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TET2 REGULATES HOMEOSTATIC TH2 IMMUNITY IN THE SMALL INTESTINE

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ABSTRACT

The intestinal immune system facilitates nutrient absorption in the presence of diverse commensal microbiota while establishing a protective barrier to prevent infection. Prototypically studied microbes induce specific immune programs and these models provide insight into how the immune system is regulated in this unique environment. Tritrichomonas species are protozoan symbionts that are common in many mouse facilities. These protozoa typically induce a type-2 immune program in the small intestine characterized by interleukin-25 (IL-25) signaling and secretory cell hyperplasia that is primarily mediated through the action of GATA3+ innate lymphocytes (ILC2s). However, unlike immunity to helminths, for which the type 2 immune program is evolved, this immune response is self-limiting; a state of tolerance is developed whereby the protozoa continue to occupy the lumen without continued immune activation or adaptive memory formation. We previously identified small intestinal barrier dysfunction correlated with increased IL-25 signaling in mice deficient for the DNA methylcytosine dioxygenase TET2. In these mice, Tritrichomonas colonization induces a population of long-lived adaptive CD4 T lymphocytes expressing GATA3 (Th2 cells) that chronically propagate this IL-25 circuit. Naïve lymphocytes typically require paracrine interleukin-4 (IL-4) from various innate populations for efficient Th2 differentiation in helminth infections. *Tet2*-deficient naïve cells are able to make increased autocrine IL-4, which results in Th2 polarization even in the absence of helminth induced innate activation. In a model of peanut allergy, loss of TET2 predisposed mice to anaphylaxis. Collectively, our findings formally demonstrate that a cell-intrinsic checkpoint can prevent exacerbated immune responses at homeostasis in the microbe- and stimulus-rich intestinal environment.

1 INTRODUCTION

1.1 The methylcytosine dioxygenase TET2

Ten-eleven translocation 2 is a DNA methylcytosine dioxygenase that plays a role in the methylation/demethylation cycle of cytosines. It was first discovered in the context of hematologic malignancies though has since been interrogated in multiple immune contexts.

1.1.1 DNA Methylation

DNA methylation is a widespread cytosine modification (5mC) in the genome and is maintained and removed through the concerted action of DNA methyltransferases and methylcytosine dioxygenases. Members of the DNA methyltransferase (DNMT) family catalyze cytosine methylation of cytosines (5mC) present in the context of 5'-CpG-3' through a conserved catalytic domain. DNMT1 is thought to be important in preserving the methylation mark during DNA replication, when the methylated sites necessarily become hemi-methylated after the synthesis of the daughter strand. DNMT3 enzymes are responsible for *de novo* methylation, usually in the context of development or differentiation. Generally speaking, DNA methylation represses transcription when the CpG dinucleotides are concentrated in promoters called CpG islands. This is either by directly preventing binding of transcription factors or by methyl binding proteins, which can actively recruit other mechanisms of gene regulation, such as recruiting histone modification enzymes.[1]

DNA methylation marks can be lost during cell division through passive demethylation or they can be actively oxidized through the activity of TET enzymes. The first step in this process creates 5-hydroxymethylcytosine (5-hmC) and these marks, like 5mC, were observed several decades ago. The characterization of TET proteins and their catalytic function have resulted in

renewed interest in investigating their roles in gene regulation.[2] 5-hmC can then also be progressively oxidized to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC).[3,4] The demethylation from this point either occurs in passive fashion during replication in which 5-fC- and 5-caC-CpGs are not recognized as methylated and therefore the daughter strand is not methylated by DNMTs. Active demethylation can also occur when 5-fC and 5-caC are removed by DNA glycosylases replaced by base excision repair enzymes.

1.1.2 Roles of TET2 in physiology and disease

TET2 mutations are common in both myeloid leukemias and lymphoid leukemias though the progression of these malignancies is distinct.[5] Clonal hematopoiesis of indeterminate potential (CHIP), first alluded to in the late 1990s[6] and formally described in the last decade[7–10], is the phenomenon by which competitively advantageous somatic mutations in hematopoietic progenitors manifest as allelic mosaicism in the peripheral blood and hematopoietic compartments. The initiating or causative agent for mutations is largely unknown but is likely a combination of environmental stressors and germline susceptibility.[11,12] The most common CHIP mutations show a significant overlap with mutations identified in myeloid leukemias, such as *DNMT3A* and *TET2*[13], and this has led to the hypothesis that CHIP represents a precursor stage for age associated myeloid leukemias.[5] *TET2* mutations are also present in lymphoid malignancies, primarily in acute lymphoid leukemias (ALL). These can be of T cell or B cell origin and are usually not age associated.

TET2 has been studied in various cell types and cell-specific roles for both its catalytic and non-catalytic functions have been described. Myeloid cells deficient for *Tet2* tend to have pro-inflammatory phenotypes, and this in part was shown to be mediated through differential

recruitment of histone deacetylases.[14] Interestingly, *Tet2* deficiency in CD8 T cells also resulted in a proinflammatory phenotype with increased cytokine production and degranulation in the context of chronic viral infection.[15] Regulatory T cells (Treg) require TET function for stabilization of the lineage defining transcription factor Foxp3 and loss of multiple TET enzymes results in inflammation and lymphoproliferation.[16] Further roles for TET2, particularly in type 2 immune effectors are discussed below.

1.1.3 Motivations for present work and study

My thesis work was built on findings made previously in the Jabri Laboratory regarding barrier dysfunction in the context of Tet2 deficiency and how this potentially contributes to systemic malignancy.[17] *Tet2*-deficient mice are characterized by an age-dependent myeloproliferation phenotype that resembles a chronic myeloproliferative neoplasm.[18–20] In our mouse facility, and in other mouse facilities, it was observed that myeloproliferation was variably penetrant in *Tet2*$^{-/-}$ mice. For example, in a given cage, one could observe littermate *Tet2*$^{+/+}$ mice with no myeloproliferation, *Tet2*$^{-/-}$ mice with myeloproliferation and intriguingly, *Tet2*$^{-/-}$ mice that phenocopy the wildtype mice in all measured hematologic parameters. In mice with myeloproliferation, live bacteria could be cultured in the periphery, and these bacteria were driving inflammatory processes that were required for myeloproliferation Antibiotic treatment reversed this phenotype as did anti-inflammatory treatment with anti-IL6. These defects were traced to changes in gene expression and permeability of the small intestine, particularly the jejunum. Interestingly, treating the myeloproliferation with anti-IL6 did not impact the intestinal phenotype and barrier defects were preserved, suggesting the peripheral immune activation and myeloproliferation could be mechanistically decoupled from the intestinal barrier changes.

Further, providing bacterial ligands in the periphery was sufficient to drive myeloproliferation in symptom-free *Tet2*$^{-/-}$ mice, but this treatment did not induce barrier changes, suggesting distinct bacterial signals were required for myeloproliferation and intestinal barrier dysfunction.[17] My focus for this thesis is primarily on the intestinal phenotype of these mice, but I have also worked a bit on pre-malignant models of *Tet2*-deficiency, especially on how they relate to the hypothesized progression of myeloid malignancies in patients. The findings below reflect my work on understanding the intestinal phenotype.

1.2 Functional units of the intestinal immune system

1.2.1 Physical organization and barrier integrity

The regional and histological organization of intestinal tissue serves to separate digestive functions as well as immune functions. The intestine is broadly divided into the small bowel and the colon. The small bowel serves primarily for nutrient uptake and digestion, whereas the large bowel serves to reabsorb water and minerals. Interestingly, the small bowel has much lower microbial presence than the large bowel, an opposing gradient to their respective digestive functions. In both mice and humans, the small bowel is significantly longer, allowing for maximum absorption of nutrients during transit.[21] The small bowel is further divided proximally to distally into the duodenum, jejunum and ileum.

Microscopically, the functional unit of the intestine is the villus or a finger like projection of tissue into the lumen. The villus is lined luminally with epithelial cells, which are separated via a basement membrane from the underlying lamina propria. The epithelial cells serve to further increase surface area digestion with membrane features called microvilli that resemble a brush. The large intestine, however, does not have villi or microvilli, which is in line with its minimal

digestive function. At the base of villi are crypts, which contain epithelial stem cells and other specialized cells. The stem cells give rise to developing epithelial cells that progressively move 'up' the villus to replace extruded cells. As they develop, epithelial cells can acquire specialized fates and functions that can be dictated by immune signaling on progenitors. The specialized cells are also distributed differently across regions. For example, goblet cells are predominantly found in the colon and Paneth cells are predominant in the distal small intestine.[21] Below the epithelial layer is the lamina propria that is characterized by connective tissue, blood vessels, lymph drainage and neurons. The lamina propria and epithelium contain the majority of the immune cells and serve distinct immunological functions. Below these two layers is the submucosa that is characterized by its plexuses of nerves and thick muscles involved in peristalsis. This basic structuring of the small intestine is visualized in Figure 1.

The intestinal tissue must strike a balance between promoting efficient digestion and priming of immune responses and the maintenance of a selective barrier. The intestinal barrier is formed by specialized protein interactions between epithelial cells, and this barrier is dynamically regulated during the course of immune responses as the expression of involved genes can change. These tight-junction associated proteins can be divided into several subgroups including claudins, peripheral plaque proteins like ZO1 and other proteins associated with the tight junction like occludin.[22] The relative roles for these proteins in maintaining barrier integrity are difficult to distinguish as genetic models using mice are embryonic lethal or show complex phenotypes beyond intestinal barrier dysfunction. This is somewhat unsurprising, because mutations in these proteins have also been documented in humans and they are usually unlinked to disease or linked to complex disorders that go beyond the gastrointestinal system.[22] Thus, understanding how these

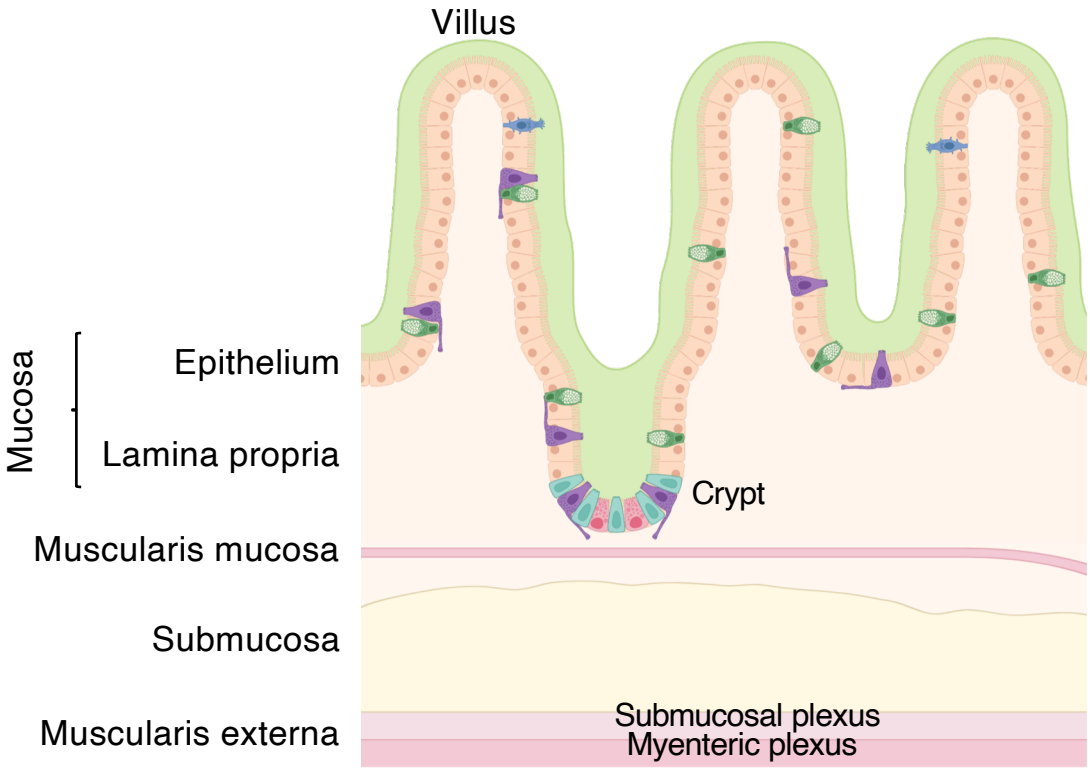

Figure 1. Microanatomy of the intestine

The intestine can be subdivided broadly into the mucosa and submucosa separated by the muscularis mucosa and bounded by the epithelial surface luminally and the muscularis externa basally. The mucosa consists of the epithelium, which coats the entire luminal surface from the villus to the crypt and over lymphoid structures such as Peyer's patches. Enterocytes and epithelial cells of specialized lineages such as tuft cells, goblet cells, Paneth cells, M cell compose the epithelial layer and serve specific functions. These cells are interspersed by intra-epithelial lymphocytes, which have function in both homeostasis and disease. The lamina propria consists of more diverse immune populations as well as stromal cells, which together help support the epithelium and more broadly the immune and digestive functions of the intestine. The submucosa consists of lymphatic vessels that drain intestinal lymphocytes and metabolites to secondary lymphoid organs such as lymph nodes and blood vessels that bring lymphocytes to the intestine and carry some nutrients and signals to the portal circulation. The muscularis layers promote motility functions of the intestine during digestion and these actions are coordinated by the submucosal and myenteric plexus, critical components of the enteric nervous system.

proteins function at homeostasis and in the context of barrier dysfunction remains an important question.[23]

1.2.2 Lymphoid organs and tissues

The functional units for induction and propagation of intestinal immune responses primarily involve Peyer's patches along the length of the intestine and mesenteric lymph nodes that drain the various segments. These structures are critical for homeostatic immunity, such as the responses to dietary antigens, as well as immunity against pathogens. Peyer's patches contain abundant B cell follicles with surrounding T cells and are characterized by M cells on their luminal surface.[21,24] Peyer's patches are a predominant source of IgA plasma cells that reside in the small intestine, which can be predicted from ongoing germinal center reactions that occur at these sites.[25] Mesenteric lymph nodes drain the intestine in segments[26,27] and their immune composition and activation largely reflect immune processes occurring in their corresponding tissue. As with other tissues, dendritic cells migrate to lymph nodes to prime naïve T cells to adopt effector programs. For example, Esterhazy and colleagues demonstrated clear segregation between where immune responses are mounted to different microbiota or pathogens and found them to be largely specific to the region where the microbe or pathogen colonizes.[27]

1.3 Type 2 Immunity

The immune system can be conceptualized as a collection of programs specialized to function in different pathogenic contexts. It is thought type 1 immunity is specialized for viral and intracellular pathogens, type 2 immunity is specialized for large parasitic and helminth pathogens and type 3 immunity is specialized for extracellular bacteria and fungi. In this section, I will introduce some of the effector cell types of type 2 immunity, and when relevant, will reference identified functions

of TET2 in these cell types. The primary focus will be the regulation of the lineage defining transcription factor GATA3 and common cytokines involved in type 2 immunity followed by discussion of the cell types that express these factors.

Type 2 immune effectors are of both myeloid and lymphoid lineages. Myeloid effectors include mast cells, basophils, and eosinophils as well as specially programmed macrophages and dendritic cells. Mast cells are tissue resident myeloid cells that are poised to release vasoactive mediators to aid in initiating immune responses. They are primarily sensitive to activation via the FcεRI, which binds to IgE. IgE-bound to allergens will crosslink multiple Fc receptors and result in potent release of preformed mediators by mast cells.[28] Basophils are circulating myeloid cells that share many characteristics of mast cells including developmental origins. They express many of the same cytokines though they have a distinct morphology and lifespan.[28,29] Eosinophils are present in both the blood and peripheral tissues and are characterized by the presence of abundant granules that contain cationic proteins like major basic protein, which function to attack large extracellular parasites.[28]

Dendritic cells are professional antigen presenting cells that are essential for priming adaptive T cell responses. They integrate a variety of signals from the tissue environment to ensure priming of appropriate effector programs and their function in type 2 immunity is further discussed in the Th2 section. Macrophages are phagocytic cells that can be either tissue resident or derived from circulating monocytes. They are somewhat like dendritic cells in that the tissue environment dictates their polarization and function. Myeloid cells have some of the highest expression of *Tet2* and some roles of *Tet2* in macrophages has already been discussed. Mastocytosis, or the expansion of mast cells, is a myeloproliferative neoplasm in which *TET2* mutations cooperate with oncogenic

KIT mutations to drive hyperproliferation and pathology associated with accumulations of mast cells.[30–32]

Lymphoid effectors include T-helper type 2 (Th2) cells, immunoglobulin E (IgE) class-switched plasma B cells, natural killer T type 2 (NKT2) cells and type 2 innate lymphocytes (ILC2s). Within the lymphoid compartment, Th2 and IgE+ plasma cells are considered adaptive as they are elicited with epitope or antigen specificity whereas NKT2s and ILC2s are innate and are pre-poised to produce effector cytokines. In B cells, TET2 appears to be required for efficient transit through germinal centers and class switch recombination, the immune processes through which B cells develop their avidity and affinity to immunogenic antigens and the process which results in mature antibody secreting plasma cells.[33] The functions of TET2 in Th2 cells and ILC2s will be discussed in their respective sections.

Th2 cells and ILC2s are defined by the expression of the transcription factor GATA3, although this expression is acquired in different contexts. Although GATA3 is required for and expressed in developing lymphocytes, it is further upregulated in Th2 cells and ILC2s to enable their effector function.[34–38] In Th2 cells, it is upregulated upon specific immune challenge and stimulation while it is linked to development for ILC2s. In addition to a dose-dependent regulation, the diverse functions of GATA3 are also accomplished through differential binding.[39] Through analyzing the ChIP-Seq profiles of GATA3 in different lymphocyte lineages and the use of a *Gata3* deletion model, Wei and colleagues identified cell type specific gene regulation mediated by GATA3. For example, GATA3 regulated critical components of signaling pathways required for T cell fate decision and development in the thymus such as *Zbtb7b* (Th-POK), *Runx1*, *Notch1*, and components of the TCR complex.[39] In Th2 cells, GATA3 binding was responsible for both gene

activation and repression of T-helper specific programs through the modification of histones.[39] GATA3 targets and function were primarily characterized in the context of development and Th2 cells, but after the characterization of ILC2s, GATA3 was also shown to be critical in ILC development and function as well as ILC2 survival.[40–43] Recently, a specific regulatory region downstream of *Gata3* was identified as an enhancer that was primarily required for the efficient development and function of ILC2s while having less pronounced effects in Th2 induction and function.[44] These data suggest the activity of GATA3 in different cell types even within the type 2 program is further controlled in a cell-type specific manner, which can be somewhat inferred from the different functions of these cell types but the mechanistic basis continues to be investigated.

The primary effector cytokines of type 2 immunity are IL-4, IL-5, and IL-13 and their transcription ultimately requires the expression of *GATA3*, but studies of their transcriptional control identified several other regulatory mechanisms.[45–54] Much like is the case for GATA3, many of the seminal findings involving regulation of this locus were made in Th2 cells and there are likely additional levels of regulation that are different in ILC2s, which will be discussed below. All three cytokines are located in the Th2 cytokine locus which is located on chromosome 5 in humans and chromosome 11 in mice.[55] *Il4* and *Il13* are adjacent and transcribed in the same direction, whereas *Il5* is positioned on the other end of this locus and is transcribed in the opposite direction.[56] The expression of this cytokine locus is controlled by a locus control region (LCR) in the 3' end of the gene *Rad50* which is located between the *Il5* locus and the *Il4* and *Il13* loci[57] as well as by several DNase I hypersensitivity (HS) sites located within (RHS) and outside (HS) of this LCR.[58] Differential binding of transcription factors at these sites results in positive and

negative regulation of the gene loci. For example, there are RHS sites that are only bound by GATA3[39] or STAT6[59], whereas others are bound by both and the Th1 transcription factors Runx3 and Tbet negatively regulate *Il4* transcription by binding to the HS IV site[60,61]. In addition to direct binding by transcription factors, this cytokine locus is also controlled through epigenetic modification of histone methylation and acetylation as well as DNA methylation. The methyltransferases MLL and Ezh2 control activating H3K4 methylation and repressive H3K27 methylation in Th2 and Th1 cells respectively to maintain cell fate by mediating proper repressive and activating marks around the *Il4/Il13* and *Ifng* loci.[62–65] In fact, a primary role of GATA3 in addition to its direct transcription factor activity for this cytokine locus, seems to be in mediating histone modification. When GATA3 is deleted from Th2 cells, ~5% of GATA3 bound Th2 genes have altered expression but almost half of GATA3 bound Th2 genes have altered histone modification.[39] DNA methylation is also an important mechanism of control for the expression of these signature cytokines. *Il4* is demethylated during Th2 commitment and deletion of methylation machinery, such as DNA methyltransferase 1 (Dnmt-1) or methyl CpG-binding domain protein 2 (MBD2), causes aberrant *Il4* expression.[66–68] Thus, the Th2 cytokine locus is ultimately controlled coordinated action of signature transcription factor expression as well as modification of epigenetic states.

In addition to transcriptional control, there is also specialized expression of cytokine receptors. IL-4 and IL-13 both signal through IL-4Rα but have distinct receptor complexes. IL-4Rα is widely expressed with many cell types expressing low levels, but the restricted expression of the second binding chains dictates sensitivity to IL-4 and IL-13.[69] IL-4 can signal through the type I IL-4 receptor complex consisting of IL-4Rα and the common gamma chain (γc) or the type

II IL-4 receptor complex consisting of IL-4Ra and IL-13Rα1.[70] The type I IL-4 receptor complex is primarily formed in hematopoietic cells, whereas the type II IL-4 receptor complex is more widely expressed. IL-4 first binds to IL-4Rα and then will complex with one of the above secondary chains. IL-13 also has two possible receptors but is distinct in that it can initially bind with one of two different chains, IL-13Rα1 and IL-13Rα2.[69] The bound IL-13-IL-13Rα1 complex recruits IL-4Rα forming the type II complex as described above. The role of the IL-13Rα2 is still the topic of investigation and functions beyond just being a decoy receptor have been described. The type 1 and type 2 complexes have both shared and distinct signaling outcomes. The chain-associated Jak kinases become activated via phosphorylation and create docking sites for intracellular signaling molecules, namely STAT6 and IRS, which further control the cellular response through gene and protein regulation.[69,70] It is important to recognize that these receptors also can have distinct signaling outcomes. For example, only the type I receptor can induce IRS molecules and which can subsequently activate signaling pathways that are independent of STAT6 such as PI3K, Akt, PKBE and mTOR.[69–72]

The functional diversity conferred by receptors and proximal signaling is concordant with different outcomes observed in genetic models of cytokine deficiency. IL-4 is typically required for Th2 induction (discussed below) and the class-switch of IgE and IgG1 plasma cells.[73] IL-13 primarily acts on myeloid cells and non-hematopoietic cells, such as epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts and is often involved in orchestrating tissue responses to type 2 insults.[74]. Both can cause alternative activation of macrophages, because macrophages are unique in that they can express both complexes.[75,76] Further unique roles are observed in helminth infection models. In *Nippostrongylus brasiliensis* infection, IL-4 deficient

animals were able to clear the worm efficiently in a similar time frame as immunocompetent mice[77], but IL-4Ra deficient mice and IL-13 deficient mice were greatly impaired in their clearance of this pathogen.[78–80] This is not common to all infections, however—both cytokines appear to play important roles in *Trichuris muris* infection models.[74] These unique targets and functional outcomes are the subject of extensive study as their roles in various type 2 pathologies, such as allergy, asthma, and atopic dermatitis need to be studied in order to develop the proper and specific biologics to modulate pathology.[81–83]

IL-5 in addition to other chemokines primarily serves to recruit eosinophils to the relevant tissue[84,85] as well as regulate their development and survival.[86] IL-5 has primarily been investigated in the context of asthma where eosinophils are prominent and clinical trials showed efficacy of IL-5 blocking therapeutics in the context of eosinophilic asthma.[87,88] More recently, novel roles of eosinophils at homeostasis have been uncovered. For example, in white adipose tissue in mice, IL-4 produced by eosinophils is important for reconstituting alternatively activated macrophages and contributing to glucose homeostasis. In the absence of eosinophils, mice were less tolerant of a high-fat diet, which could be reversed through helminth-induced eosinophilia.[89]

In addition to the prototypical cytokines encoded at the *Il4/5/13* locus, additional groups of cytokines have been linked to type 2 responses. One such group is primarily released by epithelial cells, and these include IL-25, IL-33 and TSLP.[86] These are considered alarmins as they are typically released in the context of immune activation. The specific functions of some of these cytokines on Th2 cells and ILC2s is highlighted below, but I will focus primarily on IL-25. IL-25 (IL-17E) and its receptor (IL-17RB) was first identified as a member of the IL-17 family that induced NF-kB activity.[90] It was subsequently identified to induce significant expression of other

Th2 cytokines and type 2 associated pathologies in the lung and GI tract when injected *in vivo*, a very distinct function when compared to other members of the IL-17 family.[91] Various cell types, both immune and non-immune across diverse tissues have been described to produce IL-25, but the functional role of this expression is not always well-understood. However, IL-25 has been associated with several disease pathologies in diverse tissues and therefore has remained a potential target of interest for therapeutics.[92]. In the lungs, airway brush cells have been characterized to produce IL-25 in response to sensing of cysteinyl leukotrienes upon inhalation of allergens and this drives subsequent type 2 inflammation.[93] This pathway is reminiscent of pathways regulated by tuft cells in the small intestine, discussed below in the context of type 2 immunity in the intestine.

1.3.1 T-helper type 2 cells: Differentiation and function

Initial work characterizing CD4 T cell clones found two dominant programs characterized by distinctive cytokine expression, IFNγ for Th1 and IL-4 and IL-13 for Th2.[94] Since this initial characterization several important details regarding Th2 induction, signaling and function have been elucidated along with the recognition of further specialized subclasses of T helper cells. After the identification of Th2 cells, it was discovered that they could be differentiated *in vitro* by recapitulating various requirements for effective induction: T-cell receptor stimulation and co-stimulation (or chemical analogues) along with critical cytokine signals provided by IL-4 and IL-2 result in upregulation of GATA3 and establish a Th2 program.[95,96]

TCR stimulation is provided by the presentation of peptide antigen in the context of major histocompatibility complexes (MHC). The major antigen presenting cells for T helper differentiation are dendritic cells, presenting peptide in the context of MHC-II. This is common to

all T helper lineages, but it was identified early on that the strength of this signal may impact differentiation outcomes. Early work in the laboratory of Kim Bottomly found naïve CD4 T cells of the same specificity differentiated into a Th1 lineage and produced IFNγ when exposed to high doses of antigen, but low doses of the same antigen caused Th2 differentiation and the production of IL-4.[97] Work in the O'Garra laboratory found intermediate antigen doses promoted Th1 differentiation while very low or very high doses promoted Th2 differentiation,[98] but these differences can be explained by different experimental ranges—both groups found low concentration stimulated Th2 but the high concentration in the Bottomly laboratory could have been considered intermediate by the O'Garra group. Additional biochemical studies identified low extracellular regulated kinase (Erk) activity was important for early IL-4 expression in naïve CD4 T cells and sustained Erk activation resulted in Th1 differentiation.[99] This effect could be reversed with inhibition of Erk activity, further demonstrating the importance of quantitative signaling differences in T helper polarization.[100] Low affinity peptides were found to result in early IL-4 transcription within 48 hours of priming through differential activation of NFATs, suggesting qualitative differences also result from the quantitative difference in TCR signal.[101] In *Schistosoma mansoni* infection, dendritic cell activation was suppressed by pathogen derived antigens and this resulted in lower antigen presentation and conjugation with T cells, ultimately resulting in IL-4 production and Th2 differentiation.[102] Using intravital imaging to assess T cell interactions with dendritic cells, Germain and colleagues varied the adjuvant and antigen doses to determine relative contributions to T cell polarization. They found longer T cell-DC interactions, stronger calcium flux and larger synapse sizes in the presence of Th1- versus Th2-promoting adjuvants.[103] However, in the presence of the same adjuvants, differences in antigen concentration were able to overcome

adjuvant-associated antigen presentation and T cell polarization differences with low concentrations of peptide resulting in Th2 responses even in the presence of Th1-promoting adjuvants and vice versa. Adjuvants were primarily responsible for differences in costimulatory molecules on dendritic cells, whereas antigen dose determined differential cytokine receptor expression on interacting T cells, suggesting antigen dose dependent signal strength is a primary determinant of the polarization capacity of naïve T cells.[103]

A second requirement for efficient differentiation is co-stimulation. This classically occurs through CD28 expressed on T cells, but various other co-stimulatory molecules have also been identified. CD28 ligation is required for effective Th2 priming *in vitro*[104], and CD28 deficient mice have defective Th2 responses as do mice injected with CTLA4[105,106], which blocks this costimulatory pathway. The *in vitro* defect was found to be mediated through IL-2 production and signaling (discussed below) as exogenous IL-2 could override the requirement for CD28 ligation[104], however whether this mechanism holds *in vivo* is unclear. Another costimulatory interaction, OX40/OX40L, was found to promote *in vitro* IL-4 expression by naïve T cells isolated from mice[107] and humans[108]. Additionally, dendritic cells that are primed to increase expression of OX40L are more efficient inducers of Th2 polarization[109]. Thus, signaling through the TCR as well as co-stimulation are both important aspects of determining T helper fates.

A third requirement for T helper polarization is a cytokine signal that helps upregulate helper-specific programs, so-called signal 3. For Th2 cells, this is IL-4. IL-4 exerts its effect through the type I IL-4 receptor complex, which transduces signal through phosphorylation of STAT6, which can in turn bind to GATA3, the defining transcription factor for the Th2 program.[45,110–112] STAT6 is necessary and sufficient for driving Th2 differentiation, though IL4

and STAT6 independent Th2 induction has been demonstrated *in vivo* and *in vitro*.[113–115] *In vivo*, sources of IL-4 include basophils[113,116–118], NKT cells[119], and naïve CD4 T cells[120,121] themselves, but surprisingly not dendritic cells, which are capable of making other signature polarizing cytokines such as IL-12 and IL-6.[122] Importantly, early IL-4 signaling induces increased IL-4Rα expression on CD4 T cells in a positive feedback loop that potentiates further signaling.[37]

IL-2 is a product of activated T cells that serves primarily as an autocrine cytokine. IL-2 binds to the IL-2 receptor and induces activation of STAT5, which is necessary and sufficient to induce Th2 differentiation[123–125]. IL-2 signaling, as discussed above, is a mechanism by which co-stimulation through CD28 promotes Th2 differentiation.[104] Further, low dose TCR stimulation, which is known to favor Th2 differentiation, induces expression of IL-2 and its receptor early in differentiation, suggesting another mechanism by which IL-2 is important in Th2 fate.[100] The outcome of these various signaling pathways during Th2 differentiation is the induction of GATA3, which is necessary and sufficient for Th2 polarization.[45] Further, GATA3 is required to maintain cytokine producing capabilities of Th2 cells as well as the epigenetic landscape of Th2 cells, suggesting GATA3 is also an important regulator of Th2 identity.[35,39,126] Interestingly, deletion of GATA3 in Th2 cells or ectopic expression of GATA3 in non T cells impacted IL-5 and IL-13 production significantly more than IL-4 production, suggesting an additional level of regulation beyond GATA3 in the cytokine function of Th2 cells.[45,47,83,127]

Once Th2 cells develop, their effector function is dictated by location and stimulation. For example, using mice that were able to report transcript and protein expression of IL-4 simultaneously, Mohrs and colleagues found that although IL-4 competent cells (that expressed *Il4* transcript) could be identified disseminated across tissues in worm-infected mice, IL-4 protein

was only secreted at sites where antigen was present, suggesting functional regulation of IL-4 production. Further, IL-4 protein producing cells reverted to an IL-4 competent state after antigen was removed and were again poised to produce protein after re-stimulation.[128] Although Th2 cells are broadly defined by their three signature cytokines and early theories predicted coordinated expression of these cytokines, early single cell analysis had already shown heterogeneity in which cytokines were expressed by individual Th2 cells.[129,130] Using reporter strains, Locksley and colleagues were able to segregate these functions further and attribute them to the physiology of the type 2 response. Using models in which IL-4 or IL-13 producing cells were specifically deleted, they first confirmed the importance of these cytokines for developing a humoral response and clearing *Nippostrongylus brasiliensis*, respectively.[38] Interestingly, deletion of IL-13 expressing cells also led to a decrease in IL-5 and eosinophilia, suggesting these cytokines are coregulated in this system. Further, IL-13 expressing cells were restricted to non-lymphoid tissues, which is in line with the fact that IL-13 receptors are primarily expressed in the non-hematopoietic compartment.[38] In the lungs, although there were some co-expressing CD4 T cells, the larger proportion were single expressers, further suggesting selective cytokine expression from the common Th2 locus.[38]

A critical function of type 2 immunity, as observed in various genetic models, is the production of an IgE humoral response, which requires class switch in the germinal center reaction. IgE class switching is accomplished through the production of IL-4 primarily by T follicular helper (Tfh) cells. In the same cytokine reporter models as above, IL-4 production in the lymph nodes during worm infection was primarily from GATA3 non-expressing cells, whereas IL-4 expression correlated with GATA3 expression in the tissue, suggesting bona fide Th2 cells express IL-4 in

the tissue, while lymph node IL-4 is primarily produced by Tfh cells. Although Tfh cells do not classically express *GATA3*, conditionally GATA3-deficient mice still fail to develop an IgE response.[54] However, when GATA3 is specifically deleted in IL-13 producing cells, effector Th2 and ILC2s, antibody responses are preserved.[38] This suggests Tfh cells may have a lower dependence on GATA3 for efficient IL-4 production, but there remains some role for GATA3 in establishing a Tfh population. If and how Tfh and Th2 cells have a common precursor during the course of the immune response is an open and important question as Tfh cells adopt a unique transcriptional and epigenetic state that is marked by the expression of BCL6 and the inducing signals for this program are incompletely understood. [131]

Effector helper cells primarily respond to TCR stimulation to produce cytokines, but also have been known to respond to cytokine signals. Intriguingly, many of these cytokines are of the IL-1 family and are able to signal through their respective STAT proteins in effector CD4 populaitons. IL-18 has been shown to activate Th1 cells, and IL-1β has been shown to activate Th17 cells. For Th2 cells, IL-33.[132] *In vivo* and *in vitro* differentiated Th2 cells express high levels of IL-33 receptor, ST2. Expression or function of this receptor was found to be important for development and function of an antigen-specific Th2 response to *Schistosoma mansoni* infection and in an allergic context in the lungs but did not appear to have an effect at homeostasis or in in vitro Th2 differentiation.[133,134] Recently, the Paul group posited the expression of this receptor on effector Th2 cells was important for their function in heterologous protection. *In vitro* and *in vivo* differentiated Th2 OTII cells produced IL-13 in response to IL-33. This response to IL-33 was dependent on ST2 but not on MHCII, suggesting effector cytokines can be produced in the absence of cognate antigen.[135] Further, in mice that had formed a Th2 memory compartment in response to

worm infection, house dust mite injections induced type 2 inflammation independently of MHCII but dependent on IL-33. Finally, when mice were serially infected with two unrelated nematodes, prior infection-induced CD4 T cells were necessary and sufficient to confer protection against secondary infection.[135] These data suggested that IL-33 confers Th2 cells the ability to promote immunity in an antigen-independent manner. The function of IL-25 signaling on Th2 cells is less clear and somewhat controversial. The expression of IL-25 receptor on Th2 cells is not as clear as the expression of ST2. In fact, small intestinal ILC2s are the highest expressers of *Il17rb*, the receptor for IL-25 (Immgen), although *Il17rb* transcripts were also shown to be expressed in naïve and Th2 cells. Initial characterization of IL-25 linked its signaling with the establishment of Th2-type responses. This was prior to the characterization of innate lymphocytes, however, and many of those initial phenotypes are now recognized to be mediated through the action of ILC2s (discussed below). IL-25 was shown to promote Th2 differentiation of naïve T cells *in vitro* by Dong and Foster, but Wang suggested the effect of IL-25 was primarily on differentiated Th2 cells.[136–138] Another group demonstrated IL-25 could induce Th2 differentiation of naïve human CD4 T cells.[139] However, using genetic models of IL-4 and IL-25 deficiency, the Le Gros group demonstrated IL-25 was dispensable for *in vivo* Th2 induction and for primary or secondary immunity against *Nippostrongylus brasiliensis*.[140] These data suggest IL-25 may have different roles with respect to Th2 cells in different contexts and further investigation is required.

Two separate groups have published on the role of *Tet2* in T-helper differentiation. The Dong group assessed the effect of *Tet2* deletion on classical *in vitro* T helper polarization. They first described DNA methylation changes at signature genes for different effector programs and thus decided to assess the role of active demethylation of these genes by TET2, the most

abundantly expressed demethylase in effector CD4 cells. They found *Tet2*-deficiency impaired Th1 and Th17 differentiation but not Th2 differentiation.[141] Anjana Rao and colleagues sought to determine the absolute role for active demethylation in Th2 differentiation by using Tet-triple deficient CD4 T cells and cells deficient in the enzyme required for base excision after demethylation, TDG. Interestingly, they found when all three Tet enzymes were knocked out, IL-4 production was impaired after Th2 differentiation in their culture system, in contrast to what was found when polarizing cells deficient in only *Tet2*.[141,142] This suggests there may be some compensatory role of the other TET enzymes when *Tet2* is absent in IL-4 production. However, mice deficient for TDG had no deficiencies in Th2 differentiation, suggesting active demethylation was dispensable for this differentiation and the TET enzyme-oxidized previously-methylated loci were passively diluted. In their culture systems and genetic models, they did not observe differences in GATA3 expression, suggesting acquisition of the Th2 program was not entirely impaired and in line with what Dong and colleagues reported as well as what we have found.[141,142] Importantly, both of these studies were carried out in the context of classical Th2 polarization where exogenous IL-4 was supplemented in the media. The work I will describe below describes a mechanism found in a non-polarizing culture system that assessed IL-4 production by naïve CD4 T cells.

1.3.2 Type 2 Innate lymphocytes: Signaling and function

ILC2s were formally described first in the early 2010s, though earlier studies alluded to a non-B/T lymphoid population poised to produce type 2 cytokines. In particular, it was found that administration of IL-25 was able to result in IL-5 and IL-13 production even in a *Rag2-/-* host that lacks adaptive lymphocytes.[91,143] This population was also found to be important for the expulsion

of helminth parasites.[144] In 2010, several independent groups formally characterized this population in adipose tissue, the small intestine and lymphoid tissues.[145–147] A few years later, an IL-33 responsive ILC2 population was also characterized in the lung. Since these initial studies, several salient properties of ILC2s have been described, including their dependence on the transcription factor GATA3[40–42] and developmental trajectory and how it relates to other ILC populations.[148,149]

As has been observed with tissue resident T cells, ILC2 programs are in part imparted by their tissue of residence. Locksley and colleagues profiled ILC2 populations from various tissues and found distinct transcriptional programs and surface cytokine receptor expression that was dictated by the tissue in which the ILC2 resided and presumably by the type of cytokine signal it could receive in that location. For example, lung resident ILC2s were characterized by ST2, skin resident ILC2s expressed IL-18R1 and small intestinal ILC2s express IL-25R.[150] Importantly, this tissue-specific program was not pre-encoded, but rather established after the ILC2 had seeded a tissue. Interestingly, some of these signals (*e.g.,* IL-25, IL-33 and TSLP) were not required for seeding, but rather for function, suggesting there are specific signals that are microbiota independent that tissues use to impart ILC2 identity.[150] The contribution of neonatal generated ILC2s and adult generated ILC2s in homeostasis and inflammation varies between different tissues.[151] For a long time, it was thought that once an ILC2 has seeded a tissue, it remains at that site and serves its role as a poised producer of type 2 cytokines. Parabiosis studies confirmed this fact and there was little intermixing between tissue resident ILC populations.[152] However, through two important studies, permanent tissue residency of ILC2s was challenged and revised to appreciate the role of migrating ILC2s during inflammation. Using markers of tissue resident

ILC2s, it was observed in the context of helminth infections that tissue ILC2 migrate via the blood to distal sites and have a role in priming systemic type 2 immunity.[153,154] The fact that ILC2s have been described to express a broad array of receptors that encompass several cytokine families and metabolites as well as somewhat controversial expression of MHCII further supports their potential role as key arbiters of Th2 immunity.[155] Using a mouse with impaired ILC2 differentiation and function, it was demonstrated that ILC2s were important for establishing inflammatory responses to some type 2 challenges but not others, demonstrating ILC2s have different functions depending on context.[44]

The role of TET enzymes in ILCs was investigated by profiling methylation and hydroxymethylation patterns in ILC subsets. Unsurprisingly, differentially methylated regions were enriched for genes that defined the identity and function of various ILC subsets.[156] Using *Tet2*$^{-/-}$ mice, the Colonna group found that cytokine production was somewhat impaired in ILC2s, but was impaired in the recently described inflammatory ILC2s that can produce IL-17. Thus, they described a role for TET2 in promoting the functional plasticity of inflammatory ILC2s but did not extensively explore its function in canonical ILC2s.[156] Interestingly, small intestinal ILC2s are among the highest expressers of *Tet2* (Immgen, unpublished analysis), which may reflect its role in the functional plasticity that Colonna and colleagues describe or may represent a function that is still unknown.

1.3.3 Type 2 immunity in the intestine

Many distinct changes are observed in the organization and function of intestinal tissue when a type 2 immune response is established, and these changes are primarily accomplished through the action of effector cytokines. IL-5 serves to recruit eosinophils to the tissue where they can help

expel parasites through the secretion of major basic protein. IL-13 remodels the tissue architecture through smooth muscle hyperplasia and by inducing the development of secretory and sensory epithelial lineages such as goblet cells and tuft cells, respectively. These two changes encompass the so-called 'weep and sweep' response, where increased mucus production and smooth muscle contractions help expel large parasites out of the gastrointestinal system.

Tuft cells are increasingly being recognized as serve as important regulators of immune responses. Although they were identified several decades ago, their roles in physiological processes are still being investigated. In 2008, tuft cells were found to express the transient receptor potential channel TRPM8 as well as several other taste receptor and genes involved in the biosynthesis of neuronal and inflammatory mediators such as lipid derived molecules and *Il17e*.[157] In 2016, three different reports identified a key role for this tuft cell derived IL-25 in mediated immunity to helminths and protists.[158–160] Locksley and colleagues used an IL-25 reporter and characterized epithelial cells at various surfaces, especially tuft cells in the GI tract, as primary producers of homeostatic IL-25, but not hematopoietic cells.[160] In helminth infection models, they observed an increase in tuft cells that was dependent on IL-13 signaling, which they sourced to ILC2s. With these data, they were able to propose a tuft-ILC2 circuit that was important in establishing effective type 2 immune responses. This was exemplified by the fact that deletion of IL-25 from the epithelium impaired clearance of *Nippostrongylus brasliensis*.[160] Another group, led by Wendy Garrett, showed this circuit was also relevant to homeostatic responses to protists found in the microbiota.[158] It is now recognized that there are tuft cell subtypes characterized by unique receptors ad biosynthesis pathways through which they have the capability to interface with diverse cell types and signaling circuits.[161] For example, in addition to production of IL-25,

intestinal tuft cells produce leukotrienes in the context of worm infection, which activate ILC2 cytokine production via NFAT signaling and drive clearance of pathogens.[162,163] The sensing of environmental stimuli by tuft cells is known to be important for their cytokine production and initiation of inflammatory responses, but the mechanism of this sensation has not been described in all contexts.[161] The most well-characterized is the sensing of succinate produced by protists in the commensal microbiota (described below). In the context of helminth infections, however, the initiating signal is not known.

Goblet cells are secretory cells that also differentiate from epithelial stem cells in response to type 2 cytokines. They are also present in various barrier surfaces, including the ocular surface, upper respiratory tract, and the intestinal epithelium. At steady state, they produce mucus that provides a physical barrier in the intestinal lumen. Goblet cells also promote transfer of dietary antigens from the lumen through use of goblet cell associated passages, although the role of this function in immune activated states is not well known.[164] They are more present at steady state in the colon, but type 2 immune responses induce their differentiation through the action of type 2 cytokines on stem cells in the small intestine.[160] The enhanced production of mucus in conjunction with smooth muscle hyperplasia promote clearing of worm infections. Unlike tuft cells, goblet cells have not been shown to produce cytokines, although some studies have described cytokine mRNA in goblet cells.[164] Importantly, the increased mucus production in type 2 activated intestines makes them very difficult to profile by isolating cells from the epithelium or lamina propria compartments and there is a significant amount of cell death of immune and non-immune cells.[165]

The intestine has been recognized as a potential site for the initiation of food allergy or the intolerance towards food antigens that results in type 2 activation, because it is the major route of

exposure to food antigens. The default reaction to ingested food antigens is tolerance, and this was first described in 1911[166], and this can be observed practically by the fact that humans fail to develop significant immune responses to most of the food antigens consumed during the lifespan. This phenomenon is now known to be mediated by the action of regulatory T cells as well as other passive forms of tolerance.[167,168] Additional sites implicated in allergy initiation are the skin and the respiratory epithelium. The loss of tolerance is further influenced by the type of immune response that is mounted. For example, in celiac disease, a Th1 response is mounted against the dietary antigen gluten, and this response is thought to be influenced by viral infections and interleukin signaling that promote a type 1 response.[169,170] To model food allergy in mice, adjuvants are necessary to prime an inflammatory Th2 response against the provided antigen. This usually comes in the form of two bacterial toxins: enterotoxin B or cholera toxin. This results in the formation of a Th2 response as well as high affinity IgE and IgG1 antibodies specific to the food antigen.[171,172] Exposure to food antigen after this response has developed leads to degranulation of mast cells via crosslinking of antibody receptors and release of vasoactive mediators that result in systemic anaphylaxis. Anaphylaxis is not always manifest in patients, however, many symptoms of food allergy may be more localized to abdominal discomfort and intestinal pathology driven by local immune activation. Regardless, the study of food allergy and the contributing factors is important as allergies are increasingly common.[173]

1.4 Commensal microbiota

1.4.1 Prototypical models and major findings

The commensal microbiota contains species from multiple kingdoms and collectively has been implicated in physiology and pathology of both mice and humans. The estimated species and

organism abundance of the microbiota far outpaces the host in terms of cell number and has led to the evolution and development of complex host-microbiota interactions. The development of next generation sequencing and gnotobiotic facilities in combination with classical microbiology has allowed the identification and characterization of several members of the commensal microbiota as well as direct interrogation of their roles in host physiology. Members of the microbiome interact with the host digestive and immune system in a myriad of ways including through the production of digestive and/or immunomodulatory metabolites or direct modulation of host tissues through close interactions and immune activation.

An extensively studied phenomenon is the production of butyrate and other short chain fatty acids by the microbiome. The fermentation of dietary fibers and starch by the microbiota produces millimolar quantities of short chain fatty acids in the intestinal lumen that have been demonstrated to have local and systemic effects on the host.[174] Butyrate and other SCFAs can provide energy to directly to epithelial cells by contributing to the TCA cycle. They can also alter transcription and thus function in local immune cells by serving as inhibitors of histone deacetylation.[175] Distal effects have been also described. Strikingly, many neurological phenotypes have been found to be altered by short chain fatty acids that either directly act on brain cells or via peripheral signaling that ultimately impacts brain function.[176]

Direct immune activation by the microbiota is well appreciated through the profiling of germ-free animals versus conventionally raised animals. Further, the use of mono-association models has revealed specific adaptive responses in response to specific microbes. One of the first such antigen specific circuits was described in the context of segmented filamentous bacteria (SFB), which were found to induce a Th17 response in the ileum by attaching to the epithelial

surface.[177,178] This process of inducing an antigen specific response to this one member in a complex microbiota required several critical steps including signal amplification through the coordinated action of diverse cell types.[178–180] It is unsurprising, therefore, that such highly specific responses are rare and only a handful of examples of such specificity have been described.[181] It is metabolically costly to produce such highly specific responses, especially given the high diversity of microbes and other antigen sources in the intestine. There have been prototypical models described for the induction of microbiota specific CD8 T cell, Th1, Treg and Tfh responses in the intestine, but not for Th2.[182–185]

1.4.2 Tritrichomonas species

Tritrichomonas species have been characterized in various animal models and human populations and their roles in host physiology likely vary with protozoa subspecies as well as tissue localization.[186,187] Recent studies have elucidated their roles in physiology and pathology. In 2016, three different groups described the role of these protists in physiological processes. Mallevaey and colleagues identified these symbionts as exacerbating agents in a T-cell transfer model of colitis.[188] Merad and colleagues demonstrated colonization with *Tritrichomonas* induced IL-18 production by epithelial cells in the colon that drove Th1 and Th17 responses, which were pathogenic in the context of colitis but protective in the context of infection.[189] The Garret group described induction of tuft cell hyperplasia to be dependent on these protists and demonstrated this was primarily mediated through the production of effector cytokines by ILC2s. Additionally, they observed the IL-25 response induced by these microbes was downmodulated after persistent colonization despite no changes in the amount of protist.[158] These findings are most relevant for our studies and are roughly visualized in Figure 12A. These protozoa reside in the lumen of the

intestine and ferment dietary fibers. This results in the liberation of metabolites, one of which is succinate.[190,191] Tuft cells, as described above, have the capacity to sense many environmental metabolites, including succinate. In 2018, three different groups integrated these observations and showed succinate produced by protists or bacteria can activate tuft cells and initiate type 2 immune responses. Intriguingly, this was shown again to primarily be mediated by the action of ILC2s.[192–194] The consequences of this activation for general type 2 immunity was alluded to in a model where overactivation of the circuit conferred protective immunity in the context of helminth infection, but these effects continue to be studied.

2 TET2 REGULATES INTESTINAL TH2 RESPONSES

2.1 Summary

Our laboratory has previously characterized a model of *Tet2*-deficiency where we observed intestinal barrier defects that led to hematologic pathology.[17] We further investigated the intestines of these mice and identified a strong IL-25 signaling signature exemplified by the presences of tuft and goblet cells. This signature was microbiota dependent, and we found *Tritrichomonas spp*, a member of the commensal microbiota, to be sufficient to induce IL-25 signaling, as previously described. Interestingly, although this circuit was able to be induced in both wildtype and *Tet2*-defeicient mice, we observed there was an eventual downregulation in wildtype mice that was impaired in *Tet2*$^{-/-}$ mice and resulted in persistent remodeling. We profiled the immune cells in these mice and found a striking increase in Th2 cells that was dependent on and induced by *Tritrichomonas*. These Th2 cells were required for persistent activation of this IL-25 circuit, and their induction was dependent on IL-4. *In vitro* culture systems showed a specific propensity for Th2 differentiation in the absence of definite polarizing signals and this was confirmed through

transcriptional analysis. Further, when exogenous IL-4 was provided to wildtype animals in conjunction with *Tritrichomonas*, we found this combination of signals to be sufficient to induce a strong Th2 population as observed in *Tet2*$^{-/-}$ mice. Th2 induction was comparable in helminth infection models, suggesting a specific role for *Tet2* in responding to type 2 inducing commensal microbes. The persistent IL-25 signaling in *Tet2*-deficient mice was associated with intestinal barrier dysfunction and we demonstrate a clear role for the IL-25/IL-13 circuit in mediating barrier function. Further, mice with barrier function were susceptible to allergic pathology in a model of peanut allergy and this susceptibility was dependent on IL-25.

2.2 Results

2.2.1 Tet2-deficient mice have a Tritrichomonas-dependent Il25 signature

To understand the intestinal changes observed in *Tet2*$^{-/-}$ mice better, we re-visited our regional gene expression dataset that highlighted the jejunum as the site with the most differentially expressed genes. The intestinal tissue composition is known to vary depending on predominant immune signals, and therefore, we chose to assess the composition of our bulk-sequenced tissue using gene set enrichment analysis.[195–197] Using published cell profiles from intestinal single-cell datasets, we found enrichment of a tuft and goblet cell signature specific to the jejunum of *Tet2*$^{-/-}$ mice (Figure 2A).[198] We confirmed this signature with immunofluorescent staining for DCLK1+ tuft cells and periodic acid shift (PAS) staining for goblet cells, both of which were increased in the jejunum of *Tet2*$^{-/-}$ mice (Figure 2B,C). Tuft and goblet cell hyperplasia in the small intestine has been described in the context of helminth infections and other type 2 challenges that drive IL-25 and IL-13 signaling[160], and indeed both *Il25* and *Il13* transcripts were elevated in *Tet2*$^{-/-}$ mice (Figure 2D). We previously found intestinal changes in *Tet2*$^{-/-}$ mice were dependent on the

microbiota and broad-spectrum antibiotic treatment reversed tuft and goblet cell enrichment signatures along with *Il25* in the jejunum of *Tet2*$^{-/-}$ mice (Figure 2D, E). In fact, antibiotic treatment reversed most gene expression differences observed in the jejunum of wildtype and *Tet2*-deficent mice (Figure 2F,G). Accordingly, germ-free *Tet2*$^{-/-}$ mice do not demonstrate an increased tuft cell and *Il25* signature by qPCR (Figure 2H). These data suggest the significant remodeling of jejunal tissue in *Tet2*-deficient mice is dependent on the microbiota.

Tritrichomonas species are protozoan commensals that induce tuft and goblet cell hyperplasia through *Il25* signaling and are common in the SPF microbiota of many mouse facilities, including at the University of Chicago.[186] We identified a protozoan that shared significant sequence similarity in the 28s ITS region with previously described species of *Tritrichomonas* (Figure 3A). Colonization of *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ mice with in-house isolated *Tritrichomonas* for 4-6 weeks was able to induce a significant upregulation of *Il25* and a tuft and goblet cell signature in the jejunum and colonized *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ equally (Figure 3B). When aged beyond 16 weeks of age, we found Tet2$^{+/+}$ mice had largely downregulated this axis, but *Tet2*$^{-/-}$ mice had a persistent *Il25* signature compared to littermate colonized *Tet2*$^{+/+}$ mice despite equivalent levels of *Tritrichomonas* in the microbiota (Figure 3C). We next assessed whether the continued presence of *Tritrichomonas* was required for this axis by treating chronically colonized mice with metronidazole, which efficiently depletes anaerobic bacteria as well as protozoa[187]. Metronidazole-treated *Tet2*$^{-/-}$ mice no longer had an elevated *Il25* and tuft cell signature, suggesting the continued presence of *Tritrichomonas* is required for persistent IL-25 signaling (Figure 3D). Collectively, we demonstrate *Tet2* is a host genetic factor that regulates the intestinal response to a commensal protozoan.

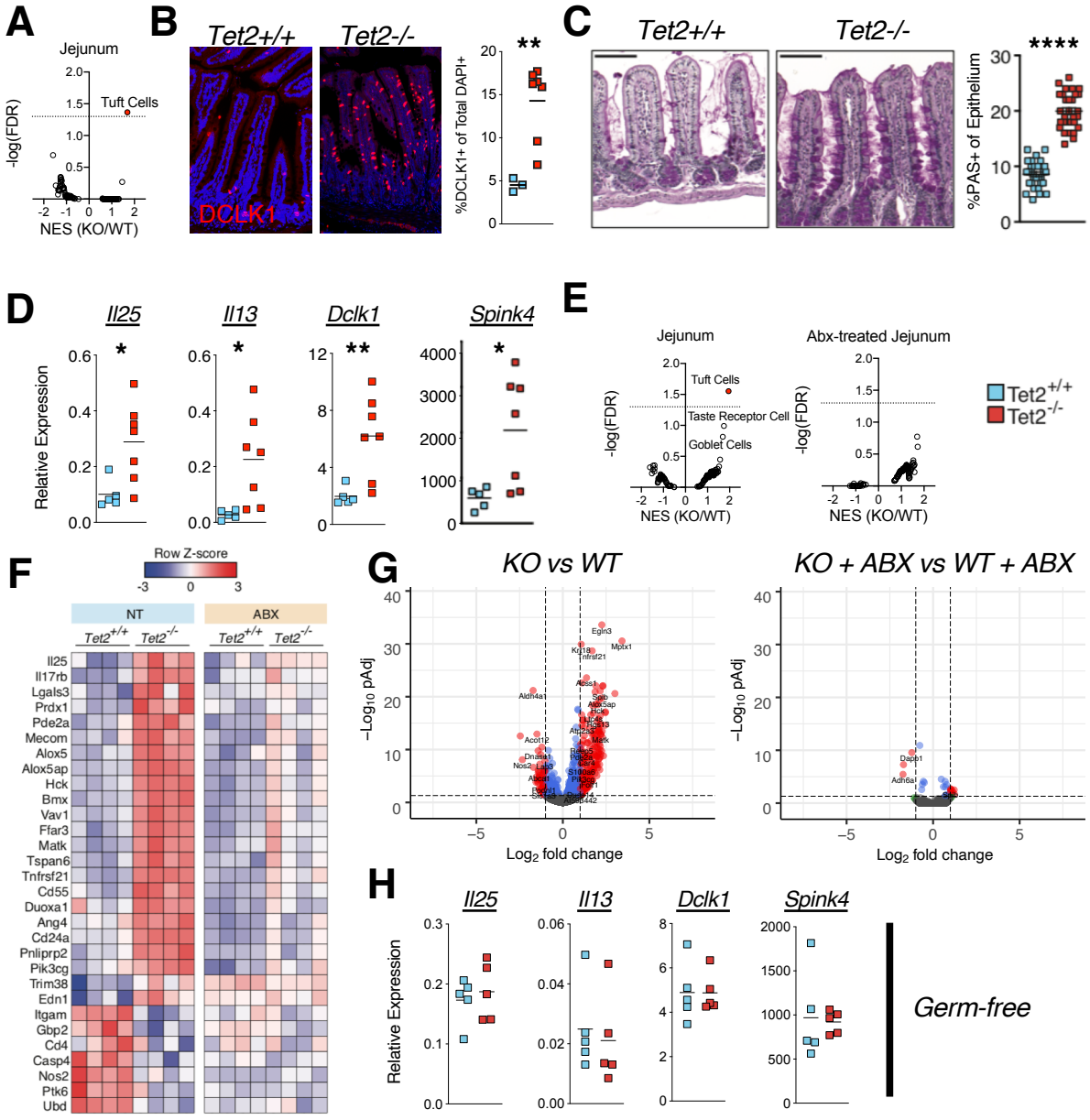

Figure 2. Microbiota-dependent IL-25 signature in the absence of *Tet2*

A. Results from GSEA for various cell types plotted as enrichment values over wildtype and FDR with significance cutoff (<0.05) indicated with the dashed line. B. Tuft cell immunofluorescence staining and quantifications. C. Goblet cell staining with periodic acid Schiff stain and quantification. D. RT-PCR of selected genes from jejunal tissue. E. GSEA results for untreated and antibiotic treated jejunum as in A. F. Heatmap curated from top differentially expressed genes that were microbiota dependent. G. Volcano plots demonstrating gene expression changes at steady state and upon antibiotic treatment of mice. H. RT-PCR of selected genes from jejunum of germ-free animals. (*,**,***,****: p<0.05, 0.01, 0.005, 0.001)

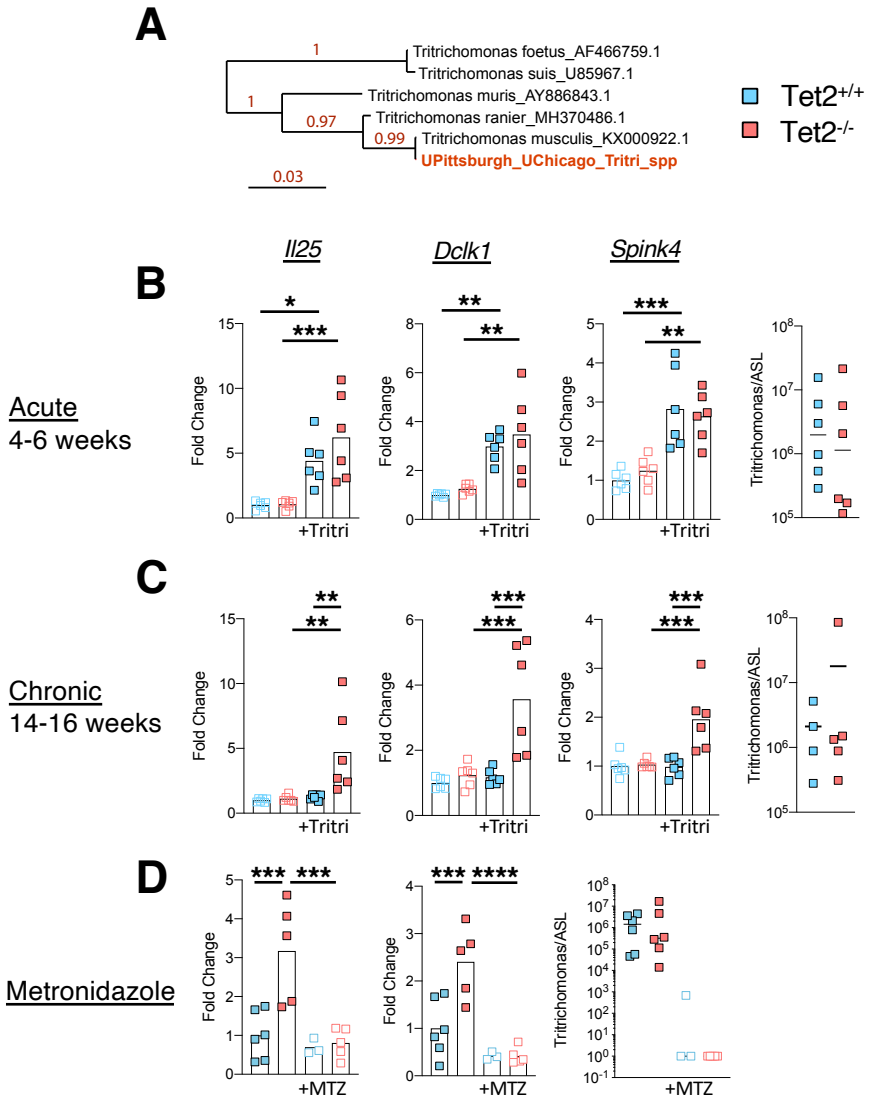

Figure 3. Tritrichomonas-dependent IL-25 signaling in acute and chronic colonization

A. The 28S ITS region was sequenced from purified Tritrichomonas protists and phylogenetic analysis was conducted using published sequences of protists. B-C. RT-PCR of selected IL-25 signaling genes in the jejunum at the acute and chronic time points along with PCR amplification results of Tritrichomonas genomes relative to host in colonic contents. D. RT-PCR of IL-25 signaling genes in the jejunum after 4 week treatment of colonized mice with 1g/L metronidazole. (*,**,***,****: $p<0.05$, 0.01, 0.005, 0.001)

2.2.2 Tet2 deficiency results in a long lived Th2 population and Il25 signaling

To assess which cell type(s) were responsible for regulating this host response, we crossed *Tet2*$^{fl/fl}$ mice with mice expressing Cre-recombinase under different promoters. We observed *Il25* upregulation relative to littermates with hematopoietic (*Vav1*-Cre), but not with epithelial (*Vil*-Cre) deletion of *Tet2*, suggesting that although tuft cells are the primary producers of IL-25 in the intestine[160], there was an immune mechanism that determined tuft cell differentiation and persistent signaling. Further, CLP-derived lymphocyte specific (*hCD2*-Cre)[199] deletion of *Tet2*, but not myeloid (*Lysm*-Cre) compartment deletion was sufficient for elevated *Il25* (Figure 4A). Interestingly, *Tet2*$^{fl/fl}$ *Il5*-Cre mice, which primarily have recombination in ILC2s[160], also did not have upregulated *Il25*, suggesting TET2 regulates this circuit in an ILC2-extrinsic manner, unlike what was recently described for the negative regulator A20 (Figure 4A).[193] Accordingly, the innate lymphocyte compartment in *Tet2*$^{-/-}$ *Rag2*$^{-/-}$ mice was not sufficient to drive increased *Il25*, suggesting adaptive lymphocytes in *Tet2*$^{-/-}$ mice drive constitutive IL-25 signaling (Figure 4B). We profiled the lamina propria compartment in chronically colonized *Tet2*$^{-/-}$ and littermate *Tet2*$^{+/+}$ along with uncolonized mice and found a significant increase in CD4+ GATA3+ FOXP3- Th2 cells in *Tritrichomonas*-colonized *Tet2*$^{-/-}$ mice (Figure 5A). A significant portion of GATA3-expressing cells in the small intestine co-express FOXP3, and this population has been shown to regulate homeostatic type 2 immune responses at barrier sites[200,201], but this subset was not different upon *Tritrichomonas* colonization or between genotypes (Figure 5A). The strong type 2 profile in the small intestine was also accompanied by an increase in eosinophils, but not by a change in proportions of ILC2s, further suggesting Th2 cells were primary drivers of this response (Figure 5B). The *Tritrichomonas*-dependent Th2 population was also present in *hCD2*-Cre mice,

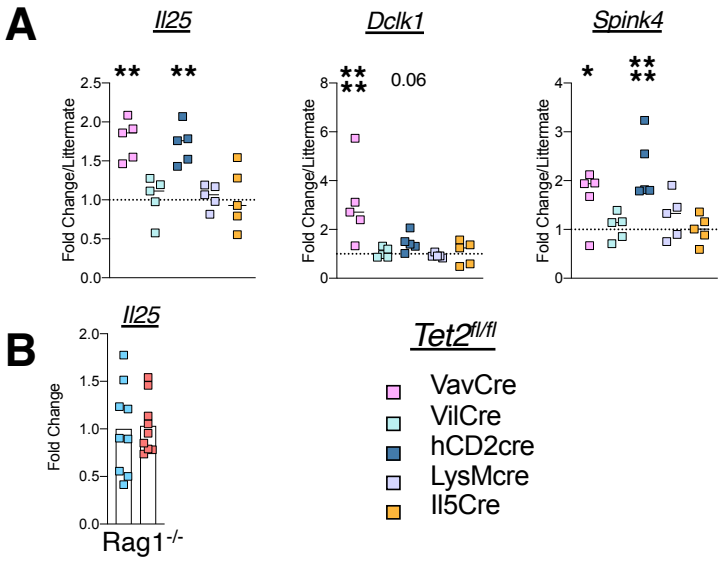

Figure 4. Chronic IL-25 signaling is a associated with *Tet2* function in lymphocytes

A. RT-PCR for IL-25 signaling genes in the jejunum from different Tet2-deficient mice. B. Il25 expression in Rag1-/- vs Rag1-/- Tet2-/- mice. (*,**,***,****: $p<0.05$, 0.01, 0.005, 0.001)

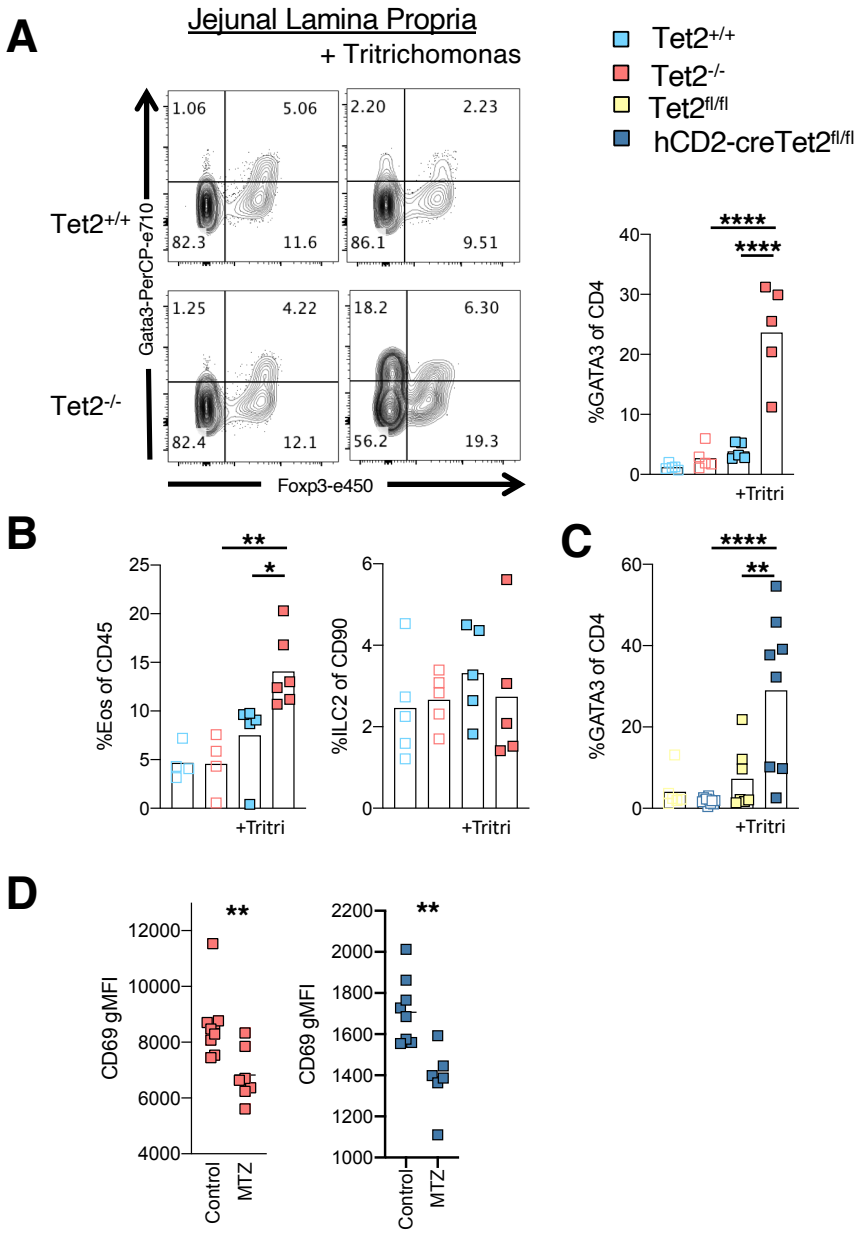

Figure 5. Tritrichomonas induces an adaptive Th2 response in the absence of Tet2

A. Quantification of Th2 frequency among CD4+ CD8a- TCRb+ CD90+ CD45+ Live cells from lamina propria samples after chronic colonization with *Tritrichomonas*. Representative FACS plot. B. Quantification of ILC2s and eosinophils as a frequency of CD45+ Live cells from jejunum LPL samples C. Quantification of Tritrichomonas dependent CD4 Th2 cells in the hCD2-Cre background. D. Mean fluorescent intensity of surface CD69 expression on Th2 cells from *Tet2*-deficient mice or *Tet2*$^{fl/fl}$ hCD2-Cre mice after 4 weeks of 1g/L metronidazole administered ad libitum in drinking water. (*,**,***,****: $p<0.05, 0.01, 0.005, 0.001$)

confirming that lymphocyte specific deletion of *Tet2* gave rise to a unique Th2 population (Figure 5D). Finally, when we depleted *Tritrichomonas* in either *Tet2*$^{-/-}$ or *Tet2*$^{fl/fl}$ *hCD2*-Cre mice, we observed a decrease in expression of CD69, a marker for lymphocyte activation and tissue residency.[202,203] These data suggest a *Tritrichomonas*-responsive CD4 Th2 population is selectively induced upon *Tet2* deficiency.

We next wanted to investigate whether this Th2 population interfaced in the intestinal IL-25 circuit as has been described for ILC2s. Depletion of CD4+ cells in *Tet2*$^{-/-}$ and in *Tet2*$^{fl/fl}$ *Vav1-Cre* abrogated *Il25* expression suggesting the Th2 cells were responsible for persistent IL-25 signaling in these mice (Figure 6A). To test whether this Th2 population also propagated the tuft cell circuit through IL-13 production as has been described for ILC2s, we depleted IL-13 and found that the IL-25 signaling associated signature was downregulated (Figure 6B). These data indicated we had a *Tritrichomonas*-induced Th2 population that propagated the IL-25 signaling circuit in the jejunum via the production of IL-13. We next deleted *Tet2* specifically in the CD4 compartment and found CD4-intrinsic *Tet2*-deficiency sufficient to drive this Th2 population and persistent IL-25 signaling (Figure 6C). When we blocked IL-25 to assess whether the Th2 population, as is the case with ILC2s, was dependent on tissue derived cytokine, we found a decrease in Th2 cells, suggesting IL-25 is an important survival factor for these cells (Figure 6D). These data suggest that when an innate lymphocyte-microbiota circuit is co-opted or replaced by an adaptive response, persistent tissue remodeling and signaling ensues in response to an otherwise tolerated commensal.

2.2.3 Tet2 regulates CD4 IL-4 production and Th2 differentiation

We next sought to understand why *Tet2*$^{-/-}$, but not *Tet2*$^{+/+}$, mice were able to mount an adaptive Th2 response in the context of *Tritrichomonas*. Th2 cells classically require IL-4 for induction and

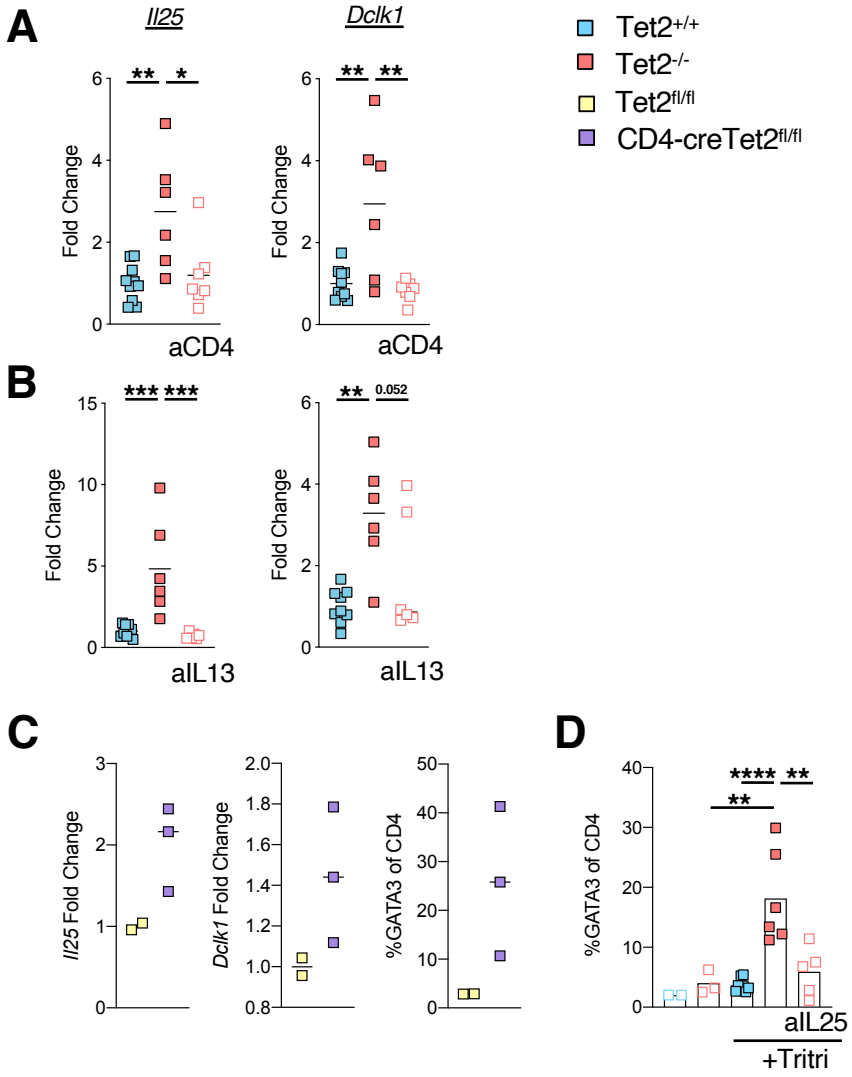

Figure 6. Adaptive Th2 responses propagate IL-25/IL-13 signaling in *Tet2*-deficient mice and CD4-intrinsic *Tet2* mediates this circuit.

A-B. RT-PCR for *Il25* and *Dclk1* in jejunum of mice depleted for CD4 (A) or with IL-13 blockade (B). C. RT-PCR for *Il25* and *Dclk1* in jejunum of CD4-Cre *Tet2 fl/fl* mice and littermates along with frequency of Th2 cells among CD4+ CD8a+ TCRb+ CD90+ CD45+ Live lymphocytes D. Quantification of Th2 frequency in mice treated with IL-25 blocking antibody. (*,**,***,****: $p<0.05$, 0.01, 0.005, 0.001)

in vivo blocking of IL-4 with neutralizing antibody prevented the induction of *Tritrichomonas*-induced Th2 cells in *Tet2*$^{-/-}$ mice (Figure 7A). These data, along with the finding that CD4-intrinsic deletion of *Tet2* sufficiently drove a Th2 population suggested differential IL-4 signaling or upregulation in *Tet2*$^{-/-}$ CD4 T cells. To test this hypothesis, we used an *in vitro* culture system and first cultured naïve CD4 T cells under Th2 polarizing conditions using recombinant IL-4. Under these conditions, *Tet2*$^{-/-}$ and *Tet2*$^{+/+}$ CD4 T cells equally differentiated to GATA3+ CD4 T-cells, which is in line with previous studies[141] and suggests the functional response to extrinsic IL-4 is not changed by the deletion of *Tet2* (Figure 7B). As extrinsic IL-4 did not differentially induce Th2 cells and lymphocyte-intrinsic deletion of *Tet2* was sufficient to drive Th2 differentiation *in vivo*, we assessed whether the CD4 T cells themselves produced IL-4, as has been described in certain contexts and genetic backgrounds.[100,120] When we cultured naïve CD4 T cells under non-polarizing conditions in which only TCR stimulation and IL2 were provided, we surprisingly saw *Tet2*$^{-/-}$ CD4 T cells had the capacity to make IL-4 and this IL-4 was necessary to drive polarization of GATA3+ cells without the need for additional exogenous signals (Figure 7B, C). The ability to make the Th1 hallmark cytokine interferon-γ (IFNγ) was not different, suggesting a specific dysregulation of IL-4 production (Figure 7B, C). To investigate the mechanism of how Th2 polarization occurred in the absence of exogenous IL-4, we performed a time course analysis of naïve CD4 T cells cultured under non-polarizing conditions. In our secondary cultures, we confirmed acquisition of Th2 program and saw a significant upregulation of GATA3 target genes, including several Th2 effector cytokines, in *Tet2*$^{-/-}$ cells (Figure 8D).

Strikingly, we also identified several genes that were already differentially expressed in unstimulated cells and in cells during the course of TCR stimulation that had not yet upregulated

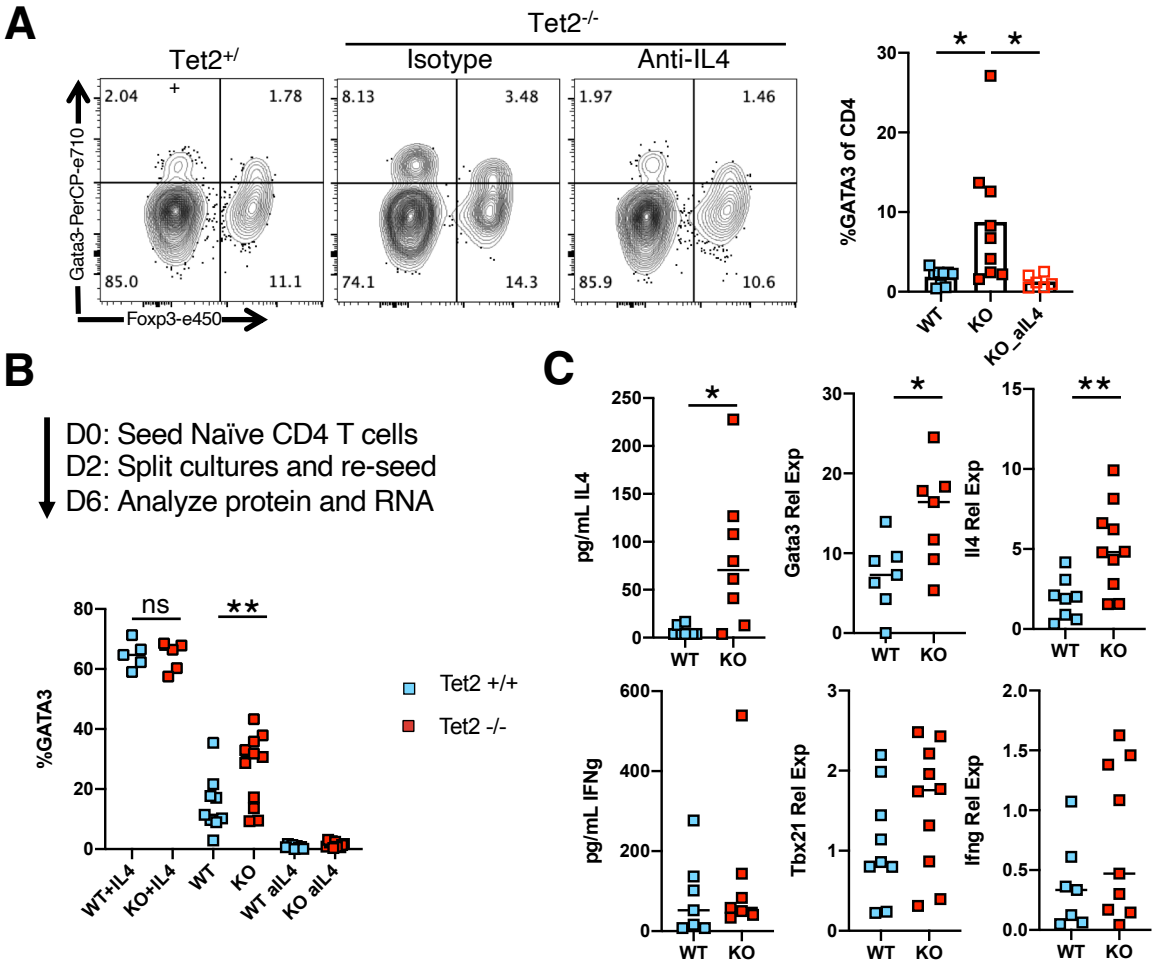

Figure 7. Intrinsic acquisition of a Th2 program

A. Th2 populations in WT or KO mice treated with anti-IL-4 for two weeks after colonization with *Tritrichomonas*. B. Expression of GATA3 in cells from secondary cultures in indicated culture systems. C. Cytokines produced in secondary cultures and genes expressed by cells from non-polarizing secondary cultures measured by ELISA and RT-PCR, respectively. (*,**,***,****: $p<0.05$, 0.01, 0.005, 0.001)

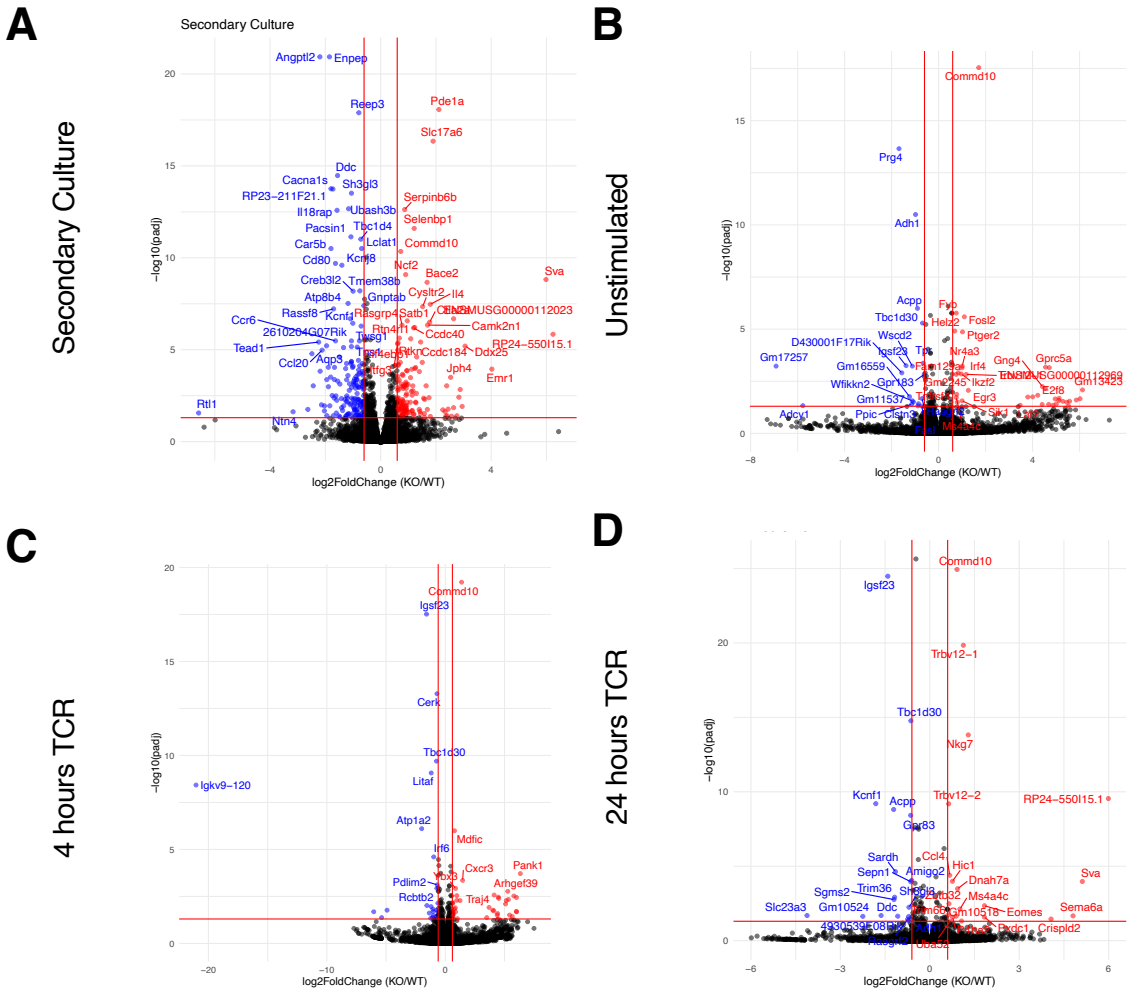

Figure 8. Differentially expressed genes in non-polarizing conditions

Volcano plots displaying genes upregulated (red) and downregulated (blue) at log2FoldChange threshold of 0.6 and FDR threshold of 0.05 in secondary cultures (A), unstimulated naïve CD4 T cells (B), or naïve CD4 T cells stimulated with 5ug/mL aCD3, 1ug/mL aCD28 and 10ng/mL IL2 for 4 hours (C) or 24 hours (D)

a Th2 program (Figure 8). This suggested a mechanism by which *Tet2*-deficient CD4 T cells are able to preferentially establish a Th2 program. Among these, we identified some genes and signatures that are crucially involved in Th2 differentiation that were already differentially expressed in the unstimulated cells, such as *Irf4* and genes regulated by STAT5 and NFKB-signaling.[123,204,205] We are actively investigating these gene programs and their associated genomic regions to assess how *Tet2*-deficiency leads to transcriptional and epigenetic changes that predispose cells towards a Th2 program.

We next tested whether IL-4 was sufficient to induce a Th2 population in the context of *Tritrichomonas* in wildtype mice. We found recombinant IL-4 complexes (rIL-4c) sufficiently drove Th2 polarization only when co-administered with *Tritrichomonas*, further suggesting that tight regulation of IL-4 production and signaling on T cells is an important checkpoint in regulating adaptive Th2 responses to the commensal microbiota (Figure 9A). To test if Th2 polarization was altered in other *in vivo* models, particularly in response to pathogens, we infected mice with the helminth pathogens *Strongyloides venezuelensis* and *Nippostrongylus brasiliensis*. Both worms induce a Th2 population in the host, however *N. brasiliensis* induced Th2 populations are thought to be IL-4 independent.[27,114] We sacrificed animals 7 days post infection and found there to be an equivalent Th2 induction in the lymph nodes of both genotypes and this response was also appropriately regionally different. Further, whereas IL-4 blockade reduced the frequency of Th2 cells in *S. venezuelensis*-infected mice, there was no appreciable difference in *N. brasiliensis*-infected mice, suggesting IL-4 dependent and independent Th2 responses to helminth pathogens are preserved and not different in *Tet2*$^{-/-}$ mice (Figure 9B). These data suggest TET2 is primarily involved in regulating cell intrinsic checkpoints to Th2 immunity in the context of commensals

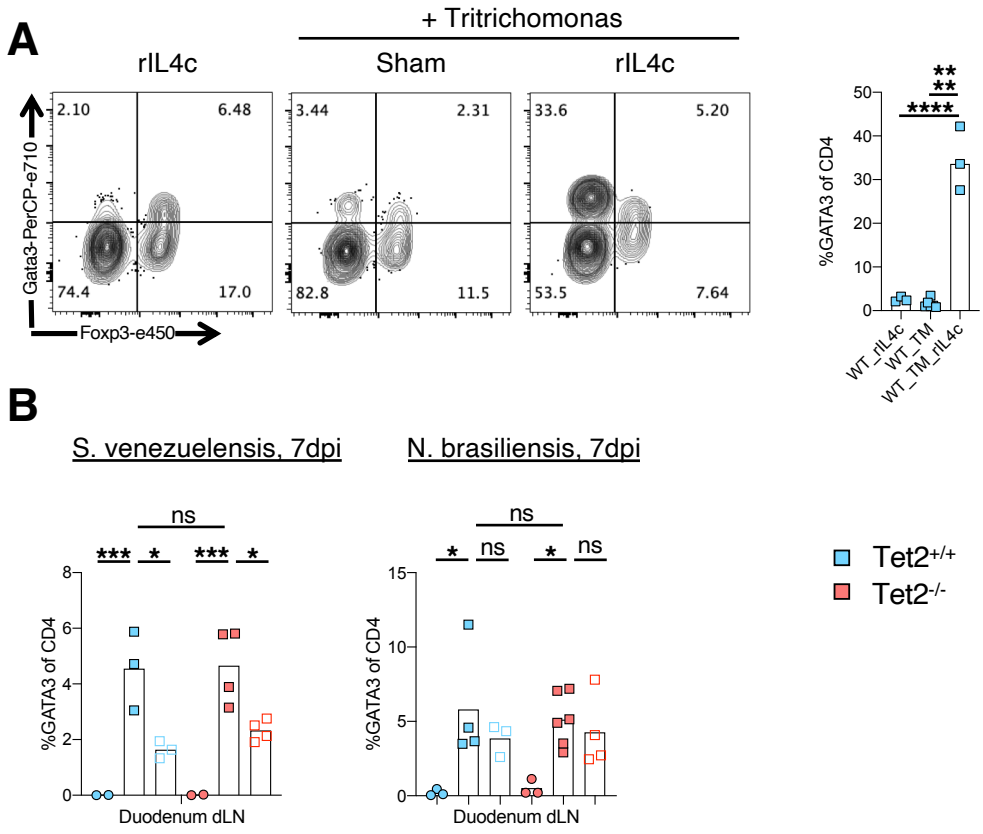

Figure 9. Control of IL-4 production in commensal and pathogen-specific adaptive responses determines Th2 polarization

A. Quantification of Th2 cells among CD4+ CD8a- TCRb+ CD90+ CD45 Live cells in the jejunum lamina propria of WT mice 4 weeks after colonization with *Tritrichomonas* in the presence or absence of weekly IL4 complex (1ug rIL4 + 5ug anti-IL-4) (IL4c) treatment . B. Th2 cells in the duodenal lymph node on day 7 of indicated helminth infection with and without IL-4 blockade (solid vs clear symbols). (*,**,***,****: $p<0.05$, 0.01, 0.005, 0.001)

and is dispensable for Th2 responses in the context of pathogens where there are strong exogenous signals to drive Th2 polarization.

2.2.4 IL-25 drives intestinal barrier function and allergic immunopathology

We previously observed microbiota dependent barrier dysfunction in the jejunum of *Tet2*$^{-/-}$ mice and sought to determine if this was also linked to IL-25 signaling. Strikingly, we found acute upregulation of IL-25 signaling in *Tet2*$^{+/+}$ and *Tet2*$^{-/-}$ mice was associated with downregulated expression of barrier function genes and a functional barrier defect as measured by FITC-dextran permeability (Figure 10A). As with IL-25 signaling, only chronically colonized *Tet2-/-* mice maintained this barrier defect, suggesting chronic IL-25 signaling is associated with chronic barrier dysfunction (Figure 10B). As *Tritrichomonas* spp ferment dietary fibers to make succinate, we assessed whether succinate was sufficient to drive barrier changes. Interestingly, while we observed upregulation of IL-25 signaling and downregulation of barrier function genes, we did not see an increased FITC-dextran permeability, suggesting either that the IL-25 signaling was not sufficiently upregulated, or there is some Tritrichomonas-derived factor responsible for this phenotype (Figure 10C). To test formally if the IL-25/IL-13 circuit can drive barrier dysfunction, we injected wildtype mice with recombinant IL-25 in combination with isotype control or IL-13 blocking antibody and found that IL-25 was sufficient to induce barrier dysfunction in an IL-13-dependent manner (Figure 10D). These findings suggest the IL-25/IL-13 circuit is a previously unappreciated pathway by which microbiota induced signals impact intestinal barrier function and further emphasizes why this pathway should be tightly regulated. In line with these findings, we observed that *Tet2*$^{fl/fl}$ *hCD2-Cre* mice, which are also characterized by chronic IL-25 signaling, had barrier dysfunction as measured by gene expression and FITC dextran (Figure 10E).

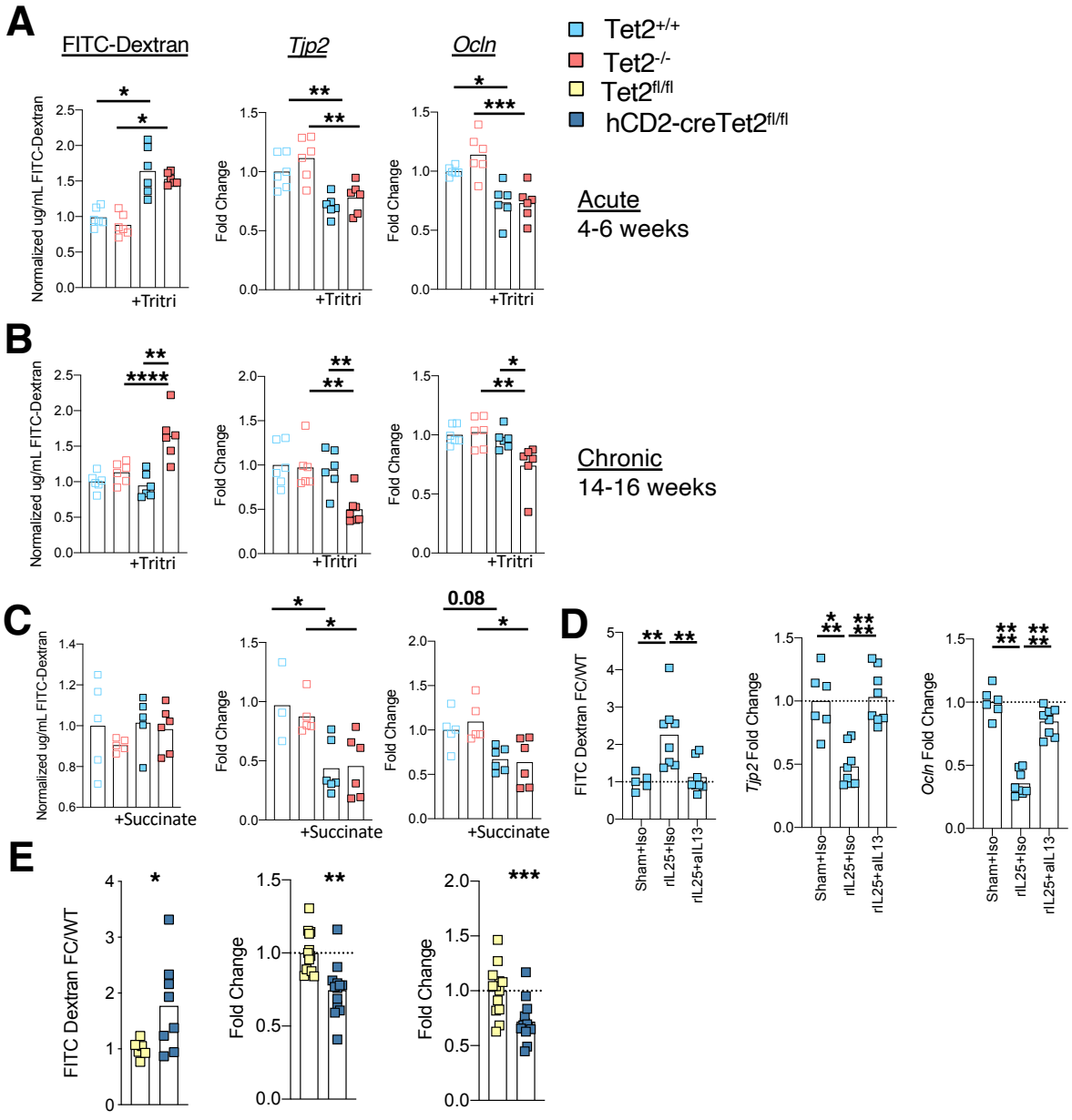

Figure 10. Chronic IL-25 signaling is associated with intestinal barrier function

Barrier function changes in mice colonized with Tritrichomonas for an acute (A) or chronic (B) timepoint as assessed by 4kDa FITC-Dextran permeability or RT-PCR of *Tjp2* or *Ocln* in the jejunum C. Barrier function readouts in mice treated with 150mM succinate in the drinking water for 6 weeks. D. Barrier function readouts in mice injected with recombinant IL-25 with or without IL-13 blocking. E. Barrier function readouts in Tritrichomonas colonized hCD2-Cre+ *Tet2*$^{fl/fl}$ mice. (*,**,***,****: $p<0.05, 0.01, 0.005, 0.001$)

Intestinal barrier function is recognized as an important mediator of tolerance and host response to intestinal derived antigens. Defects in barrier integrity have been associated with the development of allergy.[206,207] Additionally, IL-25 has been implicated as a potentiator of food allergy in pre-clinical models and increased levels of IL-25 have been reported in patients with food allergy.[208–210] Therefore, we decided to investigate whether the chronic IL-25 signaling and the barrier dysfunction observed in our *Tet2*-deficient mice was associated with the development of allergic responses. We employed a model of peanut allergy in which mice are sensitized with 6mg of crude peanut extract in the presence of 5ug cholera toxin via oral gavage 5 times over a 4-week period. One week after the final sensitization, mice are challenged intraperitoneally with 1mg of crude peanut extract and allergic responses are assessed (Figure 11A). We used $Tet2^{fl/fl}$ hCD2-Cre mice for these studies as $Tet2^{-/-}$ mice have other immune phenotypes, such as myeloproliferation, that may confound these studies. Upon antigen challenge, the body temperature of *Tet2*-deficient mice dropped significantly more than littermate controls, a sign of anaphylaxis (Figure 11B). This difference was in spite of equivalent and robust peanut-specific antibody responses in the form of IgE and IgG1 in both genotypes (Figure 11C) and also in spite of the fact we used a concentration of cholera toxin that is 2-3x lower than most published models.[211] This phenomenon is also observed between mouse genetic backgrounds, where mice of both susceptible and resistant backgrounds develop robust antibody responses to peanut antigen in this model, but only susceptible mice have overt anaphylaxis.[211,212] When we neutralized IL-25 in these mice, anaphylactic reactions were abrogated, suggesting a critical role for IL-25 signaling in the development of allergic immunopathology (Figure 11D).

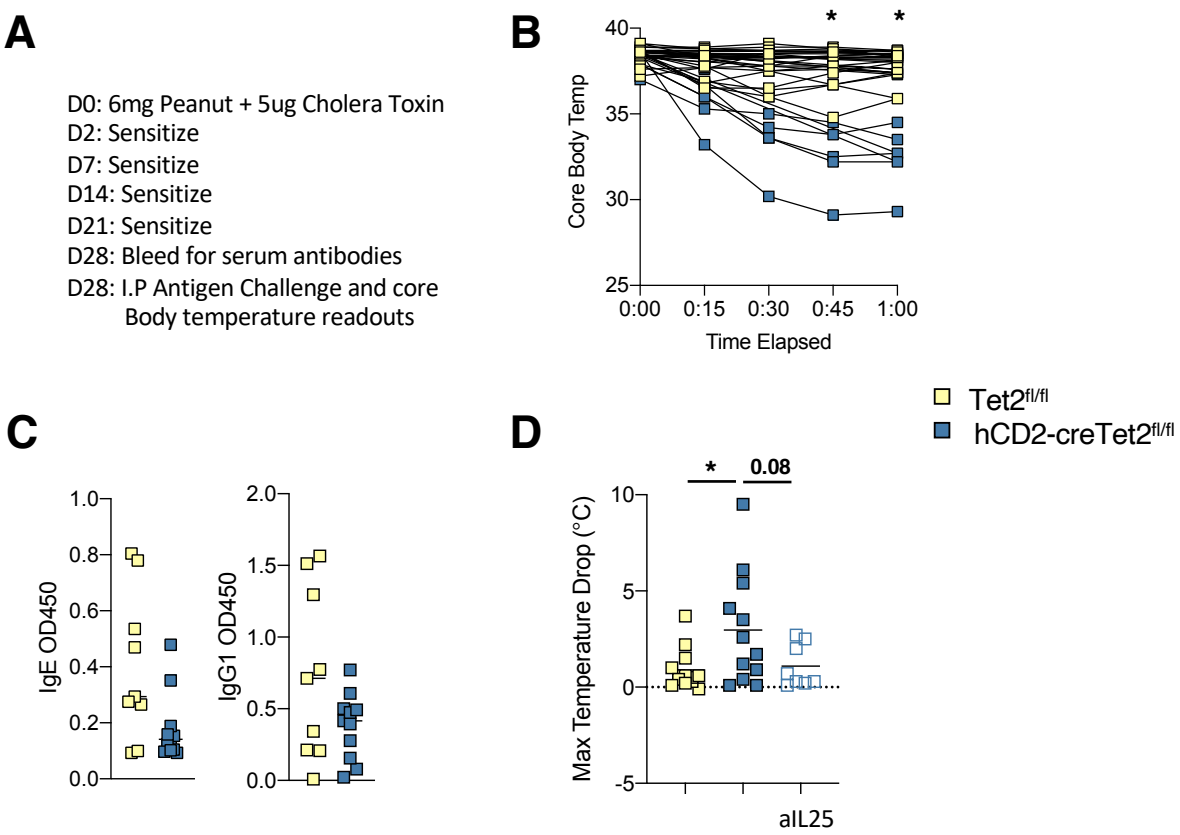

Figure 11. IL-25 dependent allergic pathology in *Tet2*-deficient mice

A. Experimental outline of allergen sensitization and challenge. B. Serial core body temperature readings after I.P antigen challenge. C. Crude peanut extract-specific antibody responses. D. Maximum temperature drop in mice treated with or without anti-IL25. (*,**,***,****: $p<0.05$, 0.01, 0.005, 0.001)

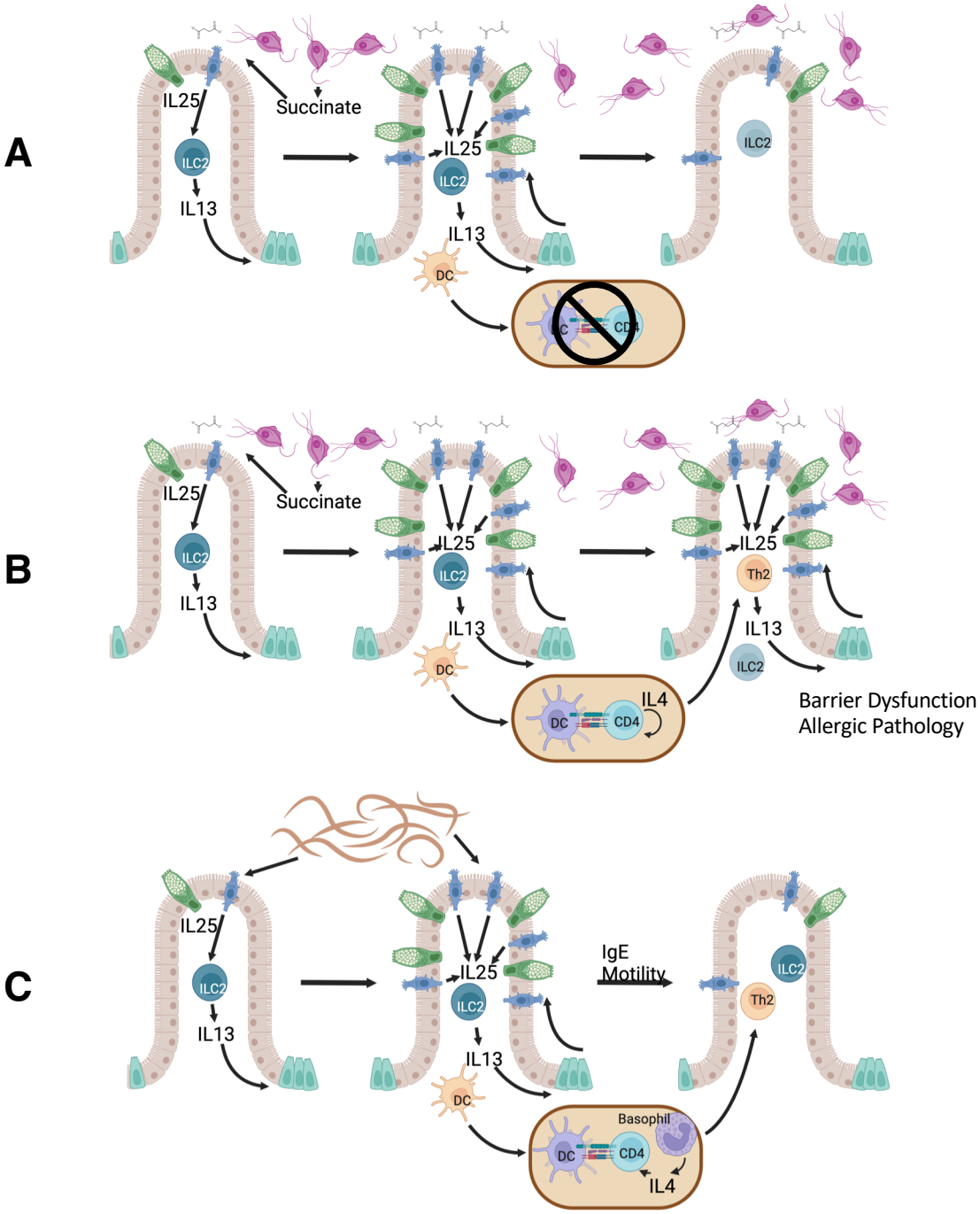

Figure 12. Type 2 responses to commensals and pathogens

A. The response to protozoa in a wildtype host. B. The response to protozoa in *Tet2*-deficient mice. C. Series of events to develop normal protective immunity to helminth infection.

Our findings collectively demonstrate the importance of host checkpoints in responses to the microbiota. Loss of one checkpoint, *Tet2*, results in a chronic adaptive response to a commensal protozoan and predisposes the host to barrier dysfunction and allergic immunopathology (Figure 12).

2.3 Materials and Methods

2.3.1 Mice and samples

Tet2$^{-/-}$ mice were obtained as previously described.[17] The following strains were purchased from Jackson lab and crossed in house: Tet2$^{fl/fl}$ (#017573), *Vav1*-Cre (#008610), hCD2-Cre (#008520), *Il5*-Cre (#030926), *Cd4*-Cre (#022071), *Rag2*$^{-/-}$ (#008449). Germ-free mice were generated previously

2.3.2 Mouse treatments

Depleting or blocking antibody treatments were injected IP in 200uL at the indicated doses every 3 days for the duration of the experiment: 500ug anti-IL25 (Amgen), 200ug anti-IL13 (Janssen), 200ug anti-CD4 (Bioxcell), 200ug anti-IL4 (Bioxcell). For rIL25 treatment, 300ng of IL25 (R&D) was injected I.P for 3 days followed by end point analysis. For metronidazole treatment, mice were provided with 2.5g/L metronidazole (Sigma) and 1% sucrose ad libitum in drinking water. Bottles were replaced weekly.

2.3.3 Tissue processing

Mouse small intestines were removed and washed in cold PBS. 1-cm segments were cut from relevant segments for RNA extraction and histology readouts. 12-cm of jejunum was measured for lamina propria isolation. Briefly, intestines were cut open longitudinally, washed in cold PBS to remove luminal contents and then were shaken 3 times in IEL medium (2mM EDTA in HBSS) at

37°C and 250rpm for 10 minutes each. Between shakes, the tissue pieces were washed with warm HBSS on 100um filters to aid in removing epithelial cells. After the IEL shakes, the tissue pieces were shaken in LPL medium (20% FBS, 0.05mg/mL DNAse I, 1mg/mL Collagenase A in RPMI) for 30 minutes. After the LPL shake, digested issue pieces were passed through a 100um filter, centrifuged and stained for FACS.

Mesenteric lymph nodes and Peyer's patches were isolated and separated by segment as previously described (source). Samples were collected in complete RMPI (10% FBS, 1% PenStrep Glutamine) and shaken in 1mL digestion medium (1mg/mL Collagenase VIII in complete RMPI) 30 minutes at 37°C at 250rpm. Digestion was halted by adding 10uL 0.5 M EDTA and placing samples on ice for 10 minutes. The media and remaining tissue were passed through 100uM filters and mashed. Digested and dissociated samples were washed once and then stained for FACS.

2.3.4 Tritrichomonas identification, isolation and colonization

Ceca from *Tritrichomonas* colonized mice were excised and their contents were removed by rinsing exposed contents in antibiotics-containing PBS (Abx-PBS). This slurry was passed through a 100um filter and spun down at 1000rpm for 7 minutes after which it was re-suspended in 5mL 40% v/v Percoll made in Abx-PBS and overlaid on 5mL 80% v/v Percoll. The percoll gradient was spun for 15 minutes at 1000g with no brakes at room temperature. The interphase was collected and washed twice in fresh PBS before sorting 2e6 protists per mouse to be colonized. *Tritrichomonas* burden was measured by extracting DNA from cecal or colonic contents using the Qiagen Fast Stool kit (Qiagen). Contents were homogenized in 1mL InhibitEx buffer (Qiagen) in 2mL screw top tubes filled with 0.5mL of 0.1mm glass beads (Biospec) using the Omni Bead

Ruptor Elite homogenizer prior to DNA extraction. PCR was performed as below using 10ng of DNA as starting material.

2.3.5 RNA isolation and Quantitative RT-PCR

Tissues stored in RNAlater for 24-48h at 4C were transferred to RLT+ containing 2-mercaptoethanol and homogenized using an equal mix of 0.5 mm and 1.0 mm zirconium oxide beads (Next Advance) and a bead homogenizer. Cells were stored directly in RLT+ containing 1% 2-mercaptoethanol and frozen at -80°C. RNA for all samples was extracted using the Qiagen RNeasy kit according to manufacturer instructions. Reverse transcription with 500ng of total RNA was performed using a GoScript Reverse Transcriptase kit (Promega) and PCR was performed on a Roche Light Cycler 480 machine using SYBR Advantage qPCR Premix (Clontech). Parameters for amplification: denature for 10s at 95C, anneal for 10s at 60c and extension for 10s at 72C. Relative expression was calculated using $1000* 2^{\wedge-\Delta CT}$ with *Gapdh* as the housekeeping gene. To account for technical variation, expression was normalized to wildtype or wildtype uncolonized samples. The primer sequences are as follows:

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Gata3	CTGGCGCCGTCTTGATAGT	GACGGTTGCTCTTCCGATCA
Tbx21	AGCAAGGACGGCGAATGTT	GGGTGGACATATAAGCGGTTC
Il4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
Ifng	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Spink4	TGCAGTCACATAGCTCACAAG	CCATGCCAAGGAGGGGAA
Dclk1	TGAACAAGAAGACGGCTCACTCC	GCTGGTGGGTGATGGACTTGG
Il13	CCTCATGGCGCTTTTGTTGAC	TCTGGTTCTGGGTGATGTTGA
Il25	ACAGGGACTTGAATCGGGTC	TGGTAAAGTGGGACGGAGTTG
Tjp1	GCCCTCCTTTTAACACATCAGA	GCCGCTAAGAGCACAGCAA
Tjp2	AAGTTCCCTGCCTACGAG	ATTCAACCGAACCACTCC
Ocln	ACTGGGTCAGGGAATATCCA	TCAGCAGCAGCCATGTACTC
Epcam	GCGGCTCAGAGAGACTGTG	CCAAGCATTTAGACGCCAGTTT
Asl	TCTTCGTTAGCTGGCAACTCACCT	ATGACCCAGCAGCTAAGCAGATCA

28s (Tritri)	GCTTTTGCAAGCTAGGTCCC	TTTCTGATGGGGCGTACCAC
16s	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC

2.3.6 Library preparation and sequencing

RNA quality and quantity were assessed using the Agilent bio-analyzer. Strand-specific RNA-SEQ libraries were prepared using a TruSEQ mRNA RNA-SEQ library protocol (Illumina provided). Library quality and quantity were assessed using the Agilent bio-analyzer, and libraries were sequenced using an Illumina NovaSEQ6000 (Illumina provided reagents and protocols).

2.3.7 Transcriptional Analysis

Raw reads were subjected to quality control checks, aligned using STAR (v2.6.1d, GRCm38, Gencode vM25) and summarized with featureCounts (subread v1.5.3). Batch correction for experimental batches was performed using ComBATseq[213], and differential expression analysis was performed using DESeq2[214]. For intestinal cell composition analysis, cell signatures were obtained from PanglaoDB[198] and used for gene set enrichment analysis (GSEA). For interrogating GATA3-regulated genes in non-polarizing cultures, we used gene lists from published ChIP-seq and RNA-seq of naïve and differentiated T helper cells.[39]

2.3.8 Cell staining and flow cytometry

Cells were stained with FC block (10 min), fixable live dead dye (15 min), and surface markers (25 min). Cells were then fixed and stained intracellularly using the eBioscience Foxp3 kit according to manufacturer instructions.

2.3.9 Helminth propagation and infection

Strongyloides venezuelensis was generously provided by Daria Esterhazy (University of Chicago) and *Nippostrongylus brasiliensis* was provided by Jakob von Moltke (University of Washington).

To propagate and maintain cultures, NSG mice (Jax #00557) were infected subcutaneously with 10,000 L3 larvae. Fecal pellets from infected mice were homogenized in water and spread onto filter paper that was partially submerged in tap water in a beaker. The beaker was loosely covered with plastic film and placed at 30°C for 4 days after which water was collected and fresh larvae were allowed to settle at room temperature. Larvae were counted and resuspended for an infection dose of 700-1000 L3 in 100uL. Experimental mice were infected and sacrificed at day 7 to assess priming of intestinal Th2 responses in mesenteric lymph nodes.

2.3.10 Naïve CD4 cultures

Naïve CD4 T cells were isolated from spleens of 6-8 week *Tet2*$^{-/-}$ or *Tet2*$^{+/+}$ mice by homogenizing spleens in RBC lysis (R&D #WL2000) and then enriching for naïve cells using MACS enrichment (Miltenyi #130-104-453). Cells were then further sort purified (CD4+ CD8- CD62L+ CD44-) and seeded at 1e5 cells/well in 96 well plates pre-coated overnight with 5ug/mL anti-CD3 (Biolegend #100302). Cells were cultured in complete RPMI (10% FBS, 1% PenStrep Glutamine) with soluble anti-CD28 (Biolegend #102102) and 10ng/mL mIL2 (Miltenyi #130-107-760) for non-polarizing cultures. For Th2 polarizing cultures, 10ng/mL IL-4 (Miltenyi #130-107-760) was added and for Th2-blocking cultures, 10ug/mL anti-IL-4 (Bioxcell #BE0045) was added. Cells were cultured for 48 hours and then split and reseeded in the same media as secondary cultures and analyzed at d4-6. For RNA, cells were collected in RLT+ and immediately stored at -80°C. Cell supernatants were frozen at -20°C before analysis of cytokines by ELISA.

2.3.11 In vivo intestinal permeability

In vivo intestinal permeability was assessed by 4kDa FITC-dextran (Sigma-Aldrich #46944). Mice were deprived of food and water for 5 hours and then orally gavaged with 60mg/kg of FITC-

dextran in PBS. Two hours later, mice were cheek bled, and the blood was spun down for 10 minutes at 10,000rpm. The serum was collected and measured for FITC-dextran using an excitation of 490nm and emission of 520nm on a fluorescent plate reader.

2.3.12 Cholera Toxin and peanut sensitization and challenge

Crude peanut extract (CPE) was prepared from roasted unsalted peanuts. Briefly, peanuts were ground to and added to 20mM Tris buffer (pH 7.2) at 25 grams peanuts per 20mL 20mM Tris. The solution was stirred at room temperature for 2 hours and then centrifuged at 3000 g for 30 minutes. The aqueous fraction below the upper most fat layer was carefully removed and measured by BCA assay for protein content. Mice were sensitized with 6mg of peanut extract and 5ug of cholera toxin in 200uL by oral gavage on day 0, 2, 7, 14 and 21. On day 28, mice were challenged I.P. with 1mg of peanut extract in 200uL. Body temperature was measured using a rectal probe prior to challenge and every 15 minutes after challenging up to one hour. Mice were bled on day 14 and day 27 to assess antibody responses in the serum and were bled after challenge on day 28 to assess systemic mast cell degranulation.

2.3.13 Enzyme-linked immunoassay

Commercial ELISA kits were used to measure IL4 production and IFNγ according to manufacturer instructions (Invitrogen). Peanut specific IgE and IgG1 was measured by first coating high-binding 96-well plates (Corning 3690) with 20µg/mL CPE in 100mM Na_2CO_3 overnight at 4C.Plates were washed three times in PBS plus 0.05% Tween-20 (PBS-T) and blocked with 150µL 1% BSA in PBS-T for 2 hours at RT and then washed 1 time with PBS-T. Mouse serum obtained via submandibular bleed was diluted in blocking buffer and added as 25µL per well and incubated for 1 hour at RT followed by 3 washes. 50 µL horseradish peroxidase-conjugated anti-mouse IgE or

IgG1 (Southern Biotech) diluted 1:1000 in blocking buffer was added to well and incubated for 45 minutes at RT and then washed 5 times. 50μL TMB substrate was added and the reaction was stopped by adding 50μL 1N H_2SO_4. Absorbance at 450nm was read immediately after stopping the reaction.

3 DISCUSSION

In the work above, I identified *Tet2* as a regulator of type 2 immunity in the intestine. *Tet2*-deficient mice had intestines with a gene signature for strong type 2 activation characterized by tuft and goblet cell expansion as well as high expression of the prototypical cytokines for type 2 activation in the intestine, IL-25 and IL-13. This circuit required the presence of the microbiota and a specific microbe, *Tritrichomonas*, was sufficient to induce activation of this circuit. The normal response to this microbe in a wildtype background involves IL-25 signaling, however this response through unknown mechanisms is eventually downregulated. We identified a *Tritrichomonas*-dependent Th2 population in *Tet2*-deficient mice as the propagators of this circuit, representing an adaptive population that co-opted a circuit normally mediated by innate lymphocytes. Due to the continued presence of *Tritrichomonas*, these tissue resident lymphocytes had continuous stimulus to which to respond and secrete effector cytokines to propagate tuft cell differentiation. This Th2 population appeared to be induced through a classical IL-4 dependent mechanism, and using *in vitro* differentiation cultures, we identified *Tet2*$^{-/-}$ CD4 T cells themselves as the source of IL-4. This autocrine IL-4 signaling was sufficient to establish an effector program even in the absence of exogenous polarizing signals. When we provided IL-4 to wildtype mice in the context of protozoa, we were able to induce a Th2 population, suggesting this is the mechanism by which *Tet2*$^{-/-}$ mice mount this commensal-specific Th2 response. Importantly, helminth infection models that are dependent on strong immune activation to induce Th2 populations did not demonstrate differential induction of Th2 responses between WT and *Tet2*$^{-/-}$ mice, suggesting this regulation of Th2 responses by *Tet2* is specific to homeostatic microbiota responses. RNA-sequencing studies confirmed these findings and elucidated several mechanisms by which naïve CD4 T cells that were

deficient for *Tet2* had a predisposition for the Th2 lineage. Given the unique intestinal remodeling that results from persistent type 2 activation and previous associations made in the lab, we assessed whether there was a link to intestinal barrier function. Indeed, we found that barrier function was acutely changed in a wildtype host in response to *Tritrichomonas*, but this response was resolved in the same way significant IL-25 signaling was downmodulated. However, the barrier function changes persisted in *Tet2*$^{-/-}$ mice, and they were predisposed to allergic immunopathology in an IL-25 dependent manner. There are several tolerance mechanisms at play in the intestine ranging from active tolerogenic responses to passive anergic responses. These can be mediated through either the innate or adaptive immune system, but they all serve to establish a state of homeostasis where the diverse microbiota and antigen rich intestinal environment exists in the context of a functional but reasonable immune response. We propose *Tet2* functions in naïve CD4 T cells as a cell intrinsic checkpoint to prevent adaptive type 2 responses in the context of type 2 promoting microbiota.

3.1 Host responses to the commensal microbiota

Although the microbiota is a continuous source of diverse immunomodulatory signals, it is somewhat paradoxical and metabolically prohibitive that a highly specific and continued immune response be developed for every new species. We hypothesize the metabolic cost of such responses is why there have been relatively few identified models in mice for which a defined microbe-induced effector T helper response has been characterized.[181] Upon colonization of a complete microbiota from Jackson Labs, for example, the immune response that develops across the small intestine and colon is relatively muted given that millions of microbes were simultaneously introduced.[215] There are several proposed ways by which the host can develop a tolerogenic

response to the microbiota such that adaptive immunity is primarily developed in the context of pathogens or novel microbes that are especially immune-stimulatory. First, the maintenance of an intestinal barrier prevents most direct interaction with the microbiota. This mechanism is especially at play in the large intestine, the site of highest microbial burden as well as the site with the most developed mucus layer. Second, active tolerogenic signals through the action of regulatory T cells can prevent active induction of adaptive responses to microbiota. These active tolerogenic responses seem to be induced upon colonization of *Clostridium*.[185] Lastly, a division of labor amongst immune subsets in the intestine can restrict initial responses to the microbiota to specific cell types that is subsequently downregulated, and a state of tolerance is established. The default response to *Tritrichomonas* in wildtype mice appears to be exemplary of the third case. However, in the context of *Tet2*-deficiency, there appears to be an induction of transient innate and chronic adaptive responses.

One example in which both innate and highly antigen specific mechanisms are at play is the host response to segmented filamentous bacterium. Although the antigen specific Th17 response to SFB has been well documented, the role of ILC3s and the innate response has increasingly been appreciated. The innate circuit in response to SFB was appreciated in *Rag*-deficient mice where the epithelium and ILC3s had a persistent phospho-STAT3 signature driven by an IL-23/IL-22 signaling axis that was dependent on the microbiota but absent in immunocompetent mice. Adaptive Th17 cells and Tregs were shown to interrupt this axis and thereby prevent persistent activation by SFB through control of its growth states and control of SFB induced cytokine signaling[216,217]. When neonatal mice were characterized over time, the authors observed initial pSTAT3 signatures upon weaning that were eventually down-regulated in

immunocompetent mice after an adaptive response was mounted, suggesting the ILC3-amplified immune activation is the first wave of response to SFB whereas Th17 and Treg mediated control is the second wave in which a more tolerant state is achieved. If this state is not achieved, as is the case in *Rag*-deficient mice that are colonized with SFB, there are metabolic defects due to epithelial programs of fat uptake being disrupted.[216,217]

First, it is important to make key distinctions between SFB and *Tritrichomonas*. SFB's primary interaction with the host occurs in the form of attachment and embedding on epithelial cells and thus results in significant antigen transfer and direct host interactions. *Tritrichomonas* primarily mediates its effects through the production of the metabolite succinate and does not have such strong attachment to the host. Given the intimate relationship between SFB and the host epithelial cell, an adaptive mechanism is how the innate circuit is resolved. With no such clonal expansion of effector T cell in response to *Tritrichomonas* in our wildtype mice, the mechanism of downregulation of the IL-25 circuit is not understood. However, this downmodulation is nonetheless important as continued IL-25 signaling, as with persistent IL-23/IL-22 signaling, results in epithelial remodeling that, when unchecked, has consequences in host physiology. This is exemplified by what occurs in *Tet2*-deficient mice. In this setting, the mice developed an adaptive *Tritrichomonas* dependent Th2 response that propagated a circuit that is usually transiently induced by ILC2s. This resulted in intestinal barrier defects and allergic pathology.

3.2 T cell effector programs and the intestinal environment

T helper programs are specialized to different pathogenic contexts depending on the nature of the pathogen. This is important as T-helper responses often dictate the subsequent responses of other immune cells as well as the tissue response. In order to establish a specific response, three signals

are required for development of a mature T-helper response. Signal 1 is provided in the form of antigen presentation by a professional antigen presenting cell. Signal 2 is a provided in the form of co-stimulation, which potentiates the TCR signal. Finally, signal 3 is a polarizing signal, usually in the form of specific cytokines. The combination of these yield an effector program for the T helper cell, and each signal individually is known to modulate the outcome. For example, the intensity of TCR signaling[99,101,103], the nature of co-stimulation[104–109] and the availability of polarizing cytokines[80,95,114,118–120,128] have all been demonstrated to be important in determining Th2 differentiation *in vivo* and *in vitro*. We observed IL-4 dependent induction of Th2 cells in response to the commensal *Tritrichomonas* and found *Tet2* was a cell intrinsic checkpoint for IL-4 derived from naïve CD4 T cells. The mechanisms by which this checkpoint exerted its function are still unclear. It is clear that naïve CD4 T cells that are deficient for *Tet2* already have gene expression differences prior to stimulation, and these expression differences likely underlie epigenetic differences that may or may not be mediated through the catalytic function of TET2. We will pursue these mechanisms through the use of CD4 T cells that express a catalytically inactive form of TET2, which will demonstrate whether the catalytic activity is required. We will also perform methylation and hydroxymethylation profiling of naïve CD4 T cells from wildtype and *Tet2*$^{-/-}$ mice to assess whether the differentially methylated regions are concordant with the gene expression differences observed. There are already some targets that appear in our expression dataset that are intriguing. *Irf4* is upregulated in *Tet2*$^{-/-}$ naïve CD4 T cells, and a NFkB signaling response signature is persistent throughout TCR stimulation of naïve CD4 T cells. IRF4 was found to regulate Th2 cytokines in naïve and effector Th2 cells.[205,218] NFkB was found to be important in promoting IL-4 expression by cooperating with TCR signaling and GATA3 expression in

allergic models, suggesting IRF4 potentiates acquisition of a Th2 fate.[204,219] The *Irf4* locus can be directly interrogated for methylation or hydroxymethlyation using targeted sequencing, whereas the NFkB response signature may be more difficult. The functional responsiveness, however, can be interrogated through *in vitro* TCR stimulation followed by assessment of phosphorylated p65 subunits. Although *Tet2* was initially demonstrated to be dispensable for *in vitro* Th2 differentiation, these studies made use of exogenous IL-4 in forced Th2 polarizing conditions.[141] Under these conditions, we also observed no difference in Th2 induction. Another group used mice deficient for all three TET proteins and actually found IL-4 production to be impaired, but GATA3 induction preserved. They posited TET enzymes play important roles in aiding passive demethylation in the process of differentiation as cells deficient in the terminal base excision process of active demethylation had no defect in IL-4 production.[142] Thus, the role of TET enzymes in Th2 differentiation is perhaps unique to differentiation and genetic contexts, and we hope to identify a specific role for *Tet2* in mediating the differentiation propensity of naïve CD4 T cells in non-polarizing contexts.

The nature of the T-helper response is known to impact the humoral response. Although the relationship between T helper subsets and T follicular helper cells is not completely understood, the role of T helper-associated cytokines in promoting class switch is well-recognized. Antibody isotypes elicit different immune outcomes and thus it is important, for example, to distinguish between IgE that will result in mast cell degranulation upon Fc receptor binding and IgA that is secreted at mucosal sites to neutralize infection. We did not observe increased total IgE (data not shown), suggesting there may be heterogeneity in T follicular helper responses and not

all effector programs even of the same subtype may be potent inducers of class-switch recombination.

T effector programs also determine which types of effector myeloid cells are recruited to the site of the immune insult, which can result in vastly different effector molecules and cytokines being secreted. For example, in type 2 responses, eosinophils produce large amounts of major basic protein. This protein is highly cationic and is intended to kill large extracellular parasites, but the cationic nature also results in some tissue destruction.[28] In the same vein, neutrophils are recruited during type 3 immune responses and have microbicidal functions. One such function is through the release of neutrophil extracellular traps (NETs). Although these structures release a lot of anti-microbial enzymes that serve to help control infection, they are also highly toxic and induce cellular damage and exacerbate inflammation.[220] The inappropriate or nonspecific recruitment and activation of these effectors would cause unnecessary tissue damage thus emphasizing the need for highly regulated determination of T effector fates. We observed eosinophilia in our tissues that is common in the acute response to *Tritrichomonas*[192] but is normally downregulated. We did not assess the direct consequence of this eosinophilia for tissue integrity, but this can be done using Siglec-F depletion antibody in the peanut allergy model we employed as well as other models.

In addition to dictating the nature of humoral and recruited innate responses, the T effector program also impacts the differentiation and function of tissue resident cells. Bystander tissue resident lymphocytes can be stimulated through the action of cytokines, particularly those that reside at epithelial sites.[135,221] The polarization of tissue resident macrophages is dictated by the T cell effector cytokines IFNγ, IL-4 and IL-13. These cells then go on to function in either promoting further inflammatory processes through the secretion of inflammatory cytokines like IL-6 or

promoting tissue healing and fibrosis through the secretion of IL-10, for example. We did not interrogate whether our persistent Th2 response led to more "M2" type macrophages, although such studies would be interesting given the role of Tet2 in regulating inflammatory gene expression in these cells.[14,222]

Finally, the nature of the T-effector response also impacts non-hematopoietic compartments. This is especially true in the intestine, a fast-dividing tissue that dynamically responds to different immune insults and signaling. Within the intestine, the epithelium has been recognized as a significant responder to immune stimulation.[196,223] This response can consist of altering differentiation fates of epithelial cells as is the case with type 2 responses driving tuft and goblet cell production.[160] This response can also impact anti-microbial peptide secretion by epithelial cells.[224] Another responding compartment of the intestine is the muscularis layer, which can proliferate and increase motility, especially in the context of worm infections.[225] Therefore, the decision of whether to mount an adaptive T helper response and the nature of the adaptive response has several consequences for host immunity and physiology. In our models of *Tet2*-deficiency, induction of an effector Th2 response through aberrant IL-4 signaling led to persistent remodeling of the tissue that involved tuft and goblet cell hyperplasia.

In addition to effector subtype, the antigen is an important determinant of how the host physiology will be modulated by the effector response. In most cases, especially in those where the antigen is pathogen derived and can be eliminated, the effector response has the opportunity to subside and result in the formation of effector memory and central memory compartments toward the specific antigen. In contexts where the antigen is persistent, however, the effector response can in theory persist. Situations where this can occur are autoimmunity, persistent pathogens or the

commensal microbiota, where self-antigens, pathogens and commensal antigens are ever present. In all situations, there are active tolerance mechanisms to prevent significant immune activation against these antigens and the lack of these tolerance mechanisms in certain genetic models, such as Aire-deficiency[226], and in diseases such as Type 1 diabetes, makes evident the necessity of this control. Persistent type 1 responses can lead to significant tissue destruction and loss of key functional units in physiology such as the case in type 1 diabetes and celiac disease. Persistent type 2 responses can lead to sustained remodeling of tissue and cell programs such that certain functions are fundamentally altered. An example of this is the chronic helminth pathogen *H polygyrus*, which can directly modulate genetically susceptible hosts to avoid clearance. This results in some changes such as metabolism that is resistant to western diet challenges[227], but others such as modulation of the microbiota such that the host is more susceptible to enteric pathogen.[228]

In our model system, we found the persistent Th2 responses to a commensal protozoan to negatively impact barrier function and cause a predisposition to allergic pathology. In contrast, Th2 responses to helminths were preserved in *Tet2*-deficient mice and helminths were cleared efficiently, suggesting the mechanism by which *Tet2* regulates Th2 induction is specific to a homeostatic response, such as responses to the microbiota, where strong innate activation is not present to provide exogenous polarizing signals. These findings, particularly the finding that chronic Th2 responses result in barrier dysfunction and allergic pathology, perhaps represent a more general principle for intestinal type 2 responses. Effector Th2 responses in the intestine are perhaps restricted to settings of strong immune activation, such as a worm infection. In other settings, there are likely checkpoints to prevent IL-4 production and Th2 induction. One such checkpoint is the requirement that type 2 myeloid and innate effectors must provide exogenous

IL-4 and other signals that drive Th2 polarization. Another such checkpoint is the control of autocrine IL-4 production by naïve CD4 T cells and this mechanism appears to be mediated by *Tet2* (Figure 13).

3.3 Host and environmental factors in human pathophysiology

Modern understanding of numerous human pathologies implicates both host genetics and environmental factors in pathogenesis. Environmental factors such as diet, pollution, and carcinogens have been identified to play a role in many chronic pathologies and systemic and public health changes have been implemented to control for and better understand these interactions. Host genetics, while classically associated with germline variation and inheritance, can also include somatic mutations in various tissues. These have been described primarily in fast-dividing tissues such as epithelial surfaces and the hematopoietic system where cell turnover is high. Increased cell division predisposes progenitor cells in these tissues to develop errors in replication and create somatic variants. The somatic variants that provide some competitive advantage in the survival or subsequent differentiation from these progenitors can be selected for and eventually represent a significant fraction of the tissue. This is the case in CHIP, where allelic frequencies of mutations can nearly reach 50%. These mutations are classically studied in the context of malignant transformation, but these somatic changes can also change how the hosts interacts with a pre-existing environment or how the host will adapt to a novel environment. For example, CHIP-associated mutations exacerbate pathology of atherosclerosis.[229,230] These new host-environment interactions have not been extensively studied and represent a potential new lens through which to study pathophysiology. As *TET2* mutations are common amongst the elderly, it

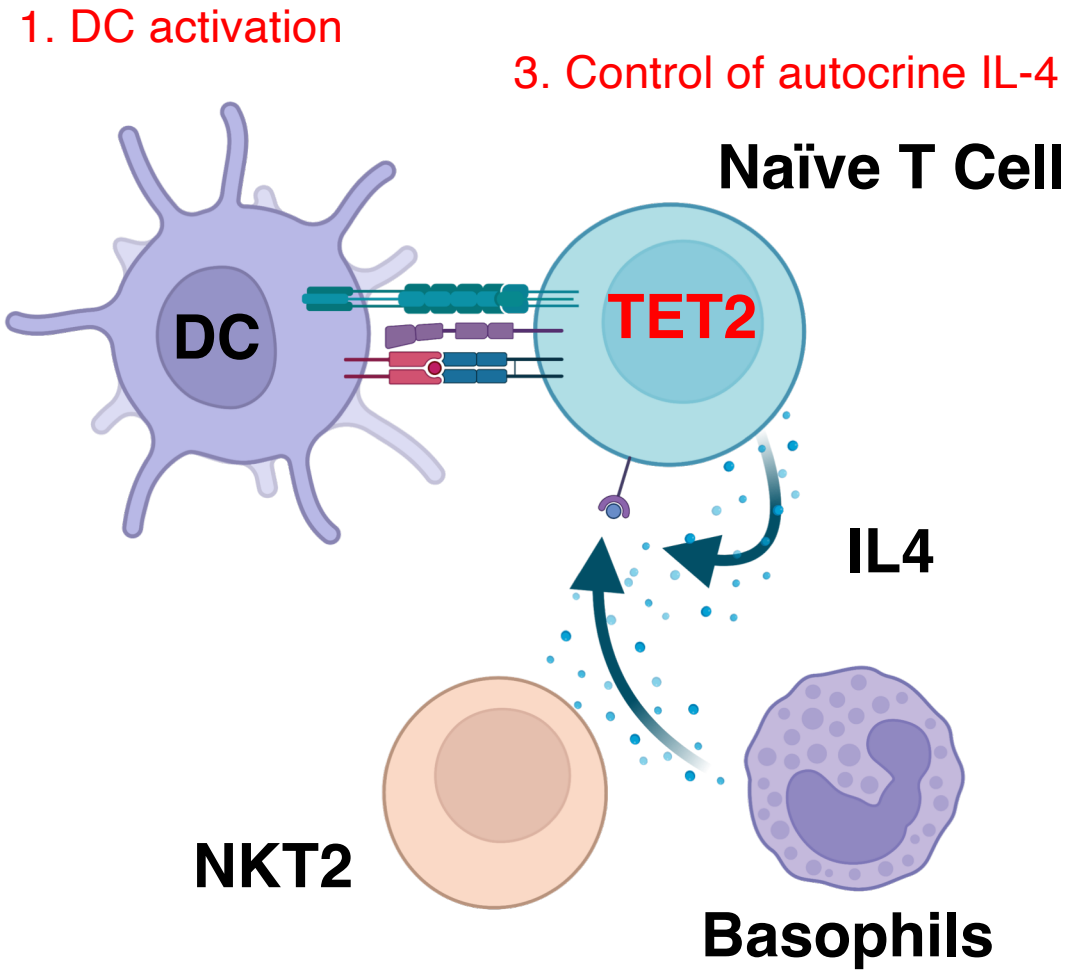

Figure 13: Checkpoints in Th2 induction

Th2 induction requires several checkpoints to be fulfilled. First, productive antigen presentation in addition to proper activation of the antigen presenting cell fulfill classical signal 1 and signal 2 requirement. For signal 3, there either must be recruitment of IL-4 competent immune cells. Their recruitment is determined by inflammatory chemokines and cytokines induced by the type 2 immune challenge. As naïve CD4 T cells are also IL4 competent, we propose an additional requirement be the selective production of autocrine IL4 as a checkpoint to Th2 induction that is controlled by the action of TET2.

is likely additional pathological processes that are altered as a result of this somatic mosaicism will be identified.

Genetic backgrounds of mouse models have long been recognized as sources of heterogeneity in infection models. Two models that are prototypical of these differences are the C57Bl/6 and the BALB/c lines, which have different susceptibilities to infection with *Leishmania major*.[231,232] The resistant line, C57Bl/6 mice, are able to develop productive Th1 responses to the pathogen and clear it, whereas BALB/c mice do not mount a Th1 response. Similar differences have been observed in models of *Francisella tularensis*, *T. cruzi*, *Encephalitozoon cuniculi*, *H. polygyrus* and *Toxoplasma gondii.*[233,234] Interestingly, many of these differences can explained by the nature of adaptive responses that are mounted towards the pathogen. Not all of the responsible genetic loci have been identified, but requirement and sufficiency experiments demonstrate clear roles for IL-12, for example, in helping susceptible BALB/c mice resist *L. major*.[231] In addition to infectious contexts, differences in adaptive immunity between mouse strains have also been observed at homeostasis. For example, homeostatic IgA differs between the Balb/c and B6 strains such that their microbiome is shaped differently.[235] These differences in adaptive immune responses may also be due to different thresholds of activation required to induce effector responses. For example, BALB/c mice have classically been considered apt for Th2 models as they have strong immunological responses to type 2 challenges. Using a system similar to the one we employed to assess autocrine IL-4 secretion by naïve CD4 T cells, Kubo and colleagues have described increased IL-4 production by BALB/c naïve cells.[236] Whether this is due to differences in Tet2 activity, as our results demonstrate, is unclear, but these findings nevertheless suggest cell

intrinsic thresholds for mounting of adaptive response are greatly impacted by genetic backgrounds and may be responsible for differences in host immunity.

4 ADDITIONAL FINDINGS AND FUTURE DIRECTIONS

4.1 Mechanisms for intestinal barrier dysfunction

My work revealed a direct role for IL-25 signaling in mediating barrier dysfunction as measured by FITC dextran permeability and the expression of barrier function genes. However, the mechanism by which this is induced is still unclear. One possible explanation is the changed composition of the epithelium after IL-25 signaling. Goblet and tuft cell hyperplasia is observed in this context and perhaps they are less efficient at establishing barrier integrity and have lower expression of barrier function associated genes and proteins. This could be tested by using models deficient in tuft or goblet cells, such as Pou2f3-deficient or Klf4-deficient mice, respectively.[237,238] If IL-25 is no longer sufficient to induce barrier dysfunction in these contexts, intestinal permeability in IL-25 signaling likely stems from an inefficient barrier when there are excessive tuft or goblet cells. If these mice still get barrier dysfunction, IL-25 signaling may induce changes in the general $Epcam^{+}$ compartment of the small intestinal epithelium involving the regulation of barrier function proteins. These data could inspire further investigation of other immune signaling pathways in the intestine and their roles in maintaining barrier integrity.[223,239]

4.2 Further checkpoints to Th2 immunity

During my thesis work, I had to work with different barrier levels of animals characterized by the presence or absence of certain members of the microbiota or pathogens. As we were studying host responses to *Tritrichomonas*, this necessitated establishing colonies of animals free of these protozoa through the use of metronidazole, and this approach generally allowed us to study the response to protozoa when it was introduced to mice after weaning, namely the expansion of Th2 cells in *Tet2*-deficient models. However, we noticed that when mice were vertically colonized with

Tritrichomonas from birth by the dam, there was an abundant Th2 population in both the wildtype mice and in our *Tet2*-deficient models, whereas significantly higher IL-25 signaling was still specific to *Tet2*-deficient models. These data suggest the microbiota plays a role in controlling Th2 responses in the intestine. When *Tritrichomonas* is present from birth, this checkpoint is not active, and there are is an expansion of Th2 cells in both wildtype and deficient models. However, when the protozoa are introduced after the mice already have a microbiota at weaning, the response is specific to *Tet2*-deficient mice. These data are concordant with the elevated type 2 responses observed in germ-free mice[240–243], and provide a model to study this microbiota checkpoint. There are several salient questions arising from these data that would be of interest to me. First, it would be interesting to do host immune and transcriptional profiling to understand perhaps the mechanistic basis of such differences. Specifically, mouse intestines could be transcriptionally profiled and immunophenotyped in the neonatal period when they are first exposed to *Tritrichomonas* from the mother, and these could be compared to neonates from *Tritrichomonas* negative dams and to the transcriptional and immune responses observed upon colonization at weaning. If such differences are identified, pathological models of allergy and helminth infections could be used to see if there is differential protection or susceptibility, as has been done with germ-free mice.[243–246] To study this model further, one could mono-colonize germ-free mice with *Tritrichomonas* to confirm whether a Th2 population is still induced. This would require either culturing this protozoan *in vitro* or mice mono-associated with sorted protozoa and placed on antibiotic treatment that would eliminate any carrier microbes. In this model, one could also test whether, in a germ-free setting, vertical colonization and colonization at the time of weaning have different outcomes in the response to *Tritrichomonas* and in the general immune composition of

the intestinal tissue. If protozoa induce robust Th2 responses in this setting, microbial consortia could be used to identify if there is a specific effect of certain microbes in preventing this Th2 response. Similarly, this could be tested in the vertical colonization setting—weanlings from dams harboring a limited consortium are colonized with *Tritrichomonas* to assess whether the consortia limit strong Th2 induction—or after weaning, where germ-free weanlings are introduced to *Tritrichomonas* and various consortia and then followed to assess the Th2 response. These series of experiments would elucidate host and microbial factors that determine Th2 responses to a specific commensal and would provide a mechanistic basis for a microbiota-dependent checkpoint for Th2 immunity.

4.3 Preleukemic myeloproliferation and clonal hematopoiesis

A defining phenotype of *Tet2*-deficient mice is myeloproliferation characterized by the expansion of mature and progenitor cells in the myeloid lineage as well as broadly increased hematopoiesis.[18–20] Previous work in the laboratory showed these phenotypes correlated with intestinal barrier dysfunction and bacterial signal-induced IL-6 production.[17] The production of IL6 and the molecular mechanisms by which IL6 drives myeloproliferation are still unknown, although *in vitro* assays suggested signaling at the level of hematopoietic progenitors was important.[17] We crossed *Tet2*$^{-/-}$ mice with *Il6*$^{-/-}$ mice to assess whether IL-6 was absolutely required for the development of myeloproliferation. *Tet2*$^{-/-}$ *Il6*$^{-/-}$ mice had attenuated myeloproliferation, confirming a critical role for IL6 in this process (Figure 14A). One major distinction between mouse models involving Tet2 and presumed pathogenesis of leukemia in patients is the

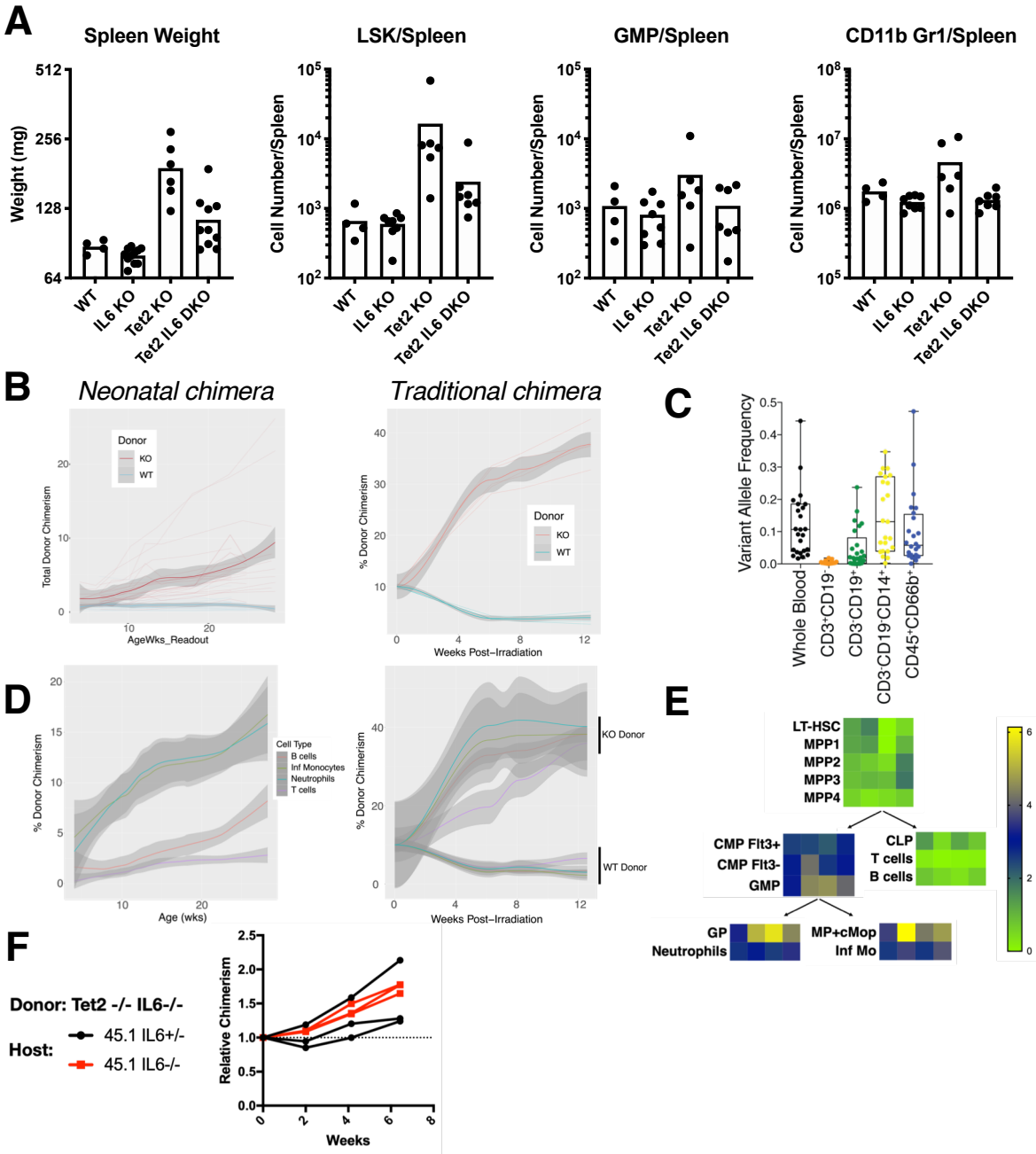

Figure 14. Myeloproliferation and CHIP

A. Myeloproliferation readouts in mice with and without IL-6. B. Clonal expansion in neonatal and traditional irradiation chimera models. C. From Arends et al 2018–lineage specific variant allele frequencies of TET2 mutations. D. Lineage biases in the peripheral blood of neonatal and traditional irradiation chimeras. E. Relative allele frequencies of Tet2-deficient cells in hematopoietic progenitors. F. Clonal expansion in IL-6 deficient and sufficient hosts represented as relative chimerism to first reading

requirement for additional mutations before dysplasia develops. In patients, a sole *TET2* mutation usually confers a condition called CHIP that is not characterized by hematologic abnormalities and has a clone size much smaller than what is reflected in knock-out mice. In CHIP, low frequency clones with mutations in genes such as *TET2* expand over time and contribute increasingly to the hematologic output.[5,9,12,247] To model this condition, groups have often used mixed bone marrow chimeras that require lethal irradiation of the host.[18–20,248] However, since radiation leads to breakdown of intestinal integrity and systemic release of inflammatory mediators[249–251], these models do not represent the steady state expansion seen in CHIP and actually are confounded by factors known to influence *Tet2*$^{-/-}$ hematopoiesis.[17] To this end, I developed a different model to understand CHIP by injecting bone marrow directly into congenically marked neonates and following clonal expansion over time (Figure 14B). These neonatal chimeras demonstrate the engraftment and expansion advantage of *Tet2*$^{-/-}$ progenitors, because this fraction engrafts better and expands over time, whereas *Tet2*$^{+/+}$ marrow does not (Figure 14C). Additionally, as with CHIP, our neonatal chimeras do not display overt hematological abnormalities or develop myeloproliferation. Further, traditional chimeras show broad expansion of *Tet2*$^{-/-}$ clones in all measured hematopoietic lineages, whereas expansion of *TET2* mutated clones in humans favors myeloid lineages (Figure 14 D,E). In my neonatal chimeras, I also capture this critical difference and observe a myeloid-biased expansion in progenitor and mature immune cells (Figure 14E,F). As myeloid progenitors were preferentially expanded, and our laboratory had demonstrated previously increased expression of IL6Ra on their surface, I tested whether IL6 was required for clonal expansion. In both genetic models and using a depleting antibody, I found IL6 was not required for steady state clonal expansion, suggesting that other mechanisms are responsible for

the steady state expansion prior to myeloproliferation (Figure 14G). The study of these factors is of great interest to me and to other groups as CHIP has also been associated with other chronic diseases in addition to myeloid leukemias, as discussed above.[229,230,247] Studying the role of other cytokines in steady state clonal expansion using blocking antibodies would be a relatively straightforward and high throughput way to screen extrinsic signals. Additionally, studying which genes are differentially expressed during clonal expansion compared to wild type non-expanding clones and to input clones may reveal clonal expansion-specific molecular pathways. This analysis may also reveal signaling pathways that implicate environmental factors or cytokines that cannot be tested using blocking antibodies. Finally, a CRISPR-Cas9 library screen in which input cells are barcoded and randomly genetically modified may test genetic targets directly and provide hypothesis generating data. Clones that no longer expand in a chimera setting would be identified using sequencing of the blood and identification of targets that were disrupted by genetic editing. This would be a significant undertaking and would require very specific controls and lots of recipient hosts for statistical power. Wildtype cells would also have to undergo the same screen, although they engraft at much lower frequencies. If there are factors that non-specifically prevent clonal expansion, such as crucial metabolic pathways, then all wildtype engraftment would be abrogated as well. If this control is not well established, there is increased likelihood many false-positive targets will be identified. Thus, mechanistic targets would be revealed by identifying disrupted genes that alter the engraftment and clonal expansion of *Tet2*$^{-/-}$ cells, but not *Tet2*$^{+/,+}$ in a neonatal chimera setting. A final question that is of interest for future studies stems from my work with Lucy Godley's group in processing marrow samples from patients undergoing hip replacement surgery. In these studies, we obtained paired bone marrow samples from the hip being

replaced as well as pre- and post-operative peripheral blood samples. Using a sequencing panel that targeted CHIP-associated mutations, we identified a high prevalence of CHIP in our patient population that was dominated by *DNMT3A* and *TET2* mutations, as has been recurrently observed. Interestingly, there were some patients that had mismatched clones such that a clone was present in the bone marrow, but not in the blood, or vice versa. This suggested that hematopoietic clones involved in CHIP were not uniformly distributed and could be site specific. Further, in patients who were seen in follow-up, variant allele frequencies had changed or even become undetectable, suggesting perioperative treatments or the removal of a significant amount of hip marrow itself altered the clone size. These findings were primarily made by Afaf Osman, Hematology fellow in Lucy Godley's laboratory, and they are now under review at *Blood Advances*. I am a co-author on this manuscript. These findings suggest that in addition to studying the signals that drive clonal expansion in neonatal chimeras, one should also study the distribution of clones across organs and sites of hematopoiesis. Variation in progenitor niches may reveal certain sites to be preferential for clonal expansion. Collectively, applying single cell approaches to our neonatal chimera model and to human samples, there is opportunity to better characterize the mechanisms underlying CHIP.

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