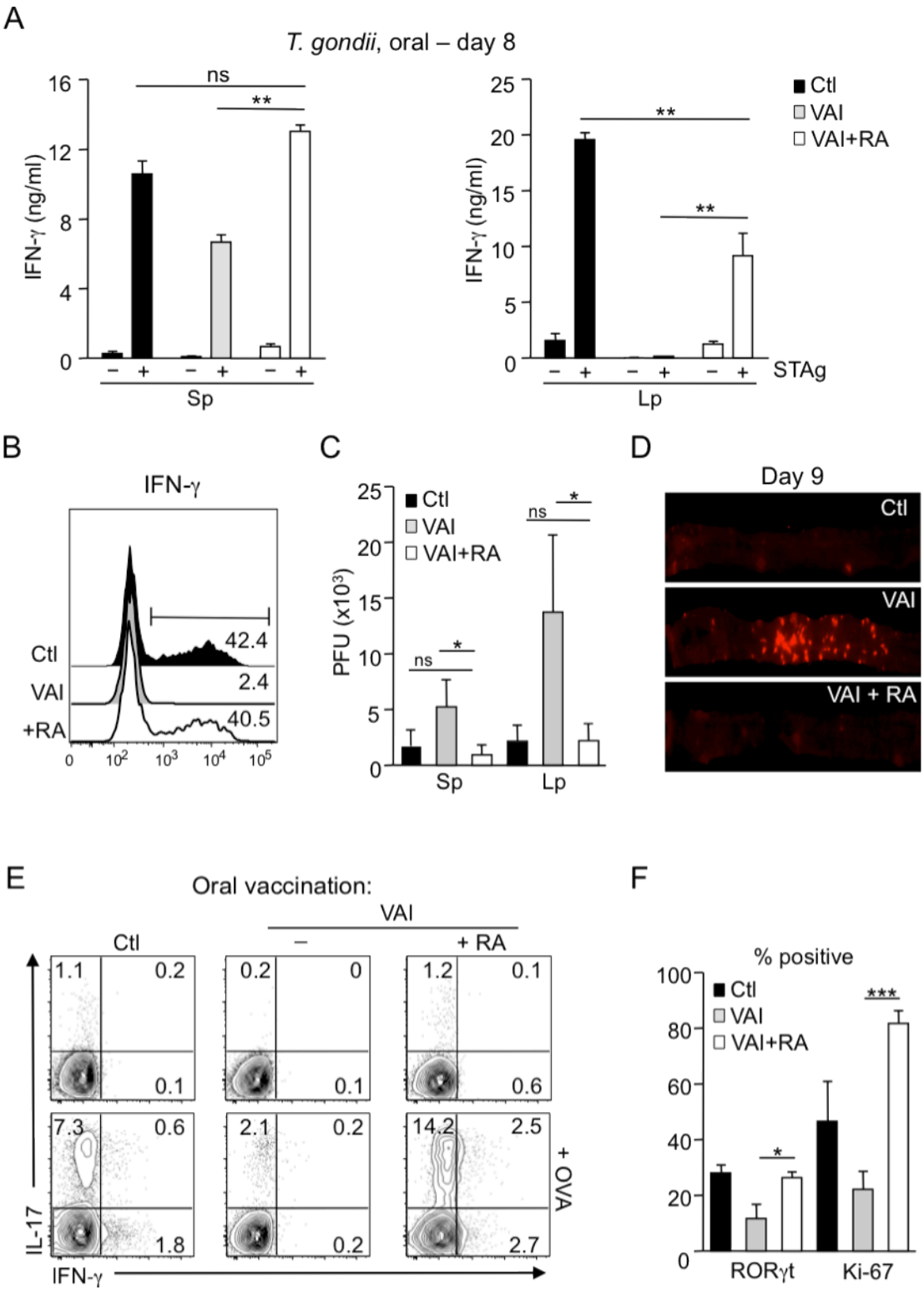
A
T. gondii, oral – day 8
IFN-γ (ng/ml)
ns
**
Sp
Lp
Ctl
VAI
VAI+RA
STAg
B
IFN-γ
Ctl
VAI
+RA
42.4
2.4
40.5
C
PFU (x10³)
ns
*
D
Day 9
Ctl
VAI
VAI + RA
E
Oral vaccination:
VAI
Ctl
–
+ RA
IL-17
IFN-γ
+ OVA
1.1
0.2
0.1
0.2
0
0.1
1.2
0.1
0.6
7.3
0.6
1.8
2.1
0.2
0.2
14.2
2.5
2.7
F
% positive
***
RORγt
Ki-67

Figure 31. Retinoic acid is required for CD4$^+$ T cell immunity
(**A-D**) Ctl and VAI mice treated with RA or vehicle were infected orally with *T. gondii*. n = 3 mice per group. (**A**) At day 8 p.i., pooled cell suspensions from Sp or Lp were enriched for T cells and cultured with irradiated BMDC +/- STAg for 48 hr. Bar graphs present the average amount of IFN-γ in duplicate or triplicate supernatants. (**B**) Lp samples treated as described in **A** were incubated for 14 hrs with STAg, and analyzed for intracellular IFN-γ via flow cytometry. Stacked histograms are gated on viable, Foxp3$^-$ CD4$^+$ T cells. (**C**) Parasite burden in Sp and Lp of individual mice was measured by PFU. (**D**) Visualization of parasite localization in duodenal-jejunal sections of individual mice on day 9 p.i. (**E-F**) Ctl and VAI mice treated with RA or vehicle were immunized orally with a mixture of OVA and LT(R129G) on day 0 and 4. n = 3 mice per group. (**E**) On day 7, suspensions pooled from Lp were enriched for T cells and cultured with SpDC +/– OVA for 14 hr and examined for intracellular IL-17A and IFN-γ. Contour plots are gated on viable, Foxp3$^-$ CD4$^+$ T cells. (**F**) Lp cells from individual mice were analyzed for intracellular RORγ(t) and Ki-67 by flow cytometry. Bar graphs depict Ki-67 and RORγ(t) as a frequency of Foxp3$^-$ CD4$^+$ T cells. (**A-F**), RA treated groups received 250mg RA intraperitoneally 5 days prior to infection or vaccine and every other day thence until takedown. Error bars in **A** depict the s.e.m.; Error bars in **C** and **F** depict the s.d. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant.

RARα regulates CD4$^+$ T cell immunity and homeostasis

As mentioned in **Chapter 1**, RA signals through several families of nuclear hormone receptors. The best characterized are RA receptors (RAR) α, β and γ, which transcriptionally regulate gene expression in partnership with retinoid X receptors (Chambon, 1996). Previously, TGF-β was shown to induce RARα expression (Schambach et al., 2007). Accordingly, it was also shown in **Chapter 2** that this receptor was required for RA to enhance Foxp3$^+$ T_{reg} differentiation *in vitro* (Hill et al., 2008; Nolting et al., 2009). Using real time quantitative PCR, naïve CD4$^+$ T cells were found to readily express mRNA for RARα. RARγ was also detected. Similar to observations in activated cells, RARβ was not detectable (#) (**Figure 32A**) (Elias et al., 2008). To deduce whether RARα played a role in RA directed immunity, RARα deficient (*Rara*$^{-/-}$) mice were orally vaccinated with LT (R129G) and OVA. Unlike VAI mice, which after 16 weeks begin to succumb to a wasting disease, these animals display no overt

impairments as adults (Lufkin et al., 1993). Notably, this vaccine regimen was unable to induce an antigen specific T_H-17 response in mice on this mixed 129 background (**data not shown**). Nevertheless, relative to WT littermates, $CD4^+$ T cells from *Rara*$^{-/-}$ mice expressed attenuated levels of the T_H-1 driving transcription factor, T-bet, after vaccination and secreted virtually no IFN-γ upon antigen recall (**data not shown and Figure 32B**), Thus, abrogation of *Rara* impaired $CD4^+$ T cell immunity.

In **Chapter 2**, disruption of RA/RAR signaling, either by antagonism *in vitro* or by loss of RA *in vivo* was shown to inhibit peripheral $Foxp3^+$ T_{reg} generation. *Other laboratories corroborated in vitro inhibition of Foxp3+ Treg generation using RAR antagonists*, as well (Coombes et al., 2007; Mucida et al., 2007). Despite the inability to generate GALT $Foxp3^+$ T_{reg} upon oral feeding (**Figure 14**), a paradoxical increase in the frequency of $Foxp3^+$ T_{reg} was observed within the Lp of VAI mice (**Figure 32C**). As noted previously, a parallel increase was also observed in the frequency of Lp $Foxp3^+$ T_{reg} in *Rara*$^{-/-}$ mice (Hill et al., 2008). However, in both VAI and *Rara*$^{-/-}$ animals, thymic production of $Foxp3^+$ T_{reg} was unperturbed (**Figure 15 and data not shown**). Thus, upon loss of RA or RARα mediated signaling, impaired GALT $Foxp3^+$ T_{reg} generation was coupled with an aberrant increase in the frequency of thymically derived $Foxp3^+$ T_{reg} in the Lp. Based on their potential to raise the threshold of immune activation, which was demonstrated in **Chapter 3** (**Figure 27**), the relative increase in thymically derived $Foxp3^+$ T_{reg} in this region was considered a potential cause of impaired $CD4^+$ T cell responses. In order to test this, VAI DEREG mice were generated (Lahl et al., 2007), in which $Foxp3^+$ T_{reg} could be selectively depleted upon injection of diphtheria toxin (DT). Oral vaccination in conjunction with DT treatment readily depleted $Foxp3^+$ T_{reg} and restored $Foxp3^-$ $CD4^+$ T cell proliferation in the Lp (**Figure 32D**). However, T_H-1 and T_H-17 cells remained undetectable in VAI mice after this treatment (**Figure 32E**).

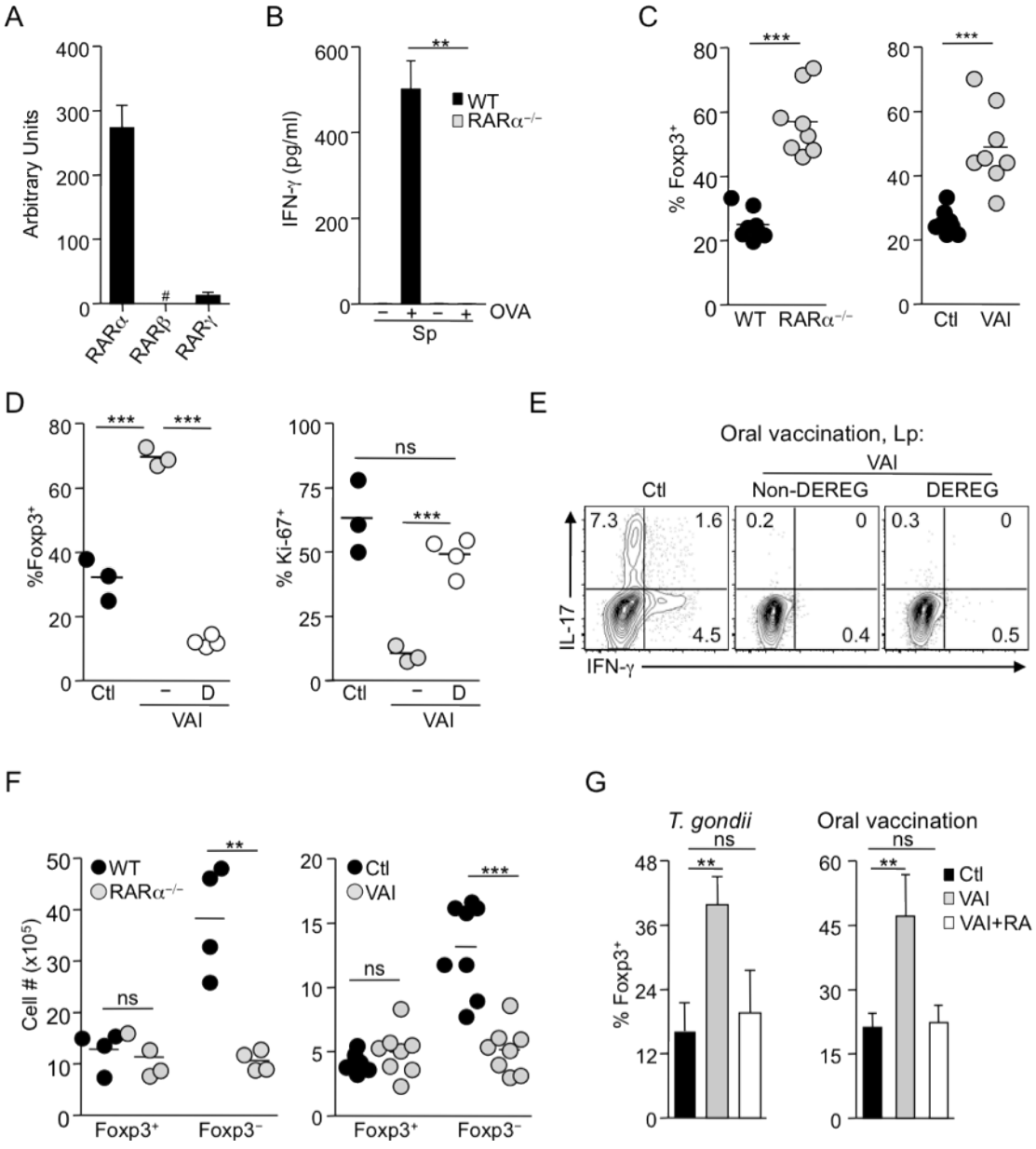
A
Arbitrary Units
400
300
200
100
0
#
RARα
RARβ
RARγ
B
IFN-γ (pg/ml)
600
400
200
0
**
WT
RARα−/−
OVA
− + − +
Sp
C
% Foxp3+
80
60
40
20
0
***
WT RARα−/−
Ctl VAI
D
%Foxp3+
***
***
Ctl − D
VAI
% Ki-67+
ns
***
Ctl − D
VAI
E
Oral vaccination, Lp:
VAI
Ctl
Non-DEREG
DEREG
7.3
1.6
4.5
0.2
0
0.4
0.3
0
0.5
IL-17
IFN-γ
F
Cell # (x10^5)
WT
RARα−/−
ns
**
Foxp3+ Foxp3−
Ctl
VAI
ns
***
Foxp3+ Foxp3−
G
T. gondii
Oral vaccination
% Foxp3+
ns
**
Ctl
VAI
VAI+RA

Figure 32. The RA/RARα signaling axis regulates CD4$^+$ T cell immunity and homeostasis.
(**A**) mRNA from sort-purified naïve CD4$^+$ T cells (Foxp3$^-$ CD25$^-$ CD44^{lo}) was assessed for RARs: α, β, and γ via quantitative RT-PCR and normalized to the house-keeping gene, hypoxanthine phosphoribosyltransferase. # = not detected. (**B**) *Rara*$^{-/-}$ and littermate control WT mice were immunized orally with a mixture of OVA and the mutant *E. coli* labile toxin, LT(R129G), once per week. On day 21, suspensions pooled from Sp were enriched for T cells and cultured with BMDC infected with recombinant vaccinia virus expressing OVA (iDC) for 72 hours. IFN-γ was quantified in triplicate supernatants. Results are representative of 2 independent experiments. (**C**) Lp cell suspensions from VAI, *Rara*$^{-/-}$ and their respective control counterparts were assessed for Foxp3$^+$ Treg via flow cytometry. Results are expressed as a proportion of viable TCRβ$^+$ CD4$^+$ T cells. (**D-E**) Ctl, VAI and VAI DEREG mice immunized orally with a mixture of OVA and LT(R129G) on days 0 and 4 were treated with 1mg of diphtheria toxin72 and 24 hrs prior to vaccination and every subsequent 48 hrs through termination of experiment. (**D**) On day 7, suspensions from individual mice were assessed for intracellular Foxp3 and Ki-67 by flow cytometry. Data are gated on CD4$^+$ T cells. Each dot represents an individual mouse. (**E**) Suspensions pooled from the Lp were enriched for T cells and cultured with purified SpDC +/– OVA for 14 hr, then assessed for intracellular IL-17A and IFN-γ via flow cytometry. Contour plots are gated on viable Foxp3$^-$ CD4$^+$ T cells. n = 3-4 mice per group (**F**) Summary of the absolute number of Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells in the Lp of mice. (**G**) Vehicle or RA treated mice were orally infected with *T. gondii* or vaccinated as described in Figure 3. A summary of the frequency of Lp Foxp3$^+$ T_{reg} ± s.d. as a proportion of viable CD4$^+$ T cells is shown. n = 3 mice per group. Data are representative of 2-3 experiments. Error bars in **A** and **B** depict the s.e.m. **, $P < 0.01$; ***, $P < 0.001$; ns = not significant.

Thus, the enhanced frequency of Foxp3$^+$ T_{reg} in the Lp was not a primary cause of impaired mucosal immune responses upon loss of vitamin A dependent signaling.

The increase in the frequency of Lp Foxp3$^+$ T_{reg} revealed that GALT T cell homeostasis was perturbed in both *Rara*$^{-/-}$ and VAI mice. Quantification of CD4$^+$T cell subsets indicated that in both these animals, the increase in Lp Foxp3$^+$ T_{reg} frequency was due to a 2 to 4 fold selective reduction in the number of Foxp3$^-$ CD44^{hi} CD4$^+$ T cells within the Lp (**Figure 32F**). Importantly, treating VAI mice with RA during *T. gondii* infection and vaccination restored GALT T cell equilibrium in addition to T_H responses (**Figure 32G**). These findings demonstrated that deficiency in RARα alone could

recapitulate the effects of vitamin A insufficiency on T cell homeostasis. Further, they identified a crucial contribution of RA/RARα signaling to vitamin A dependent $CD4^+$ T cell homeostasis and protective immunity.

RARα regulates T cell activation

Although RA mediated enhancement of protective immunity could involve multiple cellular targets, expression of RARα in naïve $CD4^+$ T cells, combined with impaired T helper immunity in *Rara*$^{-/-}$ mice, suggested a potential cell intrinsic requirement for this receptor in T cell responses. To address this, magnetically isolated naïve WT or *Rara*$^{-/-}$ $CD4^+$ $CD62L^{hi}$ T cells were stimulated in an APC-free system under T_H-1 and T_H-17 polarizing conditions, *in vitro*. After 48hrs in culture, relative to WT T cells, significant reductions of IFN-γ and IL-17A were detected in supernatants from T_H-1 and T_H-17 polarized *Rara*$^{-/-}$ T cells (**Figure 33A**). However, these cultures contained significantly fewer cells than WT cultures, indicating that differences in cytokine levels were potentially secondary to impaired cell proliferation. Confirming this hypothesis, intracellular cytokine staining revealed that surviving *Rara*$^{-/-}$ T cells were as capable as WT T cells to produce cytokine (**data not shown**). Indeed, T cells lacking RARα failed to proliferate as efficiently as their WT counterparts upon polyclonal T cell receptor (TCR) activation with anti-CD3. The lack of proliferation persisted in the presence of co-stimulation and upon exogenous provision of IL-2 (**Figure 33B**), suggesting that the loss of RARα signaling may cause a fundamental activation and/or survival defect. A potential defect in activation was explored first. After 16 hrs of stimulation, there was less upregulation of the activation markers: CD69, CD25 and the iron transferrin receptor, CD71, on *Rara*$^{-/-}$ T cells (**Figure 33C**). Expression of CD71 was recently shown to depend on activation of the mammalian target of rapamycin kinase (mTOR) (Zheng et

al., 2007), which regulates cell growth, proliferation, survival and differentiation pathways via two distinct complexes, mTORC1 and mTORC2 (Guertin et al., 2006; Loewith et al., 2002; Wullschleger et al., 2006); (Delgoffe et al., 2009; Lee et al.). mTORC1 activity regulates protein translation via activation of the ribosomal S6 kinase (S6K1) (Holz et al., 2005), while mTORC2 activity regulates cell growth, proliferation and survival via activation of AKT (kinase mediated phosphorylation of serine 473) and additional pathways. In accord with a reduction in CD71, phosphorylation of the S6K1 target, 40S ribosomal protein S6, (pS6) was decreased by as much as 50% in *Rara*$^{-/-}$ T cells relative to WT T cells, indicating a reduction in mTORC1 activity (**Figure 33D**). Suggesting a global reduction in mTOR activity, short stimulation with plate-bound anti-CD3 and anti-CD3 + anti-CD28 resulted in less efficient Akt phosphorylation at serine position 473, in *Rara*$^{-/-}$ T cells (**Figure 33E**).

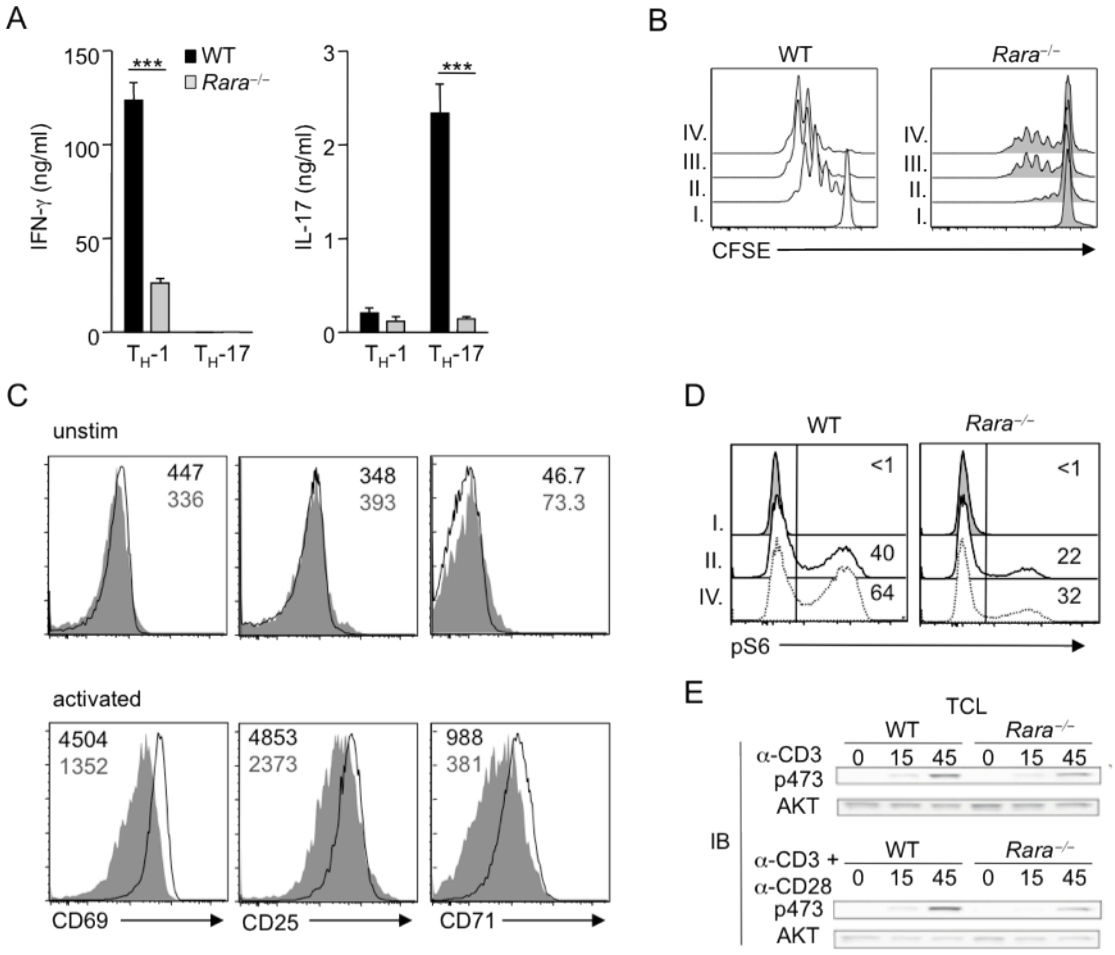

Figure 33. Role of RARα signaling for T cell activation

(**A**) $CD4^+$ $CD62L^{hi}$ T cells purified from Sp and lymph nodes of *Rar*$^{-/-}$ and littermate/WT mice were activated with plate-bound α-CD3 + soluble α-CD28, in T_H-1 or T_H-17 polarizing conditions for 48 hrs. IFN-γ and IL-17A ± s.e.m. in culture supernatants were measured by ELISA. ***, $P < 0.001$ (**B**) $CD4^+$ T cells were plated for 48 hrs in the following conditions: I. unstimulated II. α-CD3 III. α-CD3 + α-CD28 IV. α-CD3 + α-CD28 + IL-2. After 48 hrs, cells were rested overnight in IL-2 and assayed for CFSE intensity by flow cytometric analysis. Histograms are gated on viable $CD4^+$ T cells. (**C**) $CD4^+$ $CD62L^{hi}$ T cells were isolated and activated with α-CD3 + α-CD28 + IL-2 for 16 hrs, then assessed for the activation markers: CD69, CD25, and CD71. (**D**) Naïve T cells were activated as described in **C** and stained for pS6. Roman numerals indicate same conditions used in **B**. (**E**) $CD4^+$ T cells ($5x10^6$) were stimulated with plate-bound α-CD3 (2μg/ml) and/or α-CD28 (10μg/ml) for the indicated times and then lysed. Total cell lysates were immunoblotted for phosphorylated (Ser473) and total Akt.

Basal RARα activity regulates responsiveness to signaling via TCR/CD3 complex

TCR engagement results in the orchestration of signaling pathways, which promote naïve T cell transition into effector T cells. The observed decrease in activation markers at 16hrs suggested that an upstream defect might impair signal transduction pathways in *Rara*$^{-/-}$ T cells. Ca^{2+} mobilization is a critical biochemical mediator of early T cell activation events, which is initiated upon stimulation through the T cell receptor (TCR)/CD3 complex. To determine whether RARα signaling was involved in the regulation of these events, the rate and magnitude of cytoplasmic Ca^{2+} flux was

% (Cell divided)	WT	*Rara*-/-	Prolif Index	WT	*Rara*-/-
II	51	8	II	2.2	1.5
III	74.8	15.8	III	3.4	2.1
IV	76.4	15.4	IV	3.5	2.2

Table 1. Impaired proliferation of *Rara*$^{-/-}$ T cells upon activation
The percent of cells that underwent proliferation and calculated proliferation index are indicated.

assayed. Strikingly, upon anti-CD3 crosslinking, Ca^{2+} mobilization was dramatically reduced in *Rara*$^{-/-}$ $CD4^+$ T cells relative to WT counterparts (**Figure 34A**). As RA and its metabolic precursors are constitutively present in serum (Kane et al., 2008a; Kane et al., 2008b), we could not exclude the possibility that that deficiency in *Rara* may result in a long-lasting metabolic defect that may also impair T cell activation. As such, we also incubated WT cells with the pan-RAR antagonist, LE540, prior to T cell activation. Treatment with this antagonist recapitulated the *Rara*$^{-/-}$ phenotype, manifesting in impaired Ca^{2+} mobilization upon anti-CD3 crosslinking (**Figure 34B**). Supporting the idea that RARα exerts a function downstream of the TCR/CD3 complex, Ca^{2+} mobilization was unaffected in LE540 treated cells stimulated with the Ca^{2+} ionophore, ionomycin (Liu

and Hermann, 1978). These findings imply that transient blockade of RARα signaling is sufficient to impede signal transduction events upon TCR recognition. Consequently, RARα is a crucial regulator of T cell responses.

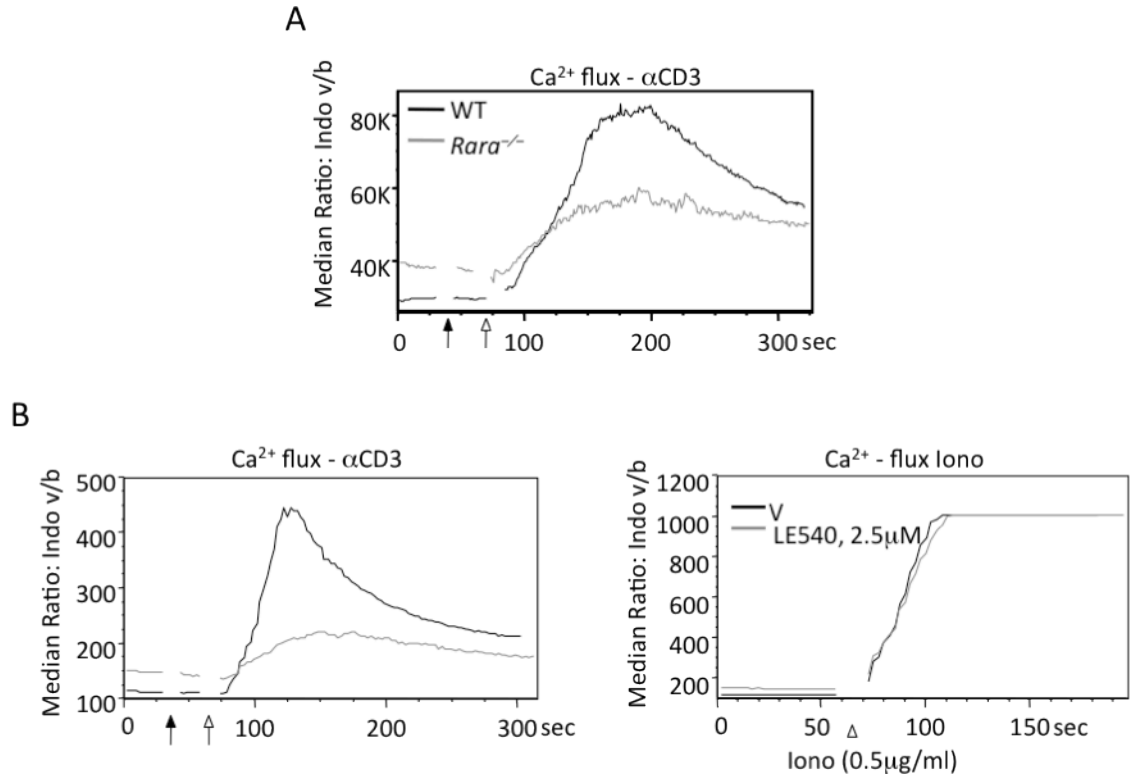

Figure 34. Loss of basal RARa signaling impairs responsiveness to TCR/CD3 engagement

(**A**) Analysis of Ca^{2+} fluxes in *Rara*$^{-/-}$ (grey line), and WT mice (black line). Cells are gated on total $CD4^+$ T cells. (**B**) Vehicle treated (black line) versus LE540 (grey line, 2.5μM) treated cells. Black-tipped arrow denotes the addition of biotin α-CD3 (10μg/ml). White-tipped arrow denotes the addition of streptavidin (20μg/ml). White arrowhead denotes addition of ionomycin. Cells are gated on total $CD4^+$ T cells. Histograms depict the median ratio of DAPI-A/Indo-1-A of Ca^{2+} fluxing cells as a function of time (sec = seconds).

Discussion:

Although vitamin A insufficiency has long been linked to impaired immunity to pathogens, the role of vitamin A metabolism in the regulation of $CD4^+$ T cell responses remains poorly understood. The experiments detailed herein indicate that the retinoic acid/RARα signaling axis is essential for adaptive $CD4^+$ T cell immunity. Specifically, mucosal T_H-1 and T_H-17 responses to oral infection and vaccination were compromised upon loss of vitamin A. These impairments were unlikely to be manifestations of a developmental defect propagated upon loss of vitamin A, as RA rapidly restored mucosal T_H-1 and T_H-17 responses. This finding, in particular, suggested that this metabolite is the cardinal mediator of vitamin A dependent immunity *in vivo.* Strikingly, genetic ablation of *Rara* was sufficient to recapitulate the phenotype of VAI mice, both at steady-state and during infection. Furthermore, T cells lacking RARα or subjected to RA receptor antagonism display early activation defects and proliferate less efficiently in response to T cell stimulation. Thus, the RA/RARα axis controls the fate of adaptive immunity, at least in part, via cell autonomous effects on $CD4^+$ T cells and reveals one potential explanation for the broad control of this pathway over various T cell fates.

RA does not impair adaptive T_H-1 responses

RA has been proposed to foster the reciprocal development of $Foxp3^+$ T_{reg} and T_H-17 cells (Mucida et al., 2007); however, the findings described here suggest that on some fundamental level RA is a physiological mediator of T_H-17 responses, as well as T_H-1 responses. Importantly, the majority of $CD4^+$ T cells that displayed a RA signature (based on $\alpha_4\beta_7$), co-expressed T-bet, the transcription factor required for T_H-1 commitment (Szabo et al., 2000). Further, *in vivo* add-back experiments demonstrated

that RA was capable of restoring T_H-1 responses in VAI mice. These data are somewhat in conflict with previous reports that have suggested that RA is a negative regulator of T_H-1 inflammation (Cui et al., 2000). For instance, VAI mice produced abnormally high levels of IFN-γ during infection with the nematode, *Trichinella Spiralis* and failed to elicit a proper and robust T_H-2 response (Carman et al., 1992). In this system, RA was shown to decrease IFN-γ production when added to *in vitro* restimulated cell suspensions and was, hence, as a T_H-1 suppressor (Cantorna et al., 1994). However, this type of "add-back" experiment is difficult to interpret physiologically, especially when considering that the cells treated in culture were likely of a heterogeneous activation status. In this regard, RA was shown to be able to inhibit effector/memory T cell cytokine production (Hill et al., 2008). Integrating these data into a working model suggests that RA signaling is potentially biphasic - driving T cell activation/differentiation during the early stages of an immune response, but regulating the amplitude at later stages. In terms of tissue immunity, this could be a particularly effective strategy to minimize tissue damage.

RA may potentiate T_H-17 through a multitude of effects in the intestinal mucosa

T_H-17 cells are elicited via the actions of multiple cytokines, including TGF-b, and any combination of IL-6, IL-21, IL-23 and/or IL-1 (Korn et al., 2009). The data described here demonstrate that diminished vitamin A prevented the acquisition of a robust T_H-17 response *in vivo*. The ability of RA to restore T_H-17 responses in VAI mice was also unexpected in light of recent studies that have reported negative effects of RA on IL-17 production *in vitro* (Elias et al., 2008; Mucida et al., 2007) and in certain animal models of autoimmune disease (Xiao et al., 2008). Nevertheless, in systems that have scrutinized the effects of RA at low doses (Wang et al.) and in conjunction with microbial stimuli, such as TLR5 ligands (Uematsu et al., 2008), T_H-17 generation was shown to be

unaffected or enhanced, respectively. Thus, in physiological settings and microbial rich environments, RA may favor the generation of T_H-17 cells. Compatible with this idea, two recent studies revealed that T_H-17 cells were virtually ablated in the Pp and Lp of VAI mice during steady-state (Cha et al.; Wang et al.). In one of these studies, the authors attributed this result to impeded migration of T_H-17 cells into the gut; however, deficiency did not result in their increase elsewhere(Wang et al.). Therefore, at the minimum, the intestinal mucosal environment is requisite for promoting T_H-17 development and/or maintenance. Moreover, the other study, which observed T_H-17 depletion, detected this defect while the mice were young and T_H homing capacity to mucosal sites would have been intact (Cha et al.). Strikingly, this study also reported large shifts in the commensal microbial phyla that were present within the intestinal ileum when mice were reared on a vitamin A deficient diet. Not only was the total amount of bacteria decreased in these animals, but SFB, which was present in the control diet fed mice, was also completely missing. This finding indicates that RA may be able to influence both the density and the composition of the commensal microbiota, which when considered alongside the findings described **in Chapter 3**, could also explain, in part, the attenuation of T_H responses in VAI mice. Supposing that the influence of RA and TLR ligands on follicular DC can extend to LpDC, another possibility is that RA may foster T_H-17 development via promotion of TGF-β production (Suzuki et al.). Importantly, these potential modes of T_H-17 generation are consistent with the proposed biphasic model of RA on T cell activation, where RA could provide a source of active TGF-β *in situ*.

Potential roles of RA/RARα in the regulation of T cell activation

Rara$^{-/-}$ mice complemented VAI mice on multiple levels. First, the number of Lp effector T cells was reduced in both animals compared to their control counterparts (~3

fold for VAI; ~2 fold for *Rara*$^{-/-}$). These findings suggest that RARα is critical for upregulation of homing receptors, which fits with the data presented in **Figure 11** of **Chapter 2**. In that experiment, *Rara*$^{-/-}$ T cells failed to upregulate $\alpha_4\beta_7$, when cultured in the presence of RA, albeit in Foxp3$^+$ T_{reg} polarizing conditions. Remarkably, transient provision of RA restored both T cell equilibrium and the CD4$^+$ T cell response within the Lp of VAI mice. Since RA restored both of these parameters, it is difficult to comment on the relative contribution of homing versus effects on T cell activation to the restoration of immunity in this tissue. However, suggesting that RA/RARα signals impart more than merely homing features to T cells, systemically infecting VAI mice with *T. gondii* still resulted in a markedly impaired T_H-1 response. This outcome could be the product of both direct and indirect actions of RA/RARα signaling on T lymphocytes.

Nevertheless, indicative that this pathway can function directly through T cells, RARα was observed to mediate signal transduction events downstream of TCR recognition, which governed T cell activation. In the absence of RARα CD4$^+$ T cells failed to respond efficiently to T cell stimulation and growth factors. While this deficiency could potentially reflect a T cell developmental defect in *Rara*$^{-/-}$ mice, retinoic acid receptor antagonism of WT cells also impaired Ca^{2+} mobilization upon TCR activation, which was similarly observed in RARα deficient T cells. Prolonged Ca^{2+} mobilization induces the dephosphorylation and subsequent translocation of a series of transcription factors collectively referred to as the nuclear factor of activated T cells (NFAT), into the nucleus (Winslow et al., 2003). Transcriptional events mediated by NFAT result in a host of genetic changes, culminating in cell growth, proliferation and effector function (Feske et al., 2001). Thus, one possibility is that loss or antagonism of RARα results in delayed translocation of NFAT.

Recent findings indicate a role for nutrient metabolism in T cell activation. For instance, vitamin D/vitamin D receptor (VDR) signaling was shown to promote the proliferation of human T cells in response to TCR stimulation via the induction of PLCγ (von Essen et al.). The DNA binding capacity of VDR presumably mediates this induction via transcription. RARα is also recognized to regulate gene expression in the same fashion (Chambon, 1996). As RA, as well as other retinoids, are present in the serum and tissues of mice (Kane et al., 2008a; Kane et al., 2008b), it is intriguing to speculate that these compounds exert constitutive effects on the phosphorylation status, localization and/or conformation of RARα in T cells, which may in turn regulate this protein's function. Short-term incubation (< 1 hr) with a pan-RAR antagonist impaired T cell Ca^{2+} mobilization in a manner similar to that observed in the absence of RARα. One explanation for this finding is that nuclear RARα regulates the expression or kinase activity of a mediator of T cell activation through its function as a transcription factor. Another possibility is that extra-nuclear RARα molecules facilitate TCR dependent signal transduction. Indeed, unconventional roles for RARα in mitogen-activated protein kinase as well as PI3K regulation have been reported in non-hematopoietic cells (Rochette-Egly and Germain, 2009). In line with these activities, RARα was shown to localize to the plasma membrane of a neuroblastoma cell line in an RA dependent manner (Masia et al., 2007).

It is also worth noting that RARα expression is not necessarily restricted to expression in T cells and may also affect the function of APC, including DC. In this vein, RARα ligands were shown to synergize with inflammatory mediators and enhance the activation of human Langerhans cell-type dendritic cells (Geissmann et al., 2003).

Therefore, it is possible that altered APC function also contributes to impaired adaptive $CD4^+$ T cell responses in VAI and *Rara*$^{-/-}$ mice.

In summary, the GI tract must be able to tolerate constant exposure to food antigen and commensals, while maintaining the capacity to rapidly respond to encounters with pathogen. These conflicting pressures confront the host immune system defending the GI tract with a unique challenge. One would predict that the most judicious strategy to respond to this spectrum of recurring challenges would involve a conserved pathway that can readily adjust to environmental cues. Here we identify the RA/RARα signaling pathway as fitting this mode of host control, promoting $Foxp3^+$ T_{reg} generation and likely tolerance during steady-state conditions, while adaptive T cell responses in the face of pathogen. As such, we propose that RA regulates adaptive immunity in a manner that is symmetrical to TGF-β, where accompanying signals dictate whether a response ultimately becomes regulatory in nature or inflammatory. An important consideration is that adaptive immune responses often involve multiple waves of antigen presenting cell recruitment. Based on the systemic RA mediated signals that we observe during infection, it will be interesting to examine how newly recruited APC contribute to the RA/RARα signaling axis during inflammation. Finally, the requirement of RARα for T cell activation suggests that this pathway may have evolved early with the development of adaptive $CD4^+$ T cell responses to coordinate host protection.

CHAPTER 5: Conclusion

Vitamin A metabolism drives Foxp3$^+$ T_{reg} generation in vitro and in response to oral antigen, in vivo

The ability of the GI tract to provide a nutrient source to the body is dependent on maintaining homeostasis in the face of constant exposure to the external environment and the intestinal microbiota. To adapt to the constant level of stimulation in this environment, the intestinal immune system has evolved a multitude of regulatory strategies. In **Chapter 2**, one of these regulatory strategies, the local generation of Foxp3$^+$ T_{reg}, was revealed to depend on a specialized population of migratory GALT dendritic cells, which was able to synthesize retinoic acid from vitamin A derivatives. RA receptor antagonism was shown to hinder LpDC-induced Foxp3$^+$ T_{reg} generation, while RA was shown to enhance SpDC-induced Foxp3$^+$ T_{reg} generation, thus, highlighting the potential importance of this metabolite in GALT Foxp3$^+$ T_{reg} development (Sun et al., 2007). Reinforcing this idea, the induction of Foxp3$^+$ T_{reg} in the GALT was abrogated in mice, which lacked vitamin A and its associated metabolites (VAI mice). Studies on VAI mice further revealed that vitamin A was required for LpDC to retain the capacity to synthesize RA. This finding indicates the intestinal immune system has adapted to take advantage of the abundant intake and remarkable storage capacity of vitamin A. Indeed, RA is also an important mediator of IgA$^+$ class switching in B cells (Mora et al., 2006; Suzuki et al.), and the upregulation of intestinal-homing surface markers on lymphocytes (Iwata et al., 2004).

At the time that the findings in **Chapter 2** were published (Sun et al., 2007), several other groups reported similar data with mesenteric lymph node DC, particularly

the $CD103^+$ subset, which derived from the intestinal lamina propria (Coombes et al., 2007; Mucida et al., 2007). A host of other groups also reported that RA could enhance $Foxp3^+$ T_{reg} generation *in vitro* under a variety of circumstances, including: during high costimulatory activity, in the presence of T_H cytokines, and in otherwise non inhibitory conditions (Benson et al., 2007; Elias et al., 2008; Hill et al., 2008; Nolting et al., 2009). Despite several demonstrations that RARα was required for RA to be able to enhance $Foxp3^+$ T_{reg} induction, the molecular mechanism by which RA/RARα signaling stimulates Foxp3 activity remains unclear (Hill et al., 2008; Nolting et al., 2009). One interesting conjecture is that RA mediates these effects through the inhibition of the transcriptional complex, activation protein – 1 (AP-1), in T cells (von Boehmer, 2007). Rao and colleagues recently demonstrated that NFAT partnering to Foxp3 rather than AP-1 favors the development of $Foxp3^+$ T_{reg} in lieu of effector T cells (Wu et al., 2006). Therefore, inhibition of AP-1 may promote $Foxp3^+$ T_{reg} generation. Indeed RA/RARα has been shown to inhibit several aspects of the AP-1 pathway, including: upstream activation of JNK (Lee et al., 1999; Xu et al., 2002), subunit expression (Fisher et al., 1998; Talmage and Lackey, 1992), and DNA binding (Schule et al., 1991).

TLR9 / Commensal microbial DNA interactions are requisite for mucosal T cell homeostasis and immunity

While GALT mediated $Foxp3^+$ T_{reg} induction may represent a default pathway to promote tolerance to food antigen and commensals, data presented in **Chapter 3** suggested that this process is tightly controlled by components of the commensal microbiota. Specifically, engagement of TLR9 by microbiota derived DNA strongly inhibited the capacity of LpDC to promote the generation of $Foxp3^+$ T_{reg} and instead drove the formation of T_H cells, including T_H-17, T_H-1 and T_H-2. Complementing this finding, *Tlr9*$^{-/-}$ mice were found to have an increased ratio of $Foxp3^+$ T_{reg} / T_H within

intestinal effector sites. Although this shift had no bearing when *Tlr9*$^{-/-}$ mice were infected systemically with *E. cuniculi*, the adaptive immune response in these animals was dramatically impaired upon infection through the oral route. Together, these findings suggest that commensal DNA sensing is a critical mediator of adaptive immune responses to pathogens that gain access to the host through the gastrointestinal tract. Previously, Nod2 was shown to affect immune responses to bacterial infection in a similar manner, i.e. *Nod2*$^{-/-}$ mice were susceptible specifically to the oral route of challenge (Kobayashi et al., 2005). However, the data described in **Chapter 3** also illustrate that perturbation of the Foxp3^{+} T_{reg} / T_H ratio can markedly influence adaptive immune responses at mucosal sites of challenge. This finding may have significant bearing on oral vaccination strategies and also provides a framework for thinking about how the composition of the microbiota could influence the ability of the host to respond to intestinal challenge. In particular, more comprehensive analyses of the commensal microbiota may facilitate the identification of immuno-stimulatory/regulatory DNA motifs that can be utilized in oral vaccine design.

Around the time that the TLR9 findings were published (Hall et al., 2008), Littman and colleagues demonstrated that T_H-17 cells were strongly diminished in the absence of the commensal microbiota (Ivanov et al., 2008). Another study showed that T_H-1 cells were also strongly diminished in germ-free conditions (Gaboriau-Routhiau et al., 2009). The fact that T_H-1 and T_H-17 cells are not altogether ablated in these conditions may be due to residual TLR ligands present in the diet fed to these animals (Hill et al.). Surprisingly, Littman and colleagues also noted in their study that the frequency of T_H-17 cells was unaffected in mice lacking both MyD88 and TRIF. However, **Chapter 3** showed that both the frequency and absolute number of T_H-1 and T_H-17 cells were decreased in mice deficient in *Tlr9*, which is known to signal through MyD88 (Hemmi et

al., 2000). One possibility is that discrete microbial components, via signaling through distinct canonical pattern recognition receptors (TLR, Nod, and C type lectin) differentially contribute to the induction, proliferation and/or maintenance of T_H-1, T_H-17 and Foxp3$^+$ T_{reg} cells. It is also possible that combinatorial signals through these receptors influence the composition of these cells in intestinal effector sites.

In regard to toll like receptor signaling, microbial sensing and the type of response that it evokes are dependent not only on the nature of the microbe, but also on the cellular compartmentalization of the TLR in question. Surface TLRs, especially those expressed on intestinal epithelial surfaces are in direct apposition to the commensal microbiota, and are thus more inclined to experience receptor engagement. With the exception of industrialized countries and areas with access to water sanitation, IECs are also commonly in apposition with intestinal dwelling parasites. These pressures have likely compelled tissue adaptations, which regulate the ability of surface TLRs to promote signal transduction cascades that terminate in pathological situations. For example, TLR4 signaling in IEC results in the down-regulation of IRAK1, and thereby prevents the induction of an inflammatory program (Chassin et al.). TLR9 is also readily present on the surface of IEC (Lee et al., 2006); however to what extent these receptors are functional is unclear, as environmental pH plays a critical role in regulating functional TLR9 activity (Engel and Barton). Other mechanisms to avoid gross activation of the epithelial barrier upon recognition of luminal derived TLR ligands also likely exist.

Hematopoietic cells in the intestinal lamina propria have also evolved mechanisms to sustain constitutive TLR engagement in the absence of an undue pathological response. For instance, expression of the ubiquitin-editing enzyme, A20, in APC prevents spontaneous reactivity to the commensal microbiota (Turer et al., 2008).

Such a pathway illustrates the importance of having regulatory mechanisms in place to modulate the activation status of APC within the intestinal tract and also demonstrates that these cells constitutively receive commensal dependent signals. This notwithstanding, constitutive recognition of commensal DNA via TLR9 endows APC with the capacity to elicit T_H responses in intestinal effector sites in the absence of overt pathology. Given that TLR9/bacterial DNA interactions are predicated on an active sensing mechanism in these cells, it stands that the ability of this pathway to drive an inflammatory response is conserved in the event of pathogen encounter.

Vitamin A metabolism promotes T_H-1 and T_H-17 adaptive immune responses via retinoic acid/RARα

A summary of the data in **Chapter 2** and **Chapter 3** indicates that signals dependent on vitamin A metabolism and TLR9 engagement contribute to T cell homeostasis in the GI tract, which in turn, shapes host responsiveness to mucosal pathogens. Although vitamin A metabolism in LpDC appears to favor $Foxp3^+$ T_{reg} development and/or maintenance in the GALT, while commensal DNA interactions with TLR9 in LpDC appear to favor T_H development and/or maintenance, how the signaling pathways downstream of these cues converge and regulate one another in T cells is unclear and merits further investigation. Studies in this regard will also help to further clarify findings in **Chapter 4**, which demonstrate that vitamin A metabolism is required for GALT $Foxp3^+$ T_{reg} differentiation and optimal T_H-1 and T_H-17 responses. Previous studies have demonstrated the importance of RA precursors, such as retinol and retinal in the maintenance of energy homeostasis (Acin-Perez et al.; Ziouzenkova et al., 2007). However, work here suggests that these precursors are dispensable for adaptive immune responses. In support of this, administration of RA to VAI mice orally challenged

with *T. gondii* or vaccinated with a mutated *E. coli* labile toxin, completely restored $CD4^+$ T cell responses to levels observed in WT animals.

RA signals are transduced by at least four known nuclear receptors including: RARα, RARβ, RARγ, and PPARβδ, which are expressed differentially in multiple cell types (Mora et al., 2008). Given the nature of the add-back experiments performed in **Chapter 4**, it is difficult to propose a precise and all-inclusive mechanism for the coordinated activities of RA, which would account for the restoration of adaptive immune responses. One receptor that appeared particularly important was RARα, which has in fact been shown to facilitate accessibility to other genes involved in vitamin A metabolism, including RARβ2, which also controls other inducible RA responsive genes (Corlazzoli et al., 2009; Ren et al., 2005). An examination of the GALT in *Rara*$^{-/-}$ mice indicated that they shared several overlapping immunological defects with VAI mice. In addition to a disrupted T cell equilibrium in GALT effector sites, the immune response upon oral vaccination was similarly impaired in *Rara*$^{-/-}$ animals. But, perhaps, the most unexpected finding in **Chapter 4** was the importance of RA/RARα signaling in T cell activation and proliferation.

RARα regulates the acquisition of effector T cell function in a cell intrinsic manner

In the absence of RARα, T cell activation, proliferation, and survival were impaired as a result of a cell intrinsic defect. Mammalian TOR activation, which regulates multiple pathways essential for cell growth, proliferation, survival and differentiation (Wullschleger et al., 2006), was noticeably reduced. Further, Ca^{2+} mobilization in response to TCR/CD3 engagement was impaired in *Rara*$^{-/-}$ T cells, as well as, WT T cells exposed to the pan RAR antagonist, LE540, prior to activation. As retinoids are

constantly present in the serum (Kane et al., 2008a; Kane et al., 2008b), and naïve T cells were observed to actively transcribe *Rara*, it is possible that steady-state RA/RARα signals constitutively modulate protein expression and/or kinase activity to facilitate T cell activation upon TCR engagement. In this regard, it may be worthwhile to examine steady-state RA activity in T cells, which could be performed using mice that have been engineered with a transgenic RA-responsive element reporter (Svensson et al., 2008). Although these findings would theoretically impact the development of all T_H subsets, including Foxp3$^+$ T_{reg}, it is important to note that the foregoing studies did not assess T_H-2 responses in VAI mice nor in *Rara*$^{-/-}$ mice. Therefore, it is still not clear precisely how defects in RA/RARα signals contribute to T_H-2 development.

Previously, it was demonstrated that the strength of signal through the TCR could function as a crucial determinant in T_H-1 versus T_H-2 polarization. In particular, weaker stimulation achieved with a low-dose of cognate peptide or an altered peptide ligand favored the development of T_H-2 cells, both *in vitro* and *in vivo* (Constant et al., 1995; Hosken et al., 1995; Pfeiffer et al., 1995). Conversely, increasing the antigen dose or strength of peptide/MHC interactions with TCR favored the development of T_H-1 cells. These findings set the stage for more recent data highlighting that T_H-1 and T_H-2 cell induction may have different signaling requirements. For instance, altered peptide ligands, which skew T cell polarization in the T_H-2 direction, were observed to induce sustained reductions and more transient spikes in Ca^{2+} mobilization (Brogdon et al., 2002). Pharmacological inhibition/reduction of extracellular signal-related kinase (ERK) activity was also shown to sensitize cells for T_H-2 polarization under normally T_H-1 polarizing circumstances (Yamane et al., 2005). As for a T_H-2 polarizing antigen, *Schistosoma mansoni* egg antigen was recently shown to contain a ribonuclease that specifically impaired conjugate formation between dendritic cells and CD4 T cells,

presumably lowering the strength of TCR signaling (Steinfelder et al., 2009). Based on these findings, it is tempting to speculate that the activation defects observed in the absence of RA/RARα may have a less pronounced impact on *in vivo* T_H-2 responses. Nevertheless, there is a notable correlation between the incidence of worm infection, which requires a T_H-2 response for effective expulsion (Paul and Zhu), and micronutrient deficiencies, particularly in vitamin A (Koski and Scott, 2001). Importantly, epithelial cells and accessory cells, such as basophils, often play critical roles in the initiation and amplification of T_H-2 responses *in vivo* (Anthony et al., 2007; Paul and Zhu). The function of these cells may be dysregulated in the absence of RA/RARα signaling. Multipotent progenitors and innate lymphoid cells, which are also important mediators of T_H-2 responses (Moro et al.; Neill et al.; Saenz et al.), may also be affected in these conditions.

Based on the data reported above, a tentative model for the influence of RA/RARα in mucosal immunity may be constructed. Antigen derived from food/environmental constituents, commensals or invasive pathogens is captured in the lamina propria by antigen presenting cells, which include both migratory and resident subsets. The migratory APC that captures antigen is the $CD103^+$ lamina propria DC, which is able to synthesize RA. The resident APC that captures antigen is $CD103^-$ and is less capable to synthesize RA. How antigens captured by these cells end up being transferred to and presented by the migratory $CD103^+$ subset remain unclear, though it may involve a mechanism such as exosomal transfer and apoptosis (Thery et al., 2009; Trombetta and Mellman, 2005). Upon entry into the draining mesenteric lymph nodes, $CD103^+$ LpDC may form a cognate interaction with a naïve T cell constitutively expressing RARα. RA secreted by LpDC may influence the phenotype of the T cell

during activation, including the upregulation of mucosal homing markers and the acquisition of Foxp3. Ultimately, however, the cytokines and costimulatory signals elicited by the antigen and environment shape the polarity of the response (**Figure 35, 1 versus 2**). There is also a possibility that autocrine/paracrine interactions with RA influence cytokine production by LpDC and contribute to the polarity of the response. For instance, RA was shown to enhance TGF-β production by follicular DC, and may potentially exert similar effects on LpDC (**Figure 35, 3)**. As TGF-β is critical for the development of both $Foxp3^+$ T_{reg} and T_H-17 cells, this could explain why the induction of both of these subsets is impaired in VAI mice. Nevertheless, these aspects are secondary to the role of RA/RARα signals in controlling early T cell activation and proliferation. The data in **Chapter 4** also suggest that RA/RARα plays a role in regulating tonic T cell signal transduction. Therefore, other sources of RA, including the serum and stroma may be critical for the coordination of early T cell activation events (**Figure 35, 4)**.

Understanding the localization of RARα will provide clues into how it regulates T cell activation.

RARα is a complexly regulated protein, making it difficult to draw any immediate conclusions on how precisely it regulates signaling events involved in T cell activation and proliferation. In addition to it's clear function as a transcriptional modifier, extra-nuclear activities of RARα have previously been noted in non-hematopoietic cells (Rochette-Egly and Germain, 2009). For example, RARα has been described to interact with the p85 subunit of phosphoinositide 3-kinase (PI3K) (Masia et al., 2007), which is involved in activating the Akt pathway. Although, little is known about the steady-state regulation and localization of RARα in T cells, a mechanism on par with this is at least

consistent with the reduction of Akt phosphorylation observed upon activation of *Rara*$^{-/-}$ T cells in **Chapter 4**. Of course, RARα has also been linked to other pathways involved in T cell activation, most notably the AP-1 pathway. In particular, RARα has been shown to induce expression of the phosphatase, MKP-1, which negatively regulates JNK activity through both transcriptional dependent and independent means (Lee et al., 1999; Xu et al., 2002). This in part explains the inhibitory effect of RA on AP-1 activity. Although it is unclear whether RA/RARα positively regulates MKP-1 in T cells, loss of this protein was shown to impair T cell activation, as well as T_H-1 and T_H-17 responses *in vivo* (Zhang et al., 2009b). Importantly, proper regulation of JNK activity has also been demonstrated to be crucial for T cell proliferation, where early over-activation promotes cell death, while lack of activation promotes proliferation (Berger et al.; Dong et al., 2000). Future studies on the role of RA/RARα in T cell function will benefit by a better understanding of the localization and regulation of RARα, in addition to the target molecular pathways.

Influence of RA on T cell responses *in situ*:

The potential inhibitory effect of RA/RARα signaling on AP-1 activation in T cells coupled with recent data showing that RA can curb cytokine production by effector/memory T cells suggest that RA may down-modulate T cell activity *in situ* (**Figure 36, 1)**. RA may support yet another regulatory circuit, as previously discussed, via activation of TGF-β in this tissue environment. In these ways, RA may contribute to the preservation of tissue integrity and barrier protection. These regulatory effects may promote the maintenance, functional differentiation, and/or expansion of Foxp3$^+$ T_{reg} and also counter the risk of excessive responsiveness to bacteria lying in close apposition to the epithelial barrier, such as SFB, the presence of which also depends on vitamin A (**Figure 36, 2 and 3)**. Although a recent report pointed out that deficiency in vitamin A

can result in perturbations to the commensal microbiota, including the loss of SFB, a comprehensive metagenomic analysis of the differences between commensal microbial communities in vitamin A replete versus deficient hosts has not been undertaken. From an evolutionary perspective, the model proposed here argues that vitamin A metabolism may not only permit the propagation and survival of commensal bacterial species with more immunostimulatory features, but also facilitates host evolutionary mechanisms to tolerate these species.

Closing Remarks:

The experiments described in this thesis highlight the importance of commensal DNA and retinoic acid in the promotion of mucosal immunity. Constitutive signals derived from both of these cues were essential to the maintenance of T cell homeostasis in the GI tract, without which adaptive mucosal immune responses could not proceed efficiently. Although TLR9 signaling was revealed to possess a non-redundant function in the GI tract, it was dispensable for immune responses to systemic challenge. In contrast, RA was required for both mucosal and systemic adaptive immune responses and was revealed to regulate the acquisition of effector T cell responses in a cell intrinsic manner. Evolutionarily, these findings correspond with the compartmentalization of these elements: where commensal DNA is typically restricted to the GI tract; while RA, through robust storage and chaperone mechanisms, is able to traverse the entire body. Given this ubiquity, the capacity of RA to induce both regulatory and inflammatory responses also likely constitutes an adaptation. Overarchingly, these data support the premise that the successful integration of commensal microbial and nutrient derived signals provides a crucial immunological advantage to the host.

Models:

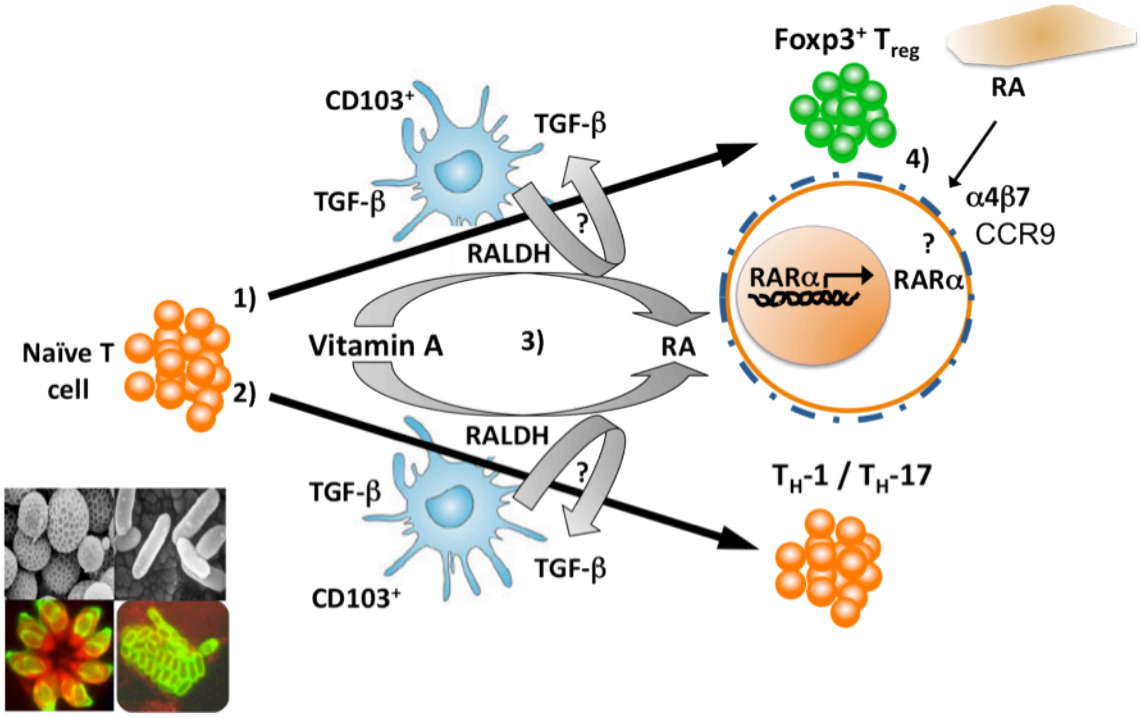

Figure 35. Retinoic acid in the induction of Foxp3$^+$ T_{reg} and helper T cell responses.

(**1**) In the absence of an inflammatory stimulus, LpDC are poised to induce Foxp3$^+$ T_{reg}. This process may occur in response to food or environmentally derived antigen (**2**) Commensal microbiota potentially alter the ability of LpDC to induce Foxp3$^+$ T_{reg}. For example, commensal derived DNA provide a potent tonic signal that favors the development of various T_H programs. Other inflammatory stimuli, especially invasive pathogens, also lead to the induction of T_H programs. These studies, in particular, were focused on the development of adaptive T_H-1 and T_H-17 responses. (**3**) Migratory CD103$^+$ lamina propria DC possess classical DC functions, including the ability to initiate T cell responses. Their polarizing capacity may be modified *in situ* via autocrine or paracrine RA signaling. (**4**) RA/RARα signaling critically controls early T cell activation and proliferation. Although LpDC derived RA is likely instrumental for upregulation of mucosal homing molecules and acquisition of Foxp3, other sources, including serum and stroma, may regulate T cell signal transduction events.

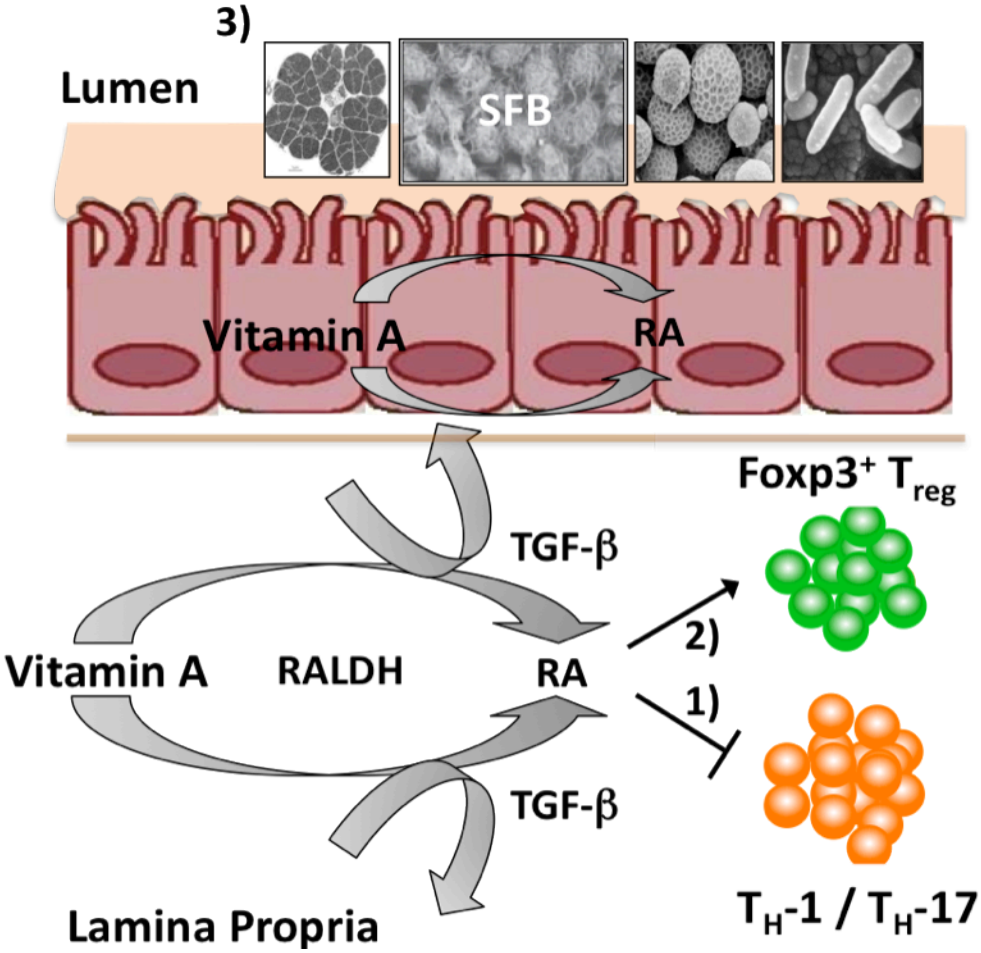

Figure 36. Retinoic acid in the regulation of adaptive T cell responses and composition in intestinal tissue

(**1**) RA can directly inhibit cytokine production by effector/memory T cells (Hill et al., 2008). This metabolite was also shown to suppress T cell expression of IL-6R and IL-23R, which may also down-modulate their effector capacity (Hill et al., 2008; Xiao et al., 2008) (**2**) The ability of RA to promote the activation of TGF-β may also limit the outgrowth of T_H cells and promote $Foxp3^+$ T_{reg} activities and/or expansion. (**3**) Vitamin A sustains the presence of certain commensal microbiota, including SFB, and may be an important factor for establishing and maintaining commensal diversity.

CHAPTER 6: Material and Methods

General methods:

Mouse strains:

C57BL/6 (WT), B6.SJL (CD45.1), OTII transgenic, and OT-II transgenic RAG-1$^{-/-}$ mice were purchased from Taconic Farms (Rockville, MD). Foxp3^{eGFP} and DEREG mice were originally obtained from Dr. M. Oukka (Bettelli et al., 2006) and Dr. T. Sparwasser (Lahl et al., 2007), respectively, and bred in house. B6.129P2-*Tlr9*^{tmAki} (*Tlr9*$^{-/-}$) (Hemmi et al., 2000) mice were obtained from Dr. S. Akira (Osaka University) via Dr. R. Seder (Vaccine Research Center, NIH) and backcrossed 11 generations onto the C57BL/6 background from Taconic Farms. OTII transgenic Foxp3^{eGFP} mice were generated by crossing the F2 progeny of OTII x Foxp3^{eGFP} breeders. *Tlr9*$^{-/-}$Foxp3^{eGFP} mice were generated by crossing the F1 progeny of *Tlr9*$^{-/-}$ x Foxp3^{eGFP} breeders. *Tlr9*$^{-/-}$B6.SJL mice were generated by crossing the F1 progeny of *Tlr9*$^{-/-}$ x B6.SJL breeders. *Rar*$\alpha^{-/-}$ mice were a generous gift of Dr. P. Chambon (Chapellier et al., 2002) and obtained via Dr. C. Benoist (Harvard Medical School), then bred in house. Experiments with *Rarb*$^{-/-}$ and *Rarg*$^{-/-}$ mice were performed under the guidance of Dr. N. Ghyselinck (igbmc, France). B6.129S6-*Il6*^{tm1Kopf} (*Il-6*$^{-/-}$) mice were purchased from the Jackson Laboratory and B6.129P2-*Myd88*^{tmAki} (*Myd88*$^{-/-}$) mice were kindly provided by Dr A. Sher.

All mice were bred and maintained under pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care accredited animal facility at the NIAID and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the

NIAID Animal Care and Use Committee. For each experiment, mice were gender, aged and/or littermate matched. All mice were used between 9 and 13 weeks of age.

Vitamin A diet studies:

Vitamin A deficient (TD.09838, yellow) and sufficient (20,000 IU vitamin A/kg, TD.09839, orange) diets were purchased from Harlan Teklad Diets. At day 14.5 of gestation, pregnant females were administered either Vitamin A deficient or sufficient diet and maintained on diet until weaning of litter. Upon weaning, females were returned to standard Harlan chow, while weanlings were maintained on special diet until use. Diet was replaced in feed hopper every 3-4 days to prevent degradation and stored at 4-8^{o}C. Diet stocks were stored in vacuum-sealed packages and discarded 3 weeks after opening. For breeding, females were rested on a standard Harlan chow diet for at least 2 weeks prior to re-mating. After 3 birth cycles under the vitamin A deficient diet, females were retired.

Tissue preparations:

Cells from Spleen (Sp) and mesenteric lymph nodes (mln), were prepared by teasing and gently smashing the tissue through a 70-μm cell strainer with the rubber end of a plunger from a 3ml syringe (BD).

In order to prepare small intestinal lamina propria (Lp) tissue cell suspensions, adventitial fat was carefully stripped away from the whole small intestine. Peyer's patches (Pp) were then carefully excised, and the remaining tissue was cut longitudinally, extensively washed in cold HBSS w/o Ca^{2+} and Mg^{2+} (cellgro), then cut into ~ 1cm segments. Segments were treated with RPMI-1640 medium (Hyclone) containing: 3% FCS, 100μg/ml Penn/Strep, 25 mM HEPES (Hyclone), 50 μM β-mercaptoethanol (β-ME)

(Gibco 1000X), 5mM EDTA, and 145 μg/ml of Dithiothreitol (DTT) for 20 min in an incubator at 37ºC/5% CO_2 with constant magnetic stirring.

IEL fraction: Tissue was then collected into a hand-held, fine mesh, sieve placed atop a collection beaker on ice. The pieces were then collected and placed into a 50ml conical containing 10-15ml of serum free media. Tissue was then further digested in serum free media containing: 25 mM HEPES and 50 μM β-ME with liberase CI (200 μg/ml, Roche) or liberase TI (100 μg/ml, Roche) in conjunction with DNase I (500 μg/ml, Sigma-Aldrich) under continuous magnetic stirring in an incubator at 37ºC/5% CO_2 for 26-30 min. Digested tissue was then immediately diluted in RPMI-1640 medium containing: 3% FCS, 100μg/ml Penn/Strep, 25 mM HEPES and 50 μM β-mercaptoethanol, and serially mashed through 70- and 40-μm cell strainers (BD Biosciences). Cell suspensions were spun down and resuspended in complete RPMI-1640 medium (Complete) containing: 10% FBS, 100μg/ml Penn/Strep, 25mM HEPES, 2mM L-glutamine, 1 mM Na Pyruvate (cellgro), 1X MEM nonessential amino acids (MEM) (cellgro) and 50 μM of β-ME – prior to use.

For Pp preparations, lymphoid aggregates were treated with RPMI-1640 medium containing: 3% FCS, 100μg/ml Penn/Strep, 25 mM HEPES, 50 μM β-ME, 5mM EDTA, and 145 μg/ml of DTT for 20 min in an incubator at 37ºC/5% CO_2. They were then washed up-and-down several times with a 1ml pipette and strained from media, which was then discarded. Next, tissue was resuspended with serum free RPMI-1640 containing: 25mM HEPES, 50 mM β-ME, liberase CI (100 μg/ml) or liberase TI (50 μg/ml) and 150 μg/ml DNase I, minced, and digested for 20 min prior to serially smashing through 70- and 40-μm cell strainers.

$CD4^+$ T cell purification for *in vitro* studies:

Sp and peripheral LNs were harvested, teased apart and gently passed through a 70μm filter. After washing, cells were RBC lysed in ACK-lysis buffer (Lonza) for 1.5 min on ice, washed again and then enriched for $CD4^+$ T cells using a negative selection $CD4^+$ T cell isolating kit and an autoMACs™ as outlined above. The enriched fraction was further labeled with a mAb cocktail containing: α-CD4 (RM4-5), α-CD25 (7D4), α-CD44, (IM7) and α-CD62L (MEL-14), where indicated. Cells were then sort purified on a FACSVantage™ or FACSAria™. When eGFP reporter strains were used, $eGFP^+$ cells were also excluded. All antibodies were purchased from eBioscience.

CFSE labeling of T cells:

Cells were labeled at a final density of $1x10^7$cell/ml in 1μM CFSE (CellTrace™ Kit, Invitrogen) dissolved in HBSS w/o Ca^{2+} and Mg^{2+} for 7 min in an incubator at $37^oC/5\%CO_2$. Labeling was quenched by washing cells 2X in complete RPMI supplemented with 30% FBS. Finally, cells were resuspended in ice-cold PBS for adoptive transfer via tail vein injection or, alternatively, in complete RPMI as described above for *in vitro* assays.

Dendritic Cell (DC) purification:

For LpDC, Lp tissue cell suspensions were obtained as described. Cells were then well resuspended in a 1.077 g/cm^3 iso-osmotic NycoPrep™ medium (Accurate Chemical & Scientific Corp.) and gently overlaid with serum free media (one intestine: 5ml NycoPrep™ medium + 2ml serum free media in 15ml conical). The low-density fraction was collected after centrifugation at 1650 x *g* for 15 min with the brake off. Note: The NycoPrep™ gradient excludes debris, red blood cells and decreases lymphocyte

numbers without significantly altering the composition of the different subsets of LpDC. Cells were then washed in 4 volumes of RPMI-1640 medium containing: 3% FCS, 100μg/ml Penn/Strep, 25 mM HEPES, 50 μM β-mercaptoethanol and incubated with a mixture of mAb containing: blocking α-CD16/32 (2.4G2) and Rat IgG (Jackson Immunoresearch); α-CD11c (HL-3), α-MHC II (I-A^b, AF6-120.1); a non-DC component including, α-NK1.1 (PK136) and α-B220 (RA3-6B2); and the DNA intercalater 7-Amino-actinomycin D (7-AAD, eBioscience) in order to exclude dead cells. α-CD103 mAb (2E7) was added in experiments where these cells were desired. All antibodies were purchased from eBioscience unless noted. LpDC were defined as $CD11c^+MHCII^+$ cells and were sort purified on a FACSVantage™ or FACSAria™. In some experiments $CD103^+$ and $CD103^-$ LpDC were separated. Purity was verified and always exceeded 90% or cells were not used.

For SpDC, Sp were thoroughly perfused with Liberase CI (100 μg/ml) and DNase I (150 μg/ml) in serum free media, prepared as described above using a tuberculin syringe (BD). Perfused Sp were then cut into fragments and digested for 25 min in an incubator at 37ºC/5% CO_2. During the last 5 min, 5mM EDTA was added. Sp cell suspensions were then gently smashed through a 70μm filter, washed, and enriched for mononuclear cells using the NycoPrep™ gradient, as detailed for LpDC. Cells were subsequently labeled using the $CD11c^+$ magnetic bead positive selection kit (Miltenyi Biotec) per the manufacturer's instructions, washed, 40μm filtered, and ran through an autoMACs™ machine using the purification program, Posseld2. Cells were then washed and incubated with a mixture of mAb containing: blocking α-CD16/32 (2.4G2), α-CD11c (HL-3), α-MHC II (AF6-120.1), and 7-AAD. Labeled cells were sorted as described above to purity resulting in > 98%.

Flow cytometry:

All antibodies used were obtained from eBioscience unless specified otherwise. All cell acquisition was performed using an LSRII machine with FACSDiVa software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). To calculate absolute numbers, the fraction of a particular subset to singlet gated, total living cells was multiplied by the total cellularity of the tissue based on trypan blue exclusion.

Statistics:

Groups were compared with Prism software (GraphPad) using the unpaired or paired Student's *t* test.

Chapter 2:

***In vivo* generation of Foxp3$^+$ T$_{reg}$ (oral feeding protocol):**

Cells were extracted from the mesenteric lymph nodes (mln) of OT-II transgenic RAG-1$^{-/-}$ mice and then adoptively transferred into B6.SJL (CD45.1) hosts. Alternatively, cells were extracted from the spleen (Sp) and secondary LNs of OTII transgenic Foxp3^{eGFP} mice (CD45.2). They were then labeled using a negative selection CD4$^+$ T cell isolating kit (Miltenyi Biotec) per the manufacturer's instructions, washed, 40μm filtered, and ran through an autoMACs™ on the sensitive mode to obtain highly enriched CD4$^+$ T cells. These cells were labeled with a non-CD4$^+$ T cell cocktail of mAb, including: blocking α-CD16/32 (2.4G2), α-CD8α (53-6.7), α-NK1.1 (PK136), and α-B220 (RA3-6B2); and α-CD25 (7D4). Cells were then sort purified on a FACSAria™ to obtain a pure CD4$^+$CD25$^-$eGFP$^-$ population, subsequently labeled with CFSE, and then adoptively transferred into 10 wk-old B6.SJL (CD45.1) hosts on diet study. In each case, recipients

received $1x10^6$ cells and were fed 1.5% OVA (grade V; Sigma-Aldrich) dissolved into autoclaved drinking water for 5 consecutive days. Solution was replaced every 48 hrs. Tissues were collected on day 6, and Foxp3 expression was assessed in transferred cells by intracellular staining as detailed below.

Assessment of DC costimulatory activity:

MHC II^+ $CD11c^+$ DC were stained with the following fluorescent dye-conjugated mAb: α-CD11b (M1/70), α-CD103 (2E7), α-CD40 (3/23, BD Biosciences), α-CD80 (16-10A1, BD Biosciences) and α-CD86 (GL-1, BD Biosciences)

***In vitro* Foxp3$^+$ T_{reg} induction assay and analysis:**

10^5 $CD4^+eGFP^-$ T cells or $CD4^+CD25^-CD62^+CD44^-$ naïve T cells from WT, OT-II, *Rara*$^{-/-}$, *Rarb*$^{-/-}$ or *Rarg*$^{-/-}$ mice were cultured with 2.5 x 10^3 – 2 x 10^4 purified Lp or SpDC in 250μl of complete medium (10% FBS, 25mM HEPES, 2mM L-glutamine, 100μg/ml Penn/Strep, 1 μM Na Pyruvate, 1X MEM, 50 μM of β-ME) with soluble α-CD3ε mAb (1 μg/ml) (145-2C11, BD Bioscience) and recombinant hTGF-β (0.6 or 3 ng/ml) (Cell Science or R&D Systems) for 5 days. For each *Rar*$^{-/-}$ mouse, the appropriate littermate controls were used. The precise ratio of T cells to DC used is provided in the text. In some experiments the following were included in the cococulture conditions:

1. All-trans retinoic acid (Sigma) or vehicle control at varying concentrations
2. Retinoic acid receptor inhibitors: LE540 (Wako Chemicals USA) and LE135 (Tocris Bioscience) or vehicle control. Each were added at 1μM
3. α-TGF-β mAb (1D11.16.8) or isotype control mAb MOPC (31C) (both from ATCC) at 10μg/ml.

All-trans retinoic acid, LE540 and LE135 were resuspended in Biotechnology Performance Certified DMSO (Sigma). Human recombinant hIL-2 (5 ng/ml) (Peprotech) was added to cultures wells every other day beginning on day 2. On day 5, cells were stained with α-CD4 mAb (RM4-5) and 7-AAD, to discriminate live/dead cells. $Foxp3^+$ cells were then detected either by eGFP expression and/or α–Foxp3 (FJK-16) following fixation and permeabilization with the kit provided by eBioscience in accordance with the manufacturer's protocol.

For viability assays, cultured cells were additionally stained with annexin V (BD Biosciences) and 7-AAD (1:50) in annexin-V binding buffer (BD Biosciences) per manufacturer's instructions to detect dying/dead cells.

For phenotypic assessment, $eGFP^+CD4^+$ cells were further stained for α-CD103 (2E7) or α-$\alpha_4\beta_7$ (DATK32) mAb, α-CD25 (PC61.5) and 7-AAD. Live cells were analyzed by flow cytometry.

***In vitro* suppression Assays:**

T_{reg} ability to suppress T cell proliferation was determined as previously reported (Thornton and Shevach, 1998). Briefly, $CD4^+GFP^+$ T_{reg} that were obtained after sorting cells from SpDC cocultures, LpDC cocultures or *ex vivo* were cultured in 96-well flat-bottom plates (Costar) with 5 x 10^4 freshly isolated $CD4^+GFP^-$ T cells used as responder T cells. For antigen presenting cells, splenocytes were depleted of $CD90^+$ cells using a positive selecting isolation kit and running through an autoMACs™. They were then irradiated and cultured at 10^5 per well. Cells were stimulated with 0.5 µg/ml α-CD3 (145-

2C11) mAb for 72 hours in an incubator at 37ºC/5% CO_2. Cultures were pulsed with [^{3}H]TdR (MP Biomedicals, Solon, OH) at 1 µCi/well for the last 8 hours of culture.

Retinal dehydrogenase (RALDH) activity in DC:

Single-cell suspensions were incubated in ice-cold HBSS with blocking α-CD16/32 (2.4G2) and 0.2mg/ml Rat IgG, and stained with the following fluorochrome-conjugated mAb: α-CD11c (N418), MHC II (M5/114.15.2), α-CD11b (M1/70), α-F4/80 (BM8), α-CD103 (2E7) and α-TCR-β (H57-597) for 15min on ice. Surface labeled cells were then washed and resuspended in 100µl of ALDEFLUOR assay buffer containing 1/100 ALDEFLUOR® reagent. Control wells were pre-incubated with (1/25) DEAB solution that came with the kit. Samples were then stored in an incubator at 37^{o}C/5% CO_2 for 30min away from light. Cells were then washed with 150ml of fresh ALDEFLUOR assay buffer, centrifuged, and resuspended in 200ml of ALDEFLUOR assay buffer containing 0.3µg/ml DAPI (Sigma Aldrich) to discriminate living/dead cells. All reagents were prepared according to the manufacturer's protocol (STEMCELL Technologies).

Chapter 3:

T cell phenotype:

Single-cell suspensions were stained with fluorochrome-conjugated to the following antibodies: α-TCR-β (H57-597), α-CD4 (RM4-5), α-CD8α (53-6.7), α-CD25 (PC61.5) in the presence of for 15min on ice. For examination of transcription factors and cellular proliferation, cells were subsequently treated with the Foxp3 staining kit (ebioscience) in accord with the manufacturer's instructions and stained for 30-45 min on ice with Ki-67 (B56, BD Pharmingen), Foxp3 (FJK-16s), T-bet (eBio4B10) and/or RORγ(t) (AFKJS-9)

or isotype controls: mouse IgG1 (BD Pharmingen) rat IgG2a (eBR2a), mouse IgG1 (clone P3) - in 1/100 α-mouse CD16/32 and 0.2mg/ml purified Rat IgG.

***In vitro* T cell/LpDC coculture assays:**

$CD4^+eGFP^-$ T cells or $CD4^+CD25^-CD62^+CD44^-$ naïve T cells from $Foxp3^{eGFP}$, *Tlr9*$^{-/-}$ $Foxp3^{eGFP}$, WT, *Tlr9*$^{-/-}$ or *Myd88*$^{-/-}$ mice were cocultured with purified LpDC at a 1:10 ratio in complete medium, as described above, in $Foxp3^+$ T_{reg} cell polarizing conditions. These conditions included soluble α–CD3 (1 μg/ml) (145-2C11) and rhTGF-β (0.6 ng/ml) (R&D Systems). 5 ng/ml of rhIL-2 was supplemented in cocultures every 2 days. In experiments detailed in **Figure 17** the following were added to co-coculture conditions: a) peptidoglycan (PGN, from *Staphylococcus aureus*), ultra-pure lipopolysaccharide (LPS, E. coli K12), flagellin (Flgn, from *S. typhimurium*), ODN 1826 (CpG, 5'-tccatgacgttcctgacgtt-3') at the indicated concentrations. All were purchased from Invivogen. In experiments detailed in **Figures 20 and 21**, various combinations of mAb including: α-IL-6 (MP5-20F3), α-IL-6R (D7715A7), α-IL12/23p40 (C17.8), α-IL27p28 (R&D) and/or α-IFN-γ (XMG1.2 or 11B11), or isotype controls α-IgG1κ (R3-34) and/or α-IgG2α (R35-95) were added at the initiation of cocultures in $Foxp3^+$ T_{reg} cell polarizing conditions containing CpG (10 μg/ml). All antibodies were purchased from BD Biosciences, excluding α-IL27p28, which was purchased from R&D Systems. On day 5, cells were surface stained with the following fluorochrome-conjugated antibodies: α-CD4 (RM4-5), α-$\alpha_4\beta_7$ (DATK32), α-CD25 (PC61.5), and the viability marker 7-AAD. $Foxp3^+$ cells were detected by eGFP expression and/or α–Foxp3 (FJK-16) following fixation and permeabilization with the kit provided by eBioscience in accordance with the manufacturer's protocol.

ELISA:

Upon harvest of coculture supernatants, IL-4, IL-10, IFN-γ and IL-17A were quantitated with the DuoSet® ELISA system (R&D Systems) per the manufacturer's instructions.

Gut flora DNA extraction:

Gut contents from the caecum and the colon of naïve C57BL/6 mice were collected and washed in cold PBS. The pellet was resuspended in lysis buffer (10 mM Tris/HCl, 50 mM EDTA, pH 8.0) containing lysozyme (0.5 mg/ml; Sigma-Aldrich). After incubation at 37°C for 2 h, 2 mg/ml of proteinase K and 1% SDS were added and the sample incubated at 60°C for 3 h. DNA was then purified by a series of 7 consecutive phenol-chloroform-isoamyl alcohol affinity extractions.

Intracellular cytokine staining of *ex vivo* isolated cells from naïve mice:

For assessment of $CD4^+$ T cells from WT $Foxp3^{eGFP}$ and *Tlr9*$^{-/-}$$Foxp3^{eGFP}$ mice, cell suspensions were enriched for T cells by incubating with CD90.2 positive selecting magnetic beads (Miltenyi Biotec) in accordance with the manufacturer's protocol. T cells were obtained by running the labeled suspension through an autoMACs™ on the program Posseld2. Enriched T cell suspensions were then mitogenically stimulated with phorbol 12-myristate 13-acetate (PMA, 50ng/ml) + ionomycin (5μg/ml) for 4 hours in U-Bottom 96-well plates containing complete (200μl/well) media and Brefeldin A (BFA, GolgiPlug™). Residual DMSO was then washed from wells and cells were incubated with 1/500 of Molecular Probes LIVE/DEAD® Fixable Blue Dead cell stain kit (Invitrogen) in HBSS according to the manufacturer's guidelines to distinguish healthy from dying cells, and subsequently fixed in 1.6% paraformaldehyde (PFA, Electron Microscopy Sciences) prior to staining intracellularly for cytokines. For intracellular cytokine staining,

cells were stained with the following fluorochrome-conjugated antibodies: α-TCR-β, α-CD4, α-CD8α, α-IFN-γ (XMG1.2), α-IL-17A (eBio17B7), α-IL-10 (JES5-16E3) or isotype controls: rat IgG1, rat IgG2a, ratIgG2b, and mouse IgG1 (clone P3) in the presence of α-mouse CD16/32 and 0.2mg/ml of Rat IgG for 45 min on ice in FACS buffer containing 0.5% saponin from Quillaja bark (Sigma).

E. cuniculi infection Protocol:

Parasite cultivation: A rabbit isolate of *E. cuniculi* was obtained from Waterborne Inc. and used throughout the study. The parasites were maintained by continuous passage in rabbit kidney (RK13) cells obtained from the American Type Culture Collection (ATCC #CCL37) and maintained as previously described (Bouladoux et al., 2003).

For infection: Spores were resuspended in sterile PBS, and immediately used for inoculation of mice or cell cultures. Mice were infected by intragastric gavage with 5×10^6 fresh spores in a volume of 200 μl. In some experiments, mice were infected i.p. using the same number of spores of *E. cuniculi*.

For $CD25^+$ cell depletion: C57BL/6 and *Tlr9*$^{-/-}$ mice were injected i.p. with 0.5 mg of anti-CD25 (clone PC61.5) or the corresponding isotype control (clone GL113) 3 days preceding and on day 0 and day 7 of infection.

Quantification of _E. cuniculi_ in tissues:

Duodenum (1cm piece starting at 3cm) and liver (weighed out piece from same lobe) were removed from infected mice and instantly snap frozen in liquid nitrogen. Tissues were next homogenized and then digested with proteinase K (Invitrogen or Qiagen).

DNA was subsequently extracted either with phenol-chloroform-isoamyl alcohol followed by ethanol precipitation or with the DNeasy® Tissue kit from Qiagen. Quantitative real-time PCR was performed in triplicates with 50 ng of total tissue DNA, the iQ SYBR Green Supermix (BioRad), and the following primers specific for a 268-bp DNA sequence of the SSU rRNA gene from *E. cuniculi* (Weiss and Vossbrinck, 1998): forward 5'-GTGAGACCCTTTGACGGTGT-3' and reverse 5'-CTCAGACCTTCCGATCTTCG-3'. Real-time PCR was conducted on a Bio-Rad iCycler under the following conditions: 3 min at 95°C; 40 cycles of: 45 s at 95°C, 60 s at 60°C, and 45 s at 72°C. Genomic DNA were extracted from known amounts of *E. cuniculi* with the QIAamp DNA stool mini-kit (Qiagen) and used as PCR standards. A standard curve was generated by linear regression on plotted cycle threshold (C_T) values of the standards against the logarithms of parasite numbers using iCycler iQ Optical System software (version 3.1; Bio-Rad).

In vitro recall assay to *E. cuniculi*

Single-cell suspensions of Sp, mln, IEL, Lp, and Pp of vaccinated or *E. cuniculi*-infected mice were prepared as described above. BMDC were generated as previously described (Lutz et al., 1999). Leukocytes (5×10^5) were incubated with 1×10^5 BMDC in 250 μl RPMI, 55 mM 2-β mercaptoethanol, and 10% FBS per well of a 96-well U-bottom plate. BMDC were previously incubated for 8 hrs with or without recombinant vaccinia virus expressing OVA protein (10 pfu / BMDC), *E. cuniculi* spores (parasite:BMDC ratio, 10:1) or OVA peptide (1 μg/ml) in the presence of 20 ng/ml GM-CSF (Peprotech) and washed before culture with leukocytes. After 3 days at 37°C in 5% CO_2, culture supernatants were collected for cytokine assays. IFN-γ, IL-4, IL-6, IL-10, and IL-17 were quantitated in culture supernatants of restimulated leukocytes using the DuoSet® ELISA system.

Antibiotic treatment:

3-wk or 8-wk-old C57BL/6 male mice were provided ampicillin (1 g/l), vancomycin (500 mg/l), neomycin trisulfate (1 g/l), and metronidazole (1 g/l) in drinking water as previously described (Rakoff-Nahoum et al., 2004). All antibiotics were purchased from Sigma-Aldrich. Where indicated, mice also orally received: 100 μg CpG ODN 1826 in sterile PBS (Coley Pharmaceutical) or 500 μg of extracted gut floral DNA in sterile water or 25 mg/kg of LPS from *Escherichia coli* (serotype 026:B6 from Sigma-Aldrich) once weekly from the start of the antibiotic course.

Chapter 4:

T cell phenotype:

Single-cell suspensions were incubated in ice-cold HBSS with 1/100 α-mouse CD16/32, 1/500 LIVE/DEAD® to exclude dead cells, and stained with the following fluorochrome-conjugated antibodies: α-TCR-β (H57-597), α-CD4 (RM4-5), α-CD8α (53-6.7), α-CD25 (PC61.5), α-CD44 (IM7), α-CD62L (MEL-14), α-CD103 (2E7), and α-$\alpha_4\beta_7$ (DATK32) for 15min on ice. For examination of transcription factors and cellular proliferation, cells were subsequently treated with the Foxp3 staining kit (eBioscience) in accord with the manufacturer's instructions and stained for 30-45 min on ice with Ki-67 (B56, BD Pharmingen), Foxp3 (FJK-16s), T-bet (eBio4B10) and/or RORγ(t) (AFKJS-9) or isotype controls: mouse IgG1 (BD Pharmingen) rat IgG2a (eBR2a), mouse IgG1 (clone P3) - in 1/100 α-mouse CD16/32 and 0.2mg/ml purified Rat IgG.

***E. coli* LT (R129G)Vaccine Protocol:**

For vaccination, mice were orally inoculated with an isotonic bicarbonate buffer (7.5%, 200μl/mouse). 10min later, mice were gavaged with a 200μl mixture of 1mg of OVA and

20 μg of the mutant form of *E. coli* LT (R129G) prepared in the same buffer. For reconstitution experiments with RA, mice were vaccinated again on day 4. Immune responses were then assessed on day 8. For all other experiments, mice were vaccinated once per week and immune responses were assessed one week after the final challenge.

***T. gondii* Infection Protocol:**

The parental ME-49 type II strain (ATCC no. 50840) (American Type Culture Collection, Manassas, VA, USA) of *T. gondii* was electroporated with RFP and selected for red fluorescence. ME-49 clone C1 was established and passed through mice. To obtain tissue cysts, brains were removed from C57BL/6 mice that were inoculated with three cysts by gavage 1–2 months prior and homogenized in 1 ml of phosphate buffer saline (pH 7.2). Cysts were counted on the basis of 2 aliquots of 20 µl. Diet study mice were infected orally with 10 cysts at 9.5-11.5 wks of age. STAg was prepared as previously described (Grunvald et al., 1996).

Parasite Burden:

Human fibroblast (Hs27; ATCC no. CRL-1634) cultures were used to quantify parasite burden as described previously (Pfefferkorn and Pfefferkorn, 1976) and (Roos et al., 1994). In brief, titrations of single-cell tissue suspensions ($1x10^4$ up to $1x10^6$ cells) were added onto confluent fibroblast monolayers cultured in DMEM (Sigma) supplemented with 100mg/ml Penn/Strep and 10% FBS (in 24-well plates). Plaques were detected by fluorescence using an Axiovert 40 inverted microscope (Zeiss) outfitted with an RFP filter. Titration results are reported in Plaque Forming Units (PFUs).

***In vivo* RA reconstitution:**

250μg of *all-trans*-RA (Sigma Aldrich), resuspended in 30μl of Biotechnology Performance Certified DMSO was administered intraperitoneally to vitamin A insufficient (VAI) mice every other day. 24hrs after the 3rd injection, mice were infected with *T. gondii* or vaccinated. Injection of RA continued until takedown of the mice. Mice not receiving RA, received DMSO vehicle instead. RA was stored at -80°C in pure DMSO in amber ependorf tubes. Aliquots were one use only.

Intracellular cytokine staining:

Tissues were harvested from infected or vaccinated mice, pooled by group, and enriched for T cells by incubating with CD90.2 selecting magnetic beads (Miltenyi) in accord with the manufacturer's protocol. T cells were obtained by running the labeled suspension through an Automacs (Miltenyi) on the program Posseld2. For *T. gondii*, T cells ($2.5x10^5$) were incubated with irradiated BMDCs ($5x10^4$) ± STAg (5mg/ml). For vaccine, T cells ($2.5x10^5$) were incubated with SpDC ($5x10^4$) ± OVA (100mg/ml) (Worthington). Cells were cultured for 14 hrs in an incubator at 37°C/5%CO_2 in 250μl/well in 96-well flat bottom plates. BFA was added for the final 7 hrs of culture. Cells were washed with FACS buffer and stained with 1/500 LIVE/DEAD® fixable Blue Dead cell stain kit in HBSS on ice for 15min. After washing 1X with FACS buffer, cells were fixed with 1.6% PFA for 20 min at room temp. For intracellular cytokine staining, cells were stained with the following fluorochrome-conjugated antibodies: α-TCR-β, α-CD4, α-CD8α, α-IFN-γ (XMG1.2), α-IL-17A (eBio17B7), α-Foxp3 (FJK-16), α-T-bet (eBio4B10), or isotype controls: rat IgG1, rat IgG2a, and mouse IgG1 (clone P3) in the presence of α-mouse CD16/32 and 0.2mg/ml purified Rat IgG for 45 min on ice in FACS buffer containing 0.5% saponin. All antibodies were purchased from eBioscience.

ELISA:

Tissues were harvested from infected or vaccinated mice, pooled by group, and enriched for T cells as described above. For *T. gondii*, T cells ($2.5x10^5$) were incubated with irradiated day-6 BMDCs ($5x10^4$) ± STAg (5mg/ml) in 250ml/well in 96-well round bottom plates for 48 hrs at 37°C, 5% CO_2. For vaccine, T cells ($2.5x10^5$) were incubated with day-7 BMDCs ($5x10^4$) that had been infected or not for 8hrs with recombinant vaccinia virus expressing OVA (MOI 10:1) for 72hrs at 37°C, 5% CO_2. Upon harvest of supernatants, IFN-γ and IL-17A was quantitated with the DuoSet® system. In initial vaccine experiments, pooled suspensions were not T cell enriched, but rather cultured at $5x10^5$ cells to $1x10^5$ infected or uninfected BMDCs.

$CD4^+$ T cell activation/ polarization assays:

Cells were extracted from the Sp and secondary LNs of *Rarα*$^{-/-}$ or littermate WT mice and enriched for $CD4^+$ $CD62L^{hi}$ T cells using the $CD4^+$ $CD62L^{hi}$ T cell isolation kit II (Miltenyi Biotec) in accordance with the manufacturer's protocol. For magnetic selection steps, cells were 40μm filtered and subsequently passed through manual columns. Cells were then CFSE labeled as described above, plated in complete media at $3.38\text{-}3.5x10^5$ cells/well into 96-well flat bottom plates, and stimulated with 1μg/ml of plate-bound α-CD3ε (BD Pharmingen, 145-2C11, coated over night in 100ml at 4°C) + soluble α-CD28 (BD Pharmingen, 37.51) + 50U of rhIL-2 (Peprotech). To assess activation, cells were harvested after 18hrs and stained for CD4, CD25, CD69 (H1.2F3), and CD71 (R17217) in HBSS containing 1/500 of the LIVE/DEAD® stain kit, 1/100 of blocking α-CD16/32, and 0.2mg/ml purified Rat IgG for 15 min on ice. Expression of markers was compared to unstimulated cells. To assess polarization, cells were further cultured in T_H-1 or T_H-17 (no rhIL-2) biased conditions, which included: purified α-IL-4 (11B11) + 10ng/ml of rmIL-

12; or purified α-IL-4 + α-IFN-γ (R4-6A2) + 20ng/ml rmIL-6 + 0.7ng/ml rhTGF-b, respectively. All cytokines, except hIL-2, were purchased from R&D Systems. 48hrs post-activation, half the supernatant from each well was removed and measured for the cytokines (IFN-γ and IL-17A) by ELISA. Complete media, including polarizing cytokines + blocking antibodies were replenished as cells were removed from αCD3ε and transferred into 96-well round bottom plates for 24hrs. CFSE proliferation was then measured.

Real-time quantitative PCR:

Naïve $CD4^+$ T cells were purified from splenic and peripheral LN of 2 mice/assay. RNA was then extracted with RNAeasy columns (QIAGEN) and analyzed by quantitative RT-PCR according to the manufacturer's instructions using primers for murine *Rara, Rarb,* and *Rarg* (QIAGEN).

Immunoblotting and Reagents:

Lysates were immunoblotted with the following antibodies: anti-phosphoSer473Akt and anti-Akt (Cell Signaling Technology).

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