UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Nod-like Receptor Protein 3 in Gut-Resident Regulatory T Cells

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Jesse Wei-Sheng Tai

Committee in Charge

Professor Li-Fan Lu, Chair
Professor Elina Zuniga, Co-Chair
Professor Wendy Huang

2020

The thesis of Jesse Wei-Sheng Tai is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____
Co-Chair

_____
Chair

University of California San Diego

2020

DEDICATION

This thesis is dedicated to the members of the Lu Lab, whom I thank for their patience and guidance over the past two years. I would especially like to thank my postdoctoral mentor, Dr. Chia-Hao Lin, for taking the time to teach me all the experimental techniques in the lab, and Professor Li-Fan Lu, for offering his advice in both the lab and in my future career.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

This thesis is coauthored with Lin, Chia-Hao and Cunha, Flavia. The thesis author is the primary author of this thesis.

ABSTRACT OF THE THESIS

The Role of Nod-like Receptor Protein 3 in Gut-Resident Regulatory T Cells

by

Jesse Wei-Sheng Tai

Master of Science in Biology

University of California San Diego, 2020

Professor Li-Fan Lu, Chair
Professor Elina Zuniga, Co-Chair

Regulatory T (Treg) cells are a subset of adaptive immune cells known for their immunosuppressive functions. Emerging evidence demonstrates that Treg exist throughout the body in tissue-specific sub-populations with specialized functions. RNA-seq analysis performed on mice during systemic autoimmune inflammation revealed high expression levels of Nod-like receptor protein 3 (NLRP3) in gut-resident Treg cells. NLRP3 is part of well-characterized inflammasome commonly associated with innate immune cells, but is beginning to be appreciated in adaptive immune cells as well. To determine the role of NLRP3 in gut-resident

Treg-mediated immunosuppression, we generated bone marrow chimeras (BMC) and found that NLRP3-deficiency in Treg cells does not contribute to Treg development or homeostasis, but does result in increased Teff production of IL-17A in the colon. Moreover, in an adoptive T cell transfer model of colitis, co-transfer of $NLRP3^{KO}$ Treg cells failed to rescue $Rag^{-/-}$ recipient mice from weight loss and led to increased Teff production of IFN-γ and IL-17A in the colon. Moreover, to gain further insights into the role of NLRP3 in Treg cells, in addition to the aforementioned studies with $NLRP3^{KO}$ Treg cells, we also generated a mouse model in which NLRP3 is constitutively-activated in a Treg-specific manner ($Foxp3^{cre}NLRP3^{iCA}$). After immunologically challenging these mice via *Citrobacter rodentium*, we have shown that colonic $Foxp3^{cre}NLRP3^{iCA}$Treg cells exhibit enhanced control over Teff production of IFN-γ which could potentially impact bacterial clearance. Collectively, by taking both loss-of-function and gain-of-function approaches, our data reveals a pivotal role of NLRP3 in gut-associated Treg cells in maintaining intestinal homeostasis.

INTRODUCTION

Chapter 1: Tissue-Specific Regulatory T Cells

Regulatory T (Treg) cells are a distinct subset of adaptive immune cells that are known for their immunosuppressive effector functions (Josefowicz et al., 2012). Treg cells are characterized by their expression of the master transcription factor Foxp3, which endows Treg cells with suppressor function (Fontenot et al., 2003). Indeed, loss-of-function mutation of the Foxp3 gene locus results in the development of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001), while causing multi-organ autoimmunity and early mortality in mice (Godfrey et al., 1991). Thus, Treg cells are essential for maintaining proper immune homeostasis and preventing autoimmune disorders.

It is well established that Treg cells are a heterogeneous population, and can upregulate certain migratory, functional, and homeostatic molecules in response to immune signaling in their environment (Campbell and Koch, 2011). Indeed, Treg cells exist throughout the body in tissue-specific niches, allowing them to employ tissue-specific transcriptional programs and effector functions to ensure immune homeostasis in their respective tissue environments (Panduro et al., 2016). For example, visceral adipose tissue (VAT)-resident Treg cells, in addition to expressing signature Treg markers such as Foxp3 and CD25, also express PPARγ, the master transcription factor of adipocyte differentiation (Cipolletta et al., 2012). PPARγ serves as a driver for the VAT-resident Treg cell phenotype, which is characterized by the differential expression of numerous genes: upregulation of genes involved in leukocyte migration and extravasation such as CCR1 and CCR2, upregulation of certain cytokines such as IL-10, and upregulation of genes involved in lipid metabolism such as CD36 and Dgat (Cipolletta et al.,

2012; Feuerer et al., 2009; Panduro et al., 2016). PPARγ is also at least partially responsible for the high expression of the ST2 receptor in VAT-resident Treg cells, which functions as a receptor for the alarmin IL-33 and contributes to the accumulation of VAT-resident Treg cells in the VAT tissue (Kolodin et al., 2015). VAT-resident Treg cells play an important role in regulating VAT inflammation, a key contributor to type 2 diabetes and obesity. Indeed, ablation of VAT-resident Treg cells results in inflammation and insulin resistance (Cipolletta et al., 2012). Moreover, PPARγ agonists, widely used to treat type 2 diabetes, required PPARγ expression in VAT-resident Treg cells to reach full efficacy, highlighting the importance of VAT-resident Treg cells in healthy VAT metabolism.

Another well-characterized group of tissue-resident Treg cells is skeletal muscle Treg cells, which have been shown to play an important role in muscle repair. This small population of Treg cells rapidly proliferate in response to muscle damage by cardiotoxin (Ctx) injection, and their proliferation coincides with the shift of macrophages from a pro-inflammatory state to an anti-inflammatory state (Burzyn et al., 2013). Such a shift is necessary for proper muscle repair. While skeletal muscle Treg cells express much of the canonical Treg gene signature, they also differentially express multiple genes that contribute to muscle repair. For instance, skeletal muscle Treg cells express higher levels of the anti-inflammatory cytokine IL-10, which plays an important role in inducing an anti-inflammatory macrophage phenotype (Villalta et al., 2011; Villalta et al., 2014). Skeletal muscle Treg cells also express high levels of the growth factor amphiregulin (Areg), which can act directly on the epithelial growth factor receptor (EGFR) on muscle progenitor cells (MPCs) and induce differentiation and muscle repair (Burzyn et al., 2013). Interestingly, similar to VAT-resident Treg cells, skeletal muscle Treg cells also express

high levels of ST2, which is important for Treg recruitment to injured skeletal muscle (Kuswanto et al., 2016).

Finally, another well-characterized group of tissue-resident Treg cells is colonic Treg cells. The colonic mucosa is a site of intense immune activity, as immune cells must simultaneously ward potential pathogens, tolerate dietary antigens, and tolerate commensal microbial communities. As such, colonic Treg cells play a major role in ensuring proper colonic homeostasis. Colonic Treg cells exist in two distinct subgroups: thymic-derived Treg cells (tTregs) which express GATA3, and peripherally-induced Treg cells (pTregs) which express retinoid-related orphan receptor gamma t (RORγt) (Ohnmacht et al., 2015; Sefik et al., 2015). GATA3 has been shown to induce ST2 expression in Th2 cells (Guo et al., 2009), and accordingly is also highly co-expressed with ST2 in colonic tTregs (Schiering et al., 2014). Functionally, ST2 was demonstrated to be important in tTreg accumulation during intestinal inflammation. Furthermore, ST2 signaling also contributed to tTreg stability and suppressor function in vivo. On the other hand, pTreg differentiation, defined as Tregs expressing RORγt, was found to be dependent on various environmental factors in the colon, namely IL-6, IL-23, and the microbiota, although conflicting results for IL-23 have been reported (Ohnmacht et al., 2015; Sefik et al., 2015). Ablation of the pTreg population, but not the tTreg population, rendered mice more susceptible to trinitrobenzenesulfonic acid (TNBS)-induced colitis, suggesting a non-redundant role of pTregs in colonic immune regulation (Sefik et al., 2015). However, the exact roles of these different subsets of Tregs remain unclear, although it has been hypothesized that pTregs primarily focus on decreasing inflammation while tTregs may have a larger role in tissue repair (Hegazy and Powrie, 2015).

While the existence and function of such tissue-specific Treg sub-populations are beginning to be explored, the precise suppressor mechanisms that each tissue-specific Treg sub-population employs to maintain immune homeostasis remains poorly described. To this end, we performed RNA-sequencing (RNA-seq) analysis on tissue-specific Treg cell subsets isolated from mice suffering from autoimmune inflammation. In brief, mice containing the coding sequence of a diphtheria toxin receptor (DTR) knocked into the 3' untranslated region of Foxp3 (Foxp3^{DTR} mice) were treated with diphtheria toxin (DT) to induce systemic Treg cell ablation as previously described (Kim et al., 2007). These mice were then rescued from the resultant autoimmune inflammation by injection of exogenous, congenically-marked Treg cells. By doing so, we hoped to induce expression of tissue-specific suppressor mechanisms in these exogenous Treg cells due to immune signaling in the inflammatory environment in which they were introduced. RNA-sequencing analysis on exogenous Treg cells extracted from different mouse organs revealed high levels of Nod-like receptor protein 3 (NLRP3) in exogenous Treg cells residing in the small intestine, suggesting that NLRP3 plays an important role in gut-resident Treg cell-mediated immunosuppression.

Chapter 2: Nod-like Receptor Protein 3

2.1. Overview of Nod-like Receptor Protein 3

The Nod-like receptor protein 3 (NLRP3) inflammasome is a well-characterized inflammasome commonly associated with the innate immune response (Swanson et al., 2019). Inflammasomes are cytosolic complexes which are generally comprised of an upstream pattern recognition receptor (PRR) sensor, the adaptor protein apoptosis-associated speck-like protein (ASC), and downstream effector caspases. Inflammasome assembly leads to the cleavage and activation of caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 into IL-1β and IL-18, respectively. To date, multiple inflammasome receptors have been characterized, including NLRP1, NLRP3, NLRC4, AIM2, and Pyrin (Kanneganti, 2015). The NLRP3 receptor is comprised of three sections: an N-terminal pyrin domain (PYD), a central nucleotide-binding and oligomerization domain (NOD), and a C-terminal leucine-rich repeat domain (LRR) (Kelley et al., 2019). During NLRP3 inflammasome activation, the NLRP3 pyrin domain oligomerizes and interacts using the ASC pyrin domain, which in turn utilizes its caspase activation and recruitment domain (CARD) to interact with the corresponding CARD domain on pro-caspase-1, stimulating its cleavage into caspase-1 (Agostini et al., 2004; Srinivasula et al., 2002). Since the initial discovery that gain-of-function mutations in NLRP3 are responsible for a family of diseases now known as cryopyrin-associated periodic syndromes (CAPS) (Aksentijevich et al., 2002; Hoffman et al., 2001), the mechanism of NLRP3 inflammasome activation has been extensively explored. In brief, NLRP3 inflammasome activation requires two signals: a priming signal which activates the transcription factor NF-κB, and an activation signal which leads to NLRP3 inflammasome assembly (Kelley et al., 2019). The priming signal can be met through the ligation of receptors such as toll-like receptors (TLRs) or Nod-like receptors (NLRs) to

pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). This signal ultimately leads to the activation of NF-κB, which then promotes the transcription of NLRP3 and pro-IL-1β, effectively licensing the cell for NLRP3 activation (Bauernfeind et al., 2009). The activation signal can be met through a wide, seemingly unrelated variety of cellular stress stimuli, including ionic flux, mitochondrial disruption, and lysosomal disruption (Swanson et al., 2019). While it is hypothesized that these signals converge into a common pathway that leads to NLRP3 activation, such a common denominator has yet to be discovered. The result of the activation signal is the full-fledged formation and activation of the inflammasome.

2.2 Nod-like Receptor Protein 3 in Intestinal Inflammation

The interleukins cleaved and released following inflammasome activation, IL-1β and IL-18, both belong to the IL-1 family of interleukins and are known to have pleiotropic roles in inflammation. Upon binding of an IL-1R family receptor, IL-1β can act on a wide range of innate and adaptive immune cell types, which is extensively reviewed elsewhere (Bent et al., 2018). Meanwhile, IL-18, originally known as IFN-γ-inducing factor, binds to the IL-18 receptor complex (Tsutsumi et al., 2014), which is also present on a wide range of innate and adaptive immune cell types, reviewed elsewhere (Yasuda et al., 2019). The roles of NLRP3, IL-1β, and IL-18 in intestinal inflammation is incredibly nuanced, as both protective and pathogenic roles have been attributed to the NLRP3 inflammasome (Mao et al., 2018). Initial studies on NLRP3-deficient mice demonstrated that mice lacking NLRP3 exhibited diminished mortality and symptoms upon induction of DSS-colitis and TNBS-colitis compared to controls, accompanied with a decrease in IL-1β (Bauer et al., 2012; Bauer et al., 2010). Moreover, gain-of-function

polymorphisms were identified in the NLRP3 gene in patients suffering from inflammatory bowel disease (IBD) (Schoultz et al., 2009; Verma et al., 2008). Additionally, high levels of IL-1β (Coccia et al., 2012; Ligumsky et al., 1990) and IL-18 (Pizarro et al., 1999) have been associated with IBD. Such data indicates that activation of the NLRP3 inflammasome may contribute to intestinal inflammation.

In contrast, other groups observed increased mortality and symptoms in NLRP3-deficient mice, suggesting a protective role of the NLRP3 inflammasome in intestinal inflammation (Dupaul-Chicoine et al., 2010; Zaki et al., 2010). Such protective effects were primarily attributed to the release of IL-18 by intestinal epithelial cells (IECs), as exogenous IL-18 rescued NLRP3-deficient mice and restored intestinal barrier function (Siegmund, 2010). Furthermore, polymorphisms within the NLRP3 gene locus, which decreased NLRP3 expression, also correlated with increased symptoms of IBD (Villani et al., 2009). Overall, such disparate findings have led to a great deal of controversy surrounding the role of the NLRP3 inflammasome and its role in regulating intestinal inflammation, and underscores the complex nature of inflammasome and interleukin biology.

2.3 Nod-like Receptor Protein 3 in Adaptive Immune Cells

While the aforementioned studies focused primarily on the role of the NLRP3 inflammasome in innate immune biology, recently the role of NLRP3 in adaptive immune cells has also begun to be appreciated. In a report on human T-helper type 1 (Th1) cells, TCR activation leads to an increased expression of an intracellular complement component C5a, which acts in an autocrine fashion and binds to the C5a receptor 1 (C5aR1). In turn, C5aR1 stimulation leads to the activation and assembly of the NLRP3 inflammasome (Arbore et al.,

2016). Following inflammasome assembly, IL-1β is released by Th1 cells and acts in an autocrine fashion to promote the Th1 phenotype, as NLRP3 inhibition attenuated IFN-γ production.

In mouse T-helper type 2 (Th2) cells, Bruchard et al. found that NLRP3 acts non-canonically as a transcription factor to support the Th2 transcriptional program in an inflammasome-independent fashion (Bruchard et al., 2015). They showed that during Th2 cell differentiation in vitro, NLRP3, and not caspase-1 or ASC, is upregulated and contributes to IL-4 expression. ChIP-seq analysis revealed that NLRP3 binds directly to Th2-related genes as a transcription factor. Nuclear localization of NLRP3 was dependent on expression of the importin protein karyopherinα (Kpna2). Furthermore, NLRP3 also uses its LRR domain to form a complex with key Th2-differentiation transcription factor IRF4. This complex then binds to the *Il4* promoter and drives transcription. Notably, Bruchard et al. also investigated the effects of NLRP3 deficiency on the differentiation of other $CD4^+$ T cell subtypes in vitro, and found that NLRP3, caspase-1, and ASC deficiency did not affect Foxp3 expression during Treg differentiation, though NLRP3 deficiency did modestly decrease expression of RORγt during T-helper type 17 (Th17) differentiation.

Another group found that during Th17 differentiation, pro-IL-1β is strongly induced, and IL-1β can be released from Th17 cells in a manner dependent on NLRP3, ASC, and caspase-8 (Martin et al., 2016). In vitro, IL-1β worked in an autocrine fashion and promoted the survival and proliferation of Th17 cells, which also expressed high levels of IL-1R. Furthermore, T-cell expression of ASC was required for Th17-mediated experimental autoimmune encephalomyelitis (EAE) pathogenesis.

Recently, a report regarding the role of NLRP3 as a transcriptional regulator in mouse Treg cells, analogous to its role in Th2 cells, was also published (Park et al., 2019). In this study, Park et al. show that NLRP3 serves as a negative regulator of Foxp3 expression in Treg cells in an inflammasome-independent manner in vitro. To this end, NLRP3-deficient Treg cells expressed higher amounts of Foxp3 on a per cell basis. This is contrary to Bruchard et al.'s report, in which no significant difference in Foxp3 expression was found during in vitro differentiation of NLRP3-deficient Treg cells (Bruchard et al., 2015). In a manner similar to the role of NLRP3 in Th2 cells as characterized by Bruchard et al., Park et al. found that co-localization of the LRR domain of NLRP3 with the importin Kpna2 in Treg cells also allowed for the translocation of NLRP3 into the nucleus in vitro. While the authors did not use ChIP-seq studies to find a consensus binding motif for NLRP3 near the Foxp3 gene locus, knockdown of the LRR domain of NLRP3 led to restoration of normal Foxp3 levels during in vitro differentiation, suggesting a transcriptional regulatory role of NLRP3 on Foxp3 expression.

In our study, we aim to continue to explore the role of NLRP3 in Treg cells in vivo, utilizing both loss-of-function and gain-of-function genetic manipulations in the NLRP3 gene locus. By utilizing bone marrow chimeras (BMCs), *Citrobacter rodentium* infection, adoptive T cell transfer colitis, and tumor models, we hope to shed light on the cell-extrinsic and cell-intrinsic role of NLRP3 in Treg cells.

RESULTS

Chapter 1: RNA-Sequencing Analysis of Tissue-Specific Regulatory T Cells in an Autoimmune Inflammation Model

Foxp3^{DTR} mice were treated with DT as previously described (Kim et al., 2007), resulting in fatal multi-organ autoimmunity characterized by hyperproliferation of CD4^{+}Foxp3^{-} cells, henceforth referred to as Teff cells (Fig. 1). Rescue of mice with injection of exogenous, congenically-marked Thy1.1^{+} Tregs resulted in a reduction of hyperproliferative Teff cells (Ki67^{+}) and hyperactivated Teff cells (CD25^{+}). Then, these activated Treg cells, as well as their Teff cell counterparts, were isolated from different anatomical regions from the mice including the spleen, lung, and small intestine lamina propria (SI LP), and utilized in RNA-seq analysis. Principal-component analysis (PCA) revealed a high degree of similarity between Treg cells, as well as Teff cells, isolated from the same tissue regardless of DT treatment status (Fig. 2a). However, DT treatment also resulted in a considerable level of similarity among the cells, regardless of cell origin (Fig. 2b). Such findings support the notion that Treg cells are greatly influenced by both environmental factors and inflammatory conditions. Next, to characterize the tissue-specific transcriptional program of intestinal Treg cells in controlling gut-associated inflammation, we used Scatter Plot analysis to compare genes that are differentially expressed in Treg cells vs Teff cells isolated from the SI LP with or without autoimmune inflammation (Fig. 3a). Our analysis of tissue-specific Treg cell transcriptome profiling data revealed high expression of *Nlrp3* specifically in Treg cells isolated from the inflammatory intestinal tissue, a phenotype which was not observed in Treg cells isolated from other inflammatory tissue (Fig. 3b). Moreover, genes involved in canonical NLRP3 function, including *Asc*, *Il1b*, and *Il18*, were also upregulated in intestinal Treg cells (Fig. 3c-e).

Chapter 2: Evaluating the Role of Nod-like Receptor Protein 3 using $NLRP3^{KO}$ Mice

2.1 Bone Marrow Chimera Studies

To evaluate the effects of NLRP3 specifically in Treg cells in vivo, we generated two different sets of bone marrow chimeras (BMC). To this end, mice with a germline deletion of NLRP3 ($NLRP3^{KO}$) were obtained. First, to evaluate the cell-extrinsic effects of Treg cells deficient in NLRP3, we mixed bone marrow (BM) cells from $Ly5.1^{+}$ mice with BM cells from either $NLRP3^{KO}$ mice or $NLRP3^{WT}$ mice. These mixtures were then injected intravenously (IV) to irradiated $Rag^{-/-}$ mice. Thus, in $Ly5.1^{+}/NLRP3^{KO}$ mice, NLRP3-deficient Treg cells are placed in a competitive environment with wild-type (WT) Treg cells. After 7-9 weeks, the animals were sacrificed and tissues were harvested for analysis. Interestingly, the absence of NLRP3 in Treg cells did not result in a change in the ratio of $Ly5.1^{-}/Ly5.1^{+}$ Treg cells in any tissue when compared to $Ly5.1^{+}/NLRP3^{WT}$ BMCs (Fig. 4). This suggests that both Treg development and homeostasis is not affected by the absence of NLRP3. Furthermore, no difference was found in the proliferation and activation of Teff cells, despite a trend for the reduction of Teff cell release of pro-inflammatory cytokines in several tissues (data not shown). However, such a reduction in pro-inflammatory cytokines was expected, as other studies have demonstrated that NLRP3 is important for Th1 and Th17 responses (Arbore et al., 2016; Martin et al., 2016).

Next, to evaluate the cell-intrinsic effect of NLRP3 in Treg cells, we generated another set of BMCs. BM cells from $Foxp3^{KO}$ mice were mixed with BM cells from either $NLRP3^{KO}$ mice or $NLRP3^{WT}$ mice, and injected IV to irradiated $Rag^{-/-}$ mice. As such, $Foxp3^{KO}/NLRP3^{KO}$ BMCs only had Treg cells that did not express NLRP3. When evaluating the Treg-specific cell-intrinsic effect of NLRP3 in $Foxp3^{KO}/NLRP3^{KO}$ BMCs, no difference in the number of Treg cells in any tissue was found when compared to $Foxp3^{KO}/NLRP3^{WT}$ BMCs (Fig. 5), further

suggesting that NLRP3 does not play a role in Treg cell homeostasis. Furthermore, the activation and proliferation of Teff cells was unchanged (data not shown). In terms of cytokine production, we observed an increasing trend in IFN-γ and TNFα released by Teff cells in some tissues, but only a statistically significant increase was observed in IL-17A production by Teff cells in the large intestine in $Foxp3^{KO}$/$NLRP3^{KO}$ BMCs (Fig. 6a). These results suggest that the absence of NLRP3 in Treg cells affects their ability to regulate the Th17 inflammatory response specifically in the large intestine.

2.2 Adoptive T Cell Transfer Colitis

Since our findings studying the $Foxp3^{KO}$/$NLRP3^{KO}$ BMC model suggest that NLRP3-deficient Treg cells have a reduced ability to control colonic inflammation, we aimed to test the functional significance of Treg-specific NLRP3-deficiency using an intestinal inflammation-driven disease model. Currently, because we lack a mouse model with a conditional knockout of NLRP3 in Treg cells ($Foxp3^{cre}NLRP3^{fl/fl}$), we utilized a previously described adoptive T cell transfer-induced intestinal inflammation model in which severe colitis is induced upon transfer of naïve $CD4^+$ Teff cells into immunodeficient $Rag^{-/-}$ recipient mice (Izcue et al., 2008). In this model, co-transfer of Treg cells rescues recipient mice from immunopathology (Mottet et al., 2003). Thus, we aimed to co-transfer either $NLRP3^{KO}$or WT Tregs to rescue recipient mice from such immunopathology. By comparing the efficacy of $NLRP3^{KO}$ or WT Tregs in rescuing recipient mice, we hoped to gain further insights on the function of NLRP3 in Treg-mediated immunosuppression. To this end, we intraperitoneally (i.p.) injected FACS-sorted $CD4^+CD45RB^{hi}$ T cells with or without WT or $NLRP3^{KO}$ Treg cells into $Rag^{-/-}$ mice and monitored for weight loss as a proxy of intestinal inflammation (Fig. 7a). As expected, we

observed a sharp decrease in body weight among $Rag^{-/-}$ recipients of Teff cells only, a phenotype which was abrogated when WT Treg cells were co-transferred. However, when co-transferring $NLRP3^{KO}$ Treg cells instead of WT Treg cells, mice once again exhibited a sharp decrease in body weight, suggesting that $NLRP3^{KO}$ Treg cells are defective in protecting mice from pathologic intestinal inflammation.

When recipient $Rag^{-/-}$ mice lost significant body weight, all mice were sacrificed, and tissues were processed for flow cytometry analysis. While Treg cell frequency, Teff activation, and Teff proliferation were not different between $Rag^{-/-}$ recipients co-transferred with WT Treg cells or $NLRP3^{KO}$ Treg cells in all tissues (data not shown), we observed an increasing trend in Teff production of both IFN-γ and IL-17A in the large intestine of $Rag^{-/-}$ recipients co-transferred with $NLRP3^{KO}$ Treg cells (Fig. 7b-c). We plan on continuing to run more of these experiments once the requisite mice are bred to gather enough data to reach a statistical significant difference. Nevertheless, just as in the $Foxp3^{KO}$/$NLRP3^{KO}$ BMC model, the data from the adoptive T cell transfer colitis model suggests that $NLRP3^{KO}$ Treg cells are deficient in their ability to control Th17 cell responses, while also possibly being deficient in their control of Th1 cell responses.

2.3 Establishing the $Vil1^{cre}APC^{fl/+}$ Model to Study the Role of Nod-like Receptor Protein 3 in Regulatory T Cells

Next, we attempted to use $NLRP3^{KO}$ Treg cells in an adoptive transfer $Vil1^{cre}Apc^{fl/+}$colorectal cancer (CRC) model (Cheung et al., 2010) to investigate whether NLRP3 may play a role in Treg-mediated immunosuppression of tumor-promoting inflammation in the gut. $Vil1^{cre}Apc^{fl/+}$ mice express cre recombinase under control of the Vil1 promoter, a gene expressed in intestinal epithelial cells, and contain a floxed *Apc* allele. Deleting the *Apc* gene

causes Vil1^{cre}Apc$^{fl/+}$ mice to spontaneously develop tumors at approximately 3 months of age. Loss-of-function of the *Apc* gene has previously been shown to cause tumorigenesis through IL-17A-mediated genotoxic inflammation (Chae et al., 2010). Thus, while generally Treg cells are thought to play a role in protecting cancer from the immune system, in the case of this CRC mouse model, Treg cells can instead prevent tumorigenesis by suppressing tumor-promoting inflammation. To this end, a previous study by Erdman et al. showed that exogenous injection of WT Treg cells can reduce tumor numbers in Apc$^{min/+}$ mice (Erdman et al., 2005). Thus, we aimed to determine whether NLRP3-deficiency would prevent NLRP3^{KO} Treg cells from rescuing Vil1^{cre}Apc$^{fl/+}$mice from spontaneous CRC. To this end, we planned to rescue 3-month old Vil1^{cre}Apc$^{fl/+}$ mice from tumor-promoting inflammation via adoptive transfer of either WT Treg cells or NLRP3^{KO} Treg cells, as previously described (Erdman et al., 2005). By monitoring tumor numbers and immune phenotype, we hoped to determine whether NLRP3 plays a role in Treg immunosuppression of tumor-promoting inflammation. However, upon adoptive transfer of WT Treg cells to Vil1^{cre}Apc$^{fl/+}$ mice, we were unable to reproduce a reduction in tumor number as found in previous studies (Erdman et al., 2005) (Fig. 8). In the future, we plan to further optimize this model by injecting a greater number of Treg cells, or by injecting mice earlier than 3 months. Alternatively, we may switch to another IL-17A-driven CRC model such as the AOM/DSS model, which we could then directly induce into Foxp3^{cre}NLRP3$^{fl/fl}$ mice once obtained (De Robertis et al., 2011; Hyun et al., 2012).

Chapter 3: Evaluating the Role of Nod-like Receptor Protein 3 using Foxp3^{cre}NLRP3^{iCA} Mice

To further our understanding of the role of NLRP3 in Treg cells, we generated a mouse line with a Treg-conditional, constitutively-active form of NLRP3 by taking advantage of the previously described NLRP3$^{A350VneoR/+}$ mouse line (Brydges et al., 2009). The NLRP3$^{A350VneoR/+}$ mouse line houses a reverse-orientation, floxed neomycin cassette upstream of a constitutively-active NLRP3^{A350V} mutant allele. The reverse-orientation neomycin cassette transcriptionally silences NLRP3^{A350V} until floxed out by cre recombinase. Thus, only cells expressing the cre recombinase protein will be induced to express a constitutively-active form of NLRP3. By crossing Foxp3^{cre} mice with NLRP3$^{A350VneoR/+}$ mice, we generated Foxp3^{cre}NLRP3$^{A350VneoR/+}$ mice, henceforth referred to as Foxp3^{cre}NLRP3^{iCA} mice. In sum, this newly-generated Foxp3^{cre}NLRP3^{iCA} mouse line has a constitutively-active form of NLRP3 specifically in Treg cells.

At steady-state (3-4 months of age), Foxp3^{cre}NLRP3^{iCA} mice were sacrificed along with age-matched Foxp3^{cre} controls, and their tissues were harvested and cells were extracted. Following ex vivo stimulation and flow cytometry analysis, we found that there was no difference in Treg frequency between Foxp3^{cre} mice and Foxp3^{cre}NLRP3^{iCA} mice in all tissues (Fig. 9, spleen and large intestine data shown). Furthermore, there was no difference in Treg or Teff activation and proliferation (data not shown). Finally, there was no statistically significant difference in cytokine production by Teff cells (Fig. 10, spleen and large intestine data shown), although there appears to be a decrease in IFN-γ production both in the large intestine and in the spleen.

Although we did not observe any differences in immune phenotype in Foxp3^{cre}NLRP3^{iCA} mice at steady-state, we speculated that because the mice were healthy, any deficiencies or

advantages Foxp3^{cre}NLRP3^{iCA} Treg cells had in immunosuppression would not be detected because Foxp3^{cre}NLRP3^{iCA} Treg cells were not being actively used to suppress pathogenic immune activation. Thus, we decided to immunologically challenge Foxp3^{cre}NLRP3^{iCA} mice, and because the BMC and adoptive T cell transfer colitis models suggested that NLRP3 is important for Treg-regulation of Th17 cells, we planned to infect Foxp3^{cre}NLRP3^{iCA} mice with *Citrobacter rodentium*. *C. rodentium* is a bacterial pathogen used in mice to model the pathogenesis of human *Escherichia Coli* infection (Bouladoux et al., 2017). *C. rodentium* infection manifests in the colon of mice, and is known to elicit a strong IL-17A response, which is important in host clearance of the bacteria (Ishigame et al., 2009). To first confirm that NLRP3 in Treg cells is indeed important for controlling *C. rodentium* infection-mediated inflammation, we infected wild-type mice with *C. rodentium* and sorted out wild-type Treg and Teff cells isolated from the spleen and large intestine. Then, ten days following induction of infection, we used quantitative polymerase chain reaction (qPCR) to check for NLRP3 gene expression in these cell subsets. As expected, following *C. rodentium* infection, Treg cells specifically from the large intestine upregulated NLRP3 gene expression (Fig. 11). Thus, we concluded that NLRP3 is important in colonic Treg cell-mediated immunosuppression of *C. rodentium* infection, and proceeded to infect Foxp3^{cre}NLRP3^{iCA} mice and Foxp3^{cre} controls with *C. rodentium*.

Ten days following infection, mice were sacrificed and tissues were harvested for flow cytometry analysis. Following *C. rodentium* infection, the trend in decreasing Teff production of IFN-γ became statistically significant in the large intestine of Foxp3^{cre}NLRP3^{iCA} mice (Fig. 12a). Meanwhile, there was no difference in Teff production of IFN-γ in the spleen (Fig. 12b), nor was there any difference in IL-17A production both in the large intestine or spleen (Fig. 12 c-d).

Fecal samples from the mice were also gathered on Day 10 to check the bacterial burden of *C. rodentium*. Interestingly, we found that there is an increasing trend in colony-forming units (CFU) in Foxp3^{cre}NLRP3^{iCA} mice compared to Foxp3^{cre} controls, a trend that is weakened by the presence of three Foxp3^{cre}NLRP3^{iCA} outliers with exceptionally low CFU/g fecal matter (Fig. 13). Initially, we did not anticipate such a trend because there was no difference in IL-17A production in the two mouse lines, as IL-17A plays an established role in clearance of the *C. rodentium* infection (Ishigame et al., 2009). However, other studies have also demonstrated a protective role for IFN-γ during *C. rodentium* infection as well, as IFN-γ-deficient mice exhibited slower clearance of *C. rodentium* (Shiomi et al., 2010; Simmons et al., 2002). This effect has previously been attributed to a decrease in mouse β-defensin 3 (mBD-3) production in intestinal epithelial cells in IFN-γ-deficient mice (Simmons et al., 2002) as well as a decreased efficiency in macrophage phagocytosis (Shiomi et al., 2010). Thus, we conclude that Treg cells with a constitutively-active form of NLRP3 are more able to control IFN-γ release from Teff cells, which leads to the delay of *C. rodentium* clearance.

DISCUSSION

Treg cells exist throughout the colon in two distinct subpopulations: tTregs which express GATA3, and pTregs which express RORγt (Ohnmacht et al., 2015; Sefik et al., 2015). While it is well-known that colonic Treg cells play diverse roles in preventing intestinal inflammation (Boden and Snapper, 2008; Panduro et al., 2016) and promoting commensal tolerance (Izcue et al., 2009), the precise suppressor mechanisms that colonic Treg cells employ remain poorly described. Here, we report a role for NLRP3 in colonic Treg-mediated immunosuppression. During intestinal inflammation, Treg cells in the intestine upregulate genes related to the canonical function of the NLRP3 inflammasome, including *Nlrp3, Asc, Il1b,* and *Il18*. During BMC experiments, irradiated $Rag^{-/-}$ mice reconstituted with BM cells from both $Foxp3^{KO}$ and $NLRP3^{KO}$ mice lack Treg cells expressing NLRP3, and consequently display an increase in IL-17A production by Teff cells. This phenotype was limited to the large intestine, suggesting that NLRP3 plays an important role in colonic Treg cell-mediated immunosuppression of Th17 cells. This phenotype is unlikely to be due to changes in Treg stability in NLRP3-deficient Treg cells, as $Foxp3^{KO}$/$NLRP3^{KO}$ BMCs had similar numbers of Treg cells in all tissues when compared to $Foxp3^{KO}$/$NLRP3^{WT}$ BMCs. Moreover, $Ly5.1^{+}$/$NLRP3^{KO}$ BMCs had a similar ratio of $Ly5.1^{-}$/$Ly5.1^{+}$ Treg cells as $Ly5.1^{+}$/$NLRP3^{WT}$ BMCs, further ruling out the possibility that NLRP3 deficiency in Treg cells leads to a change in stability of Foxp3 expression and Treg homeostasis.

Previously, another report suggests that NLRP3 expression can suppress in vitro-induced Treg (iTreg) generation by acting as a transcriptional regulator of the Foxp3 gene (Park et al., 2019). This report also showed an in vivo increase in the Treg cell population in $NLRP3^{KO}$ mice; however, it is unclear whether such an increase is due to Treg-intrinsic NLRP3 expression or due to environmental factors altered due to NLRP3-deficiency in other cell types. Regardless, in our

aforementioned experiments, we did not find any decrease in Foxp3 expression due to NLRP3-deficiency. It is unclear whether this is due to the lymphopenic environment within $Rag^{-/-}$ recipient mice, which may obscure any survival advantages that $NLRP3^{KO}$ Treg cells may hold. Future studies on $Foxp3^{cre}NLRP3^{fl/fl}$ mice will help clarify the role of NLRP3 as a transcriptional regulator of Foxp3 expression in vivo. The same report (Park et al., 2019) also shows a decreased immunosuppressive ability in $NLRP3^{KO}$iTreg cells, which we were unable to replicate (data not shown).

To further study the effects of NLRP3-deficiency on Treg cell-mediated immunosuppression, we utilized a previously established adoptive T cell transfer model to induce colitis (Izcue et al., 2008; Mottet et al., 2003). We found that when Teff cells were co-transferred with $NLRP3^{KO}$ Treg cells instead of WT Treg cells, recipient mice succumbed to severe body weight loss, just as in recipients of Teff cells only. Further analysis revealed that mice injected with a co-transfer of $NLRP3^{KO}$ Treg cells exhibited increased Teff production of IFN-γ and IL-17A in the large intestine.

While we are in the process of obtaining a mouse model with a Treg-conditional deletion of NLRP3 ($Foxp3^{cre}NLRP3^{fl/fl}$) to further our studies on NLRP3-deficiency in Treg cells, in the meantime we have obtained a mouse model with a Treg-conditional constitutively-active form of NLRP3 ($Foxp3^{cre}NLRP3^{iCA}$). At steady-state, we found no changes in immune phenotype in these mice when compared to $Foxp3^{cre}$ controls. Following *C. rodentium* infection, $Foxp3^{cre}NLRP3^{iCA}$ mice appeared to have a greater bacterial burden. Since IL-17A is important in clearance of *C. rodentium* infection (Ishigame et al., 2009), and because $NLRP3^{KO}$ Treg cells were deficient in their control of Th17 responses, we hypothesized that $Foxp3^{cre}NLRP3^{iCA}$ Treg cells were more capable of suppressing IL-17A secretion, which led to the greater bacterial

burden. However, after flow cytometry analysis, Foxp3^{cre}NLRP3^{iCA} mice showed no change in IL-17A production and instead showed a decrease in IFN-γ production. Previous works have also implicated IFN-γ in clearance of *C. rodentium* infection. Such studies have attributed this role to IFN-γ's ability to induce the antimicrobial peptide mBD-3 as well as to induce efficient phagocytosis by macrophages (Shiomi et al., 2010; Simmons et al., 2002). Overall, we propose a model in which NLRP3 is upregulated in colonic Treg cells upon intestinal inflammation, which endows Treg cells with suppressive function to act on such inflammation with possible implications during bacterial infection (Fig. 14).

Future directions for this project include mechanistic studies on the function of NLRP3 in Treg cells in vivo. Although our RNA-seq data indicates that genes involved in the canonical function of NLRP3 are upregulated, Park et al. and Bruchard et al. show that NLRP3 inflammasome assembly is not necessary for the role of NLRP3 as a transcriptional regulator in Treg cells and Th2 cells, respectively (Bruchard et al., 2015; Park et al., 2019). Thus, utilizing ChIP-seq experiments on colonic Treg cells during inflammation may yield insights into which genes NLRP3 may be influencing. To this end, a Treg-conditional mutation in the LRR domain, the domain in NLRP3 necessary for association with the Kpn2 importin and nuclear localization, may prove to be useful. However, because genes involved in canonical NLRP3 function are upregulated during autoimmune inflammation, and because we found shifts in immune phenotype in Foxp3^{cre}NLRP3^{iCA} mice, which harbor an NLRP3^{A350V} mutation resulting in hyperactivity of the NLRP3 inflammasome, we hypothesize that NLRP3 inflammasome assembly occurs in colonic Treg cells during inflammation as well. It is possible that a combination of canonical NLRP3 inflammasome assembly as well as the function of NLRP3 as a transcription factor both contribute to colonic Treg cell-mediated immunosuppression. Staining

for inflammasome speck formation, and flow cytometry analysis for IL-1β and IL-18 production in Treg cells ex vivo from mice suffering from intestinal inflammation may help test this hypothesis.

Since intestinal Treg cells have been implicated in the pathogenesis of CRC (Erdman and Poutahidis, 2010) and have previously been shown to prevent CRC-promoting inflammation (Erdman et al., 2005), in the future we hope to either optimize the $Vil1^{cre}Apc^{fl/+}$ adoptive transfer model or establish the AOM/DSS CRC model to investigate the effects of NLRP3 in Treg cell-mediated immunosuppression of CRC-promoting inflammation. We plan to modify our adoptive transfer protocol by injecting more Treg cells than previously reported, or by injecting mice earlier than 3 months. Otherwise, inducing the AOM/DSS model directly on $Foxp3^{cre}NLRP3^{fl/fl}$ and $Foxp3^{cre}NLRP3^{iCA}$ mice will also allow us to study the effects of NLRP3 in Treg cells in the context of CRC. Hopefully, our studies will open therapeutic avenues to prevent CRC progression through manipulation of NLRP3 in Treg cells.

MATERIALS & METHODS

Mice

Mice containing a germline deletion of NLRP3 ($NLRP3^{KO}$) were provided by Dr. Hal Hoffman of UCSD. $Vil1^{cre}ApcMin^{fl/+}$ were provided by Dr. Wendy Huang of UCSD. $NLRP3^{A350V}$, $Rag^{-/-}$, Ly5.1, and C57BL/6J mice were obtained from Jackson Laboratories. All mice were maintained and handled in accordance with the Institutional Guidelines for Animal Care and Use of UCSD and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the ARRIVE Guide.

Ex Vivo Phenotyping and Flow Cytometry

Single-cell suspensions of the thymus, lymph nodes, and spleen were prepared using slides and nytex filters. To isolate lymphocytes in the lung and small intestine and large intestine lamina propria, tissues were harvested and washed in RPMI-1640, and epithelial cells were removed (lamina propria- 5 mM EDTA and 1 mM DTT), followed by enzymatic digestion (0.16 U/ml Liberase TL; Roche), and 47% Percoll gradient centrifugation to enrich the lymphocytes.

Cells were labeled in FACS buffer (5% FBS in PBS) and then stained with Ghost Dye Red 780 (Tonbo Biosciences) to label dead cells. Then, cell surface markers were stained with antibodies against CD4, CD8α, CD44, CD62L, CD45, and Ly5.1 (eBioscience). For intracellular staining, a Foxp3/Transcription Factor Staining kit (Tonbo Biosciences) was used according to the manufacturer's instructions. Intracellular staining of Foxp3, Ki67, T-bet, RORγt, IFN-γ, and IL-17A (eBioscience) was performed after fixation and permeabilization.

DT-Ablation of Regulatory T Cells and RNA-sequencing

Foxp3^{DTR} mice (3-4 months) were treated with DT and Thy1.1^{+}-labeled Treg cells every other day for 11 days. At the end of 11 days, the tissues were harvested and Treg cells and Teff cells were isolated by sorting. RNA-seq was then performed to characterize altered gene expression.

Bone Marrow Chimera Generation

BM cell suspensions were extracted from femurs and tibias. These suspensions were then depleted of T cells using magnetic beads coated with the Thy1.1 antibody. Next, either Ly5.1^{+} or Foxp3^{KO} BM was mixed with either NLRP3^{WT} or NLRP3^{KO} BM in a 1:1 ratio. The resulting mixtures were injected IV in irradiated Rag$^{-/-}$ mice. The mice were maintained with water with neomycin (20 mg/mL) and sacrificed 7 to 9 weeks post-injection for tissue analysis.

Adoptive T Cell Transfer Colitis

Naïve, FACS sorted CD4^{+}CD45RB^{hi} T cells were isolated from spleens of wild-type mice. Briefly, following CD4^{+} T cell enrichment, cell suspensions were stained with anti-CD4, anti-CD25, and anti-CD45RB (eBioscience). Then, CD4^{+}CD25^{-}CD45RB^{hi} T cells were sorted. Furthermore, CD4^{+}CD25^{+}CD45RB^{lo} Treg cells were also sorted from wild-type or NLRP3^{KO} mice. Sex-matched Rag$^{-/-}$ recipient mice were then given an i.p. injection with 4×10^{5} CD4^{+}CD25^{-}CD45RB^{hi} T cells, with or without co-injection of 10^{6} WT or NLRP3^{KO} Treg cells. Then, the body weights of recipient mice were monitored until severe weight loss was observed, upon which mice were sacrificed and tissues were harvested for analysis.

Quantitative Real-Time PCR

Treg cells from Foxp3^{GFP} mice 10 days after *C. rodentium* infection were FACS-sorted from the large intestine of mice. Total RNA was extracted using the RNeasy kit (QIAGEN), and complementary DNAs (cDNAs) were generated by the iScript cDNA synthesis kit (Bio-Rad Laboratories). Real-time PCR was performed using SYBR Green PCR kits (Applied Biosystems). All real-time reactions were run on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). Primer sequences are as follows: GAPDH: F: 5'-CGTCCCGTAGACAAAATGGT-3', R: 5'-TCAATGAAGGGGTCGTTGAT'3'; NLRP3: F: 5'-AGAAGAGACCACGGCAGAAG-3', R: 5'-CCTTGGACCAGGTTCAGTGT-3'.

Citrobacter rodentium Infection

On Day 1, the DBS100, sodium iodide (NaI)-resistant strain of *C. rodentium* was streaked out on an LB and NaI plate. Our strain was obtained from Dr. Manuela Raffatellu in the School of Medicine, UCSD. The plate was then incubated at 37°C overnight. On Day 2, a single colony was used to form an overnight culture in LB and NaI with aeration. Approximately 2 mL of overnight culture was generated per mouse infected. On Day 3, each culture was diluted in 1:10 LB, and then the OD_{600} of the culture was measured. Next, 5.0 x 10^9 CFU/mouse was gathered and spun down at 3200 x g for 20 min at 4°C. The pellet was re-suspended in PBS so that there was 100 μl per mouse to be infected. Then, mice were infected by oral gavage. During this time, mouse cages were unchanged so that *C. rodentium* could be transmitted via ingestion of mouse feces. At Day 10 post-infection, mice were sacrificed, tissues were harvested, colon length was measured, and feces were gathered for analysis.

Adoptive Transfer in Vil1^{cre}ApcMin$^{fl/+}$ Mice

Treg cells were isolated from the spleen of wild-type mice, as described above, and were injected i.p. into Vil1^{cre}ApcMin$^{fl/+}$ mice at 3 months of age. Then, three weeks later, Vil1^{cre}ApcMin$^{fl/+}$ mice were sacrificed, and the duodenum, ileum, jejunum, and colon were harvested. All sections of the intestine were subsequently cut open lengthwise, and tumor numbers were counted.

Statistical Analysis

The unpaired Student t test or the non-parametric Mann-Whitney test were used. P values below 0.05 were considered significant.

This thesis is coauthored with Lin, Chia-Hao, Cunha, Flavia, and Lu, Li-Fan. The thesis author is the primary author of this thesis.

FIGURES & FIGURE LEGENDS

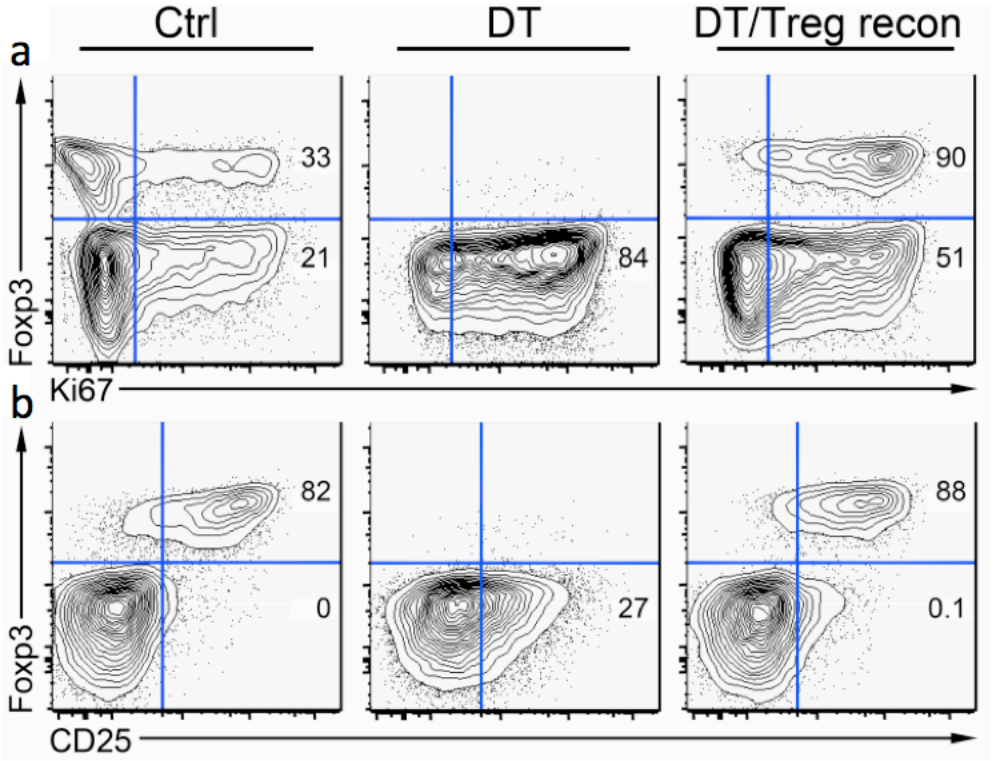

Figure 1. Mice with autoimmune inflammation following Treg cell ablation were rescued with transfer of exogenous Treg cells. The frequency of $Ki67^{+}$ (a) and $CD25^{+}$(b) cells within the $CD4^{+}$ T cell compartment isolated from $Foxp3^{DTR}$ mice treated with PBS, DT, and DT with transfer of congenically marked Treg cells.

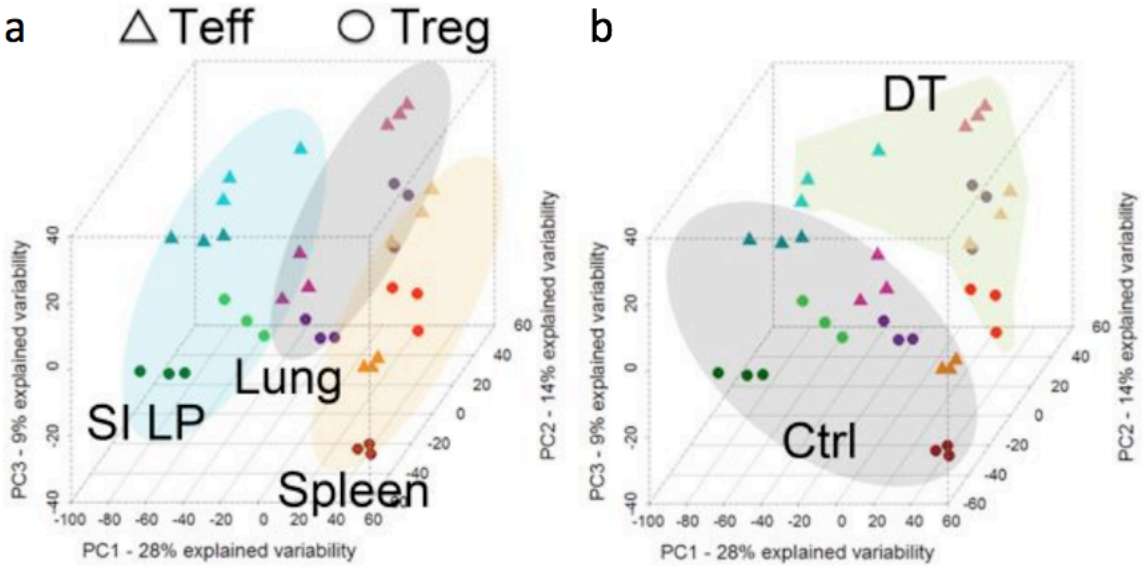

Figure 2. PCA of gene expression by different Treg and Teff cell subsets. Grouping was made by anatomical location (a) or DT treatment status (b). Axes indicate the relative scaling of the principal variables.

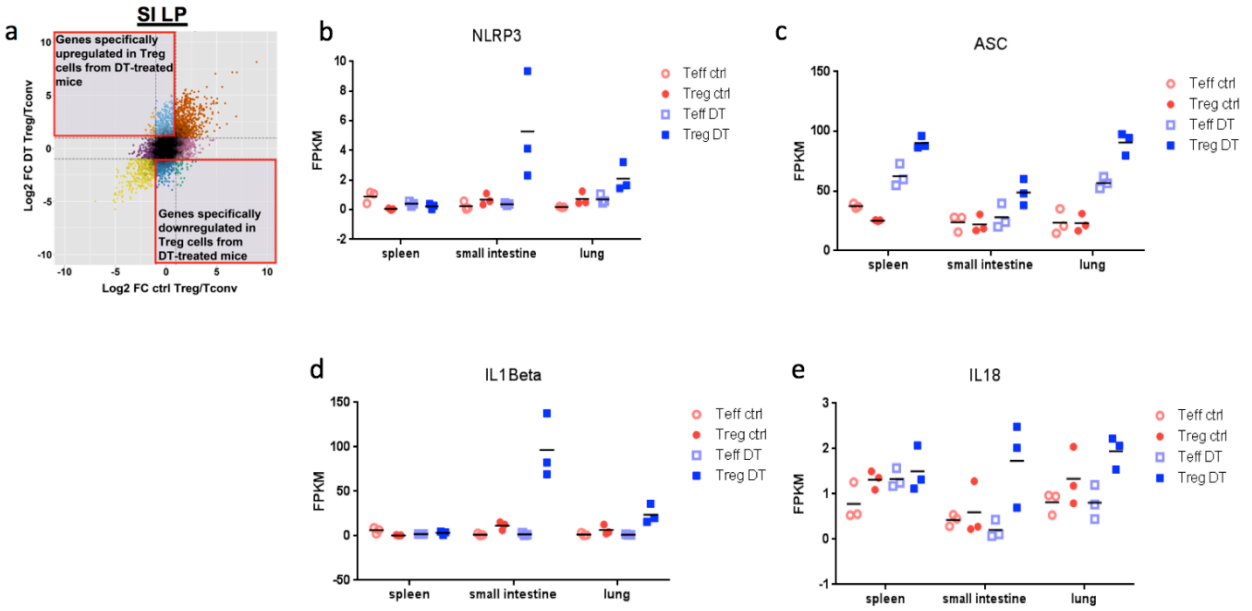

Figure 3. Elevated NLRP3 expression in intestinal Treg cells isolated from the inflammatory environment. Scatter plot analysis depicting log2 fold changes of genes differentially-expressed in SI LP Treg cells over Teff cells in DT-treated mice versus those in PBS-treated controls (a). mRNA expression of NLRP3 (b), ASC (c), IL-1β (d), and IL-18 (e) in tissue-specific Treg and Teff cell subsets, isolated from DT-treated mice and PBS-treated controls.

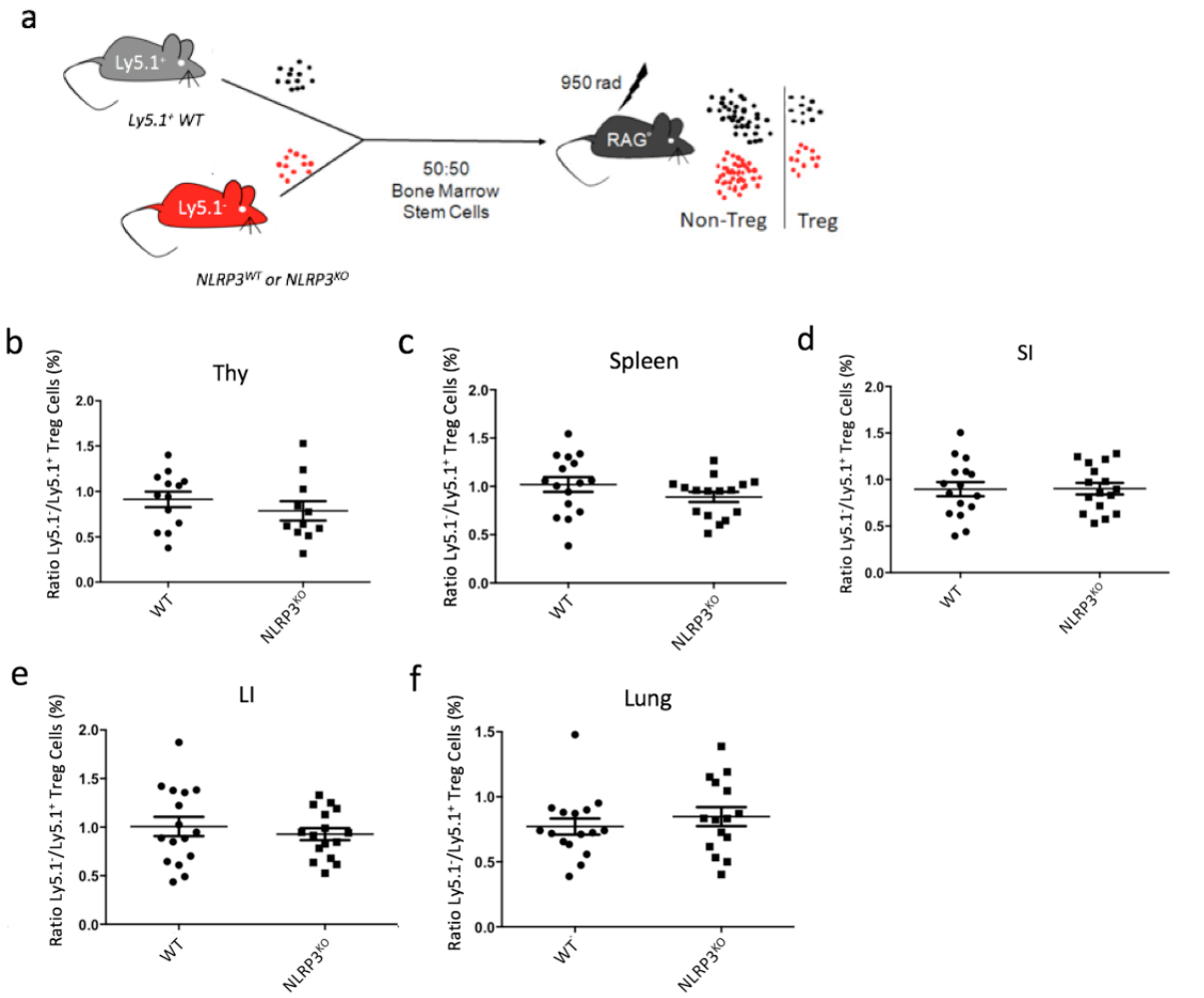

Figure 4. Evaluation of NLRP3-deficiency on Treg homeostasis in a competitive environment. BM cells from $Ly5.1^{+}$ and $NLRP3^{KO}$ or $NLRP3^{WT}$ mice were mixed and transferred IV to irradiated $Rag^{-/-}$ mice as shown (a). Tissues were harvested after 7-9 weeks. Cells were extracted, stimulated ex vivo, stained, and analyzed via FACS. The number of Treg cells was normalized calculating a ratio between $Ly5.1^{-}$ and $Ly5.1^{+}$ cells. This ratio was evaluated in different tissues, including the thymus (Thy) (b), spleen (c), small intestine (SI) (d), large intestine (LI) (e), and lung (f).

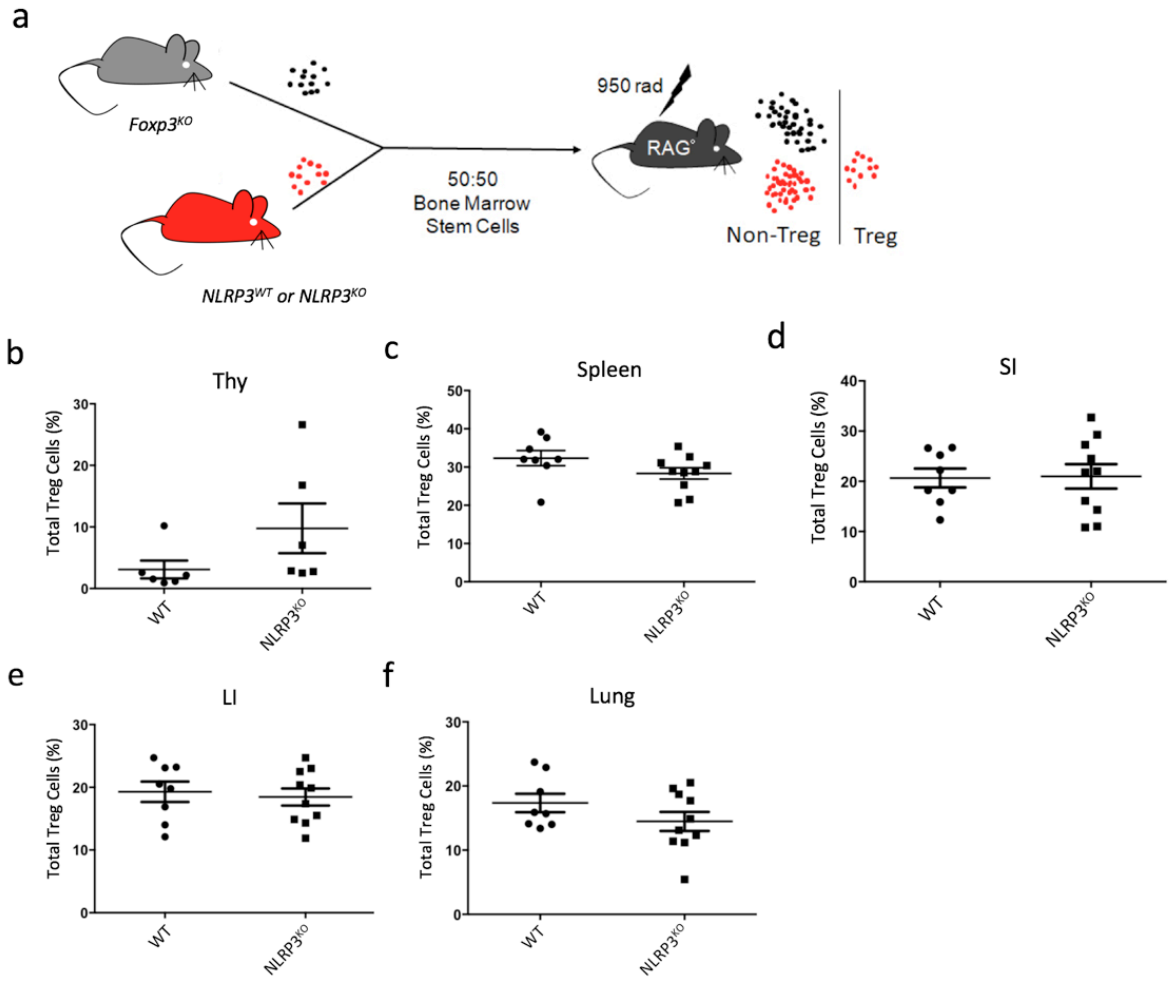

Figure 5. Evaluation of Treg-specific NLRP3-deficiency on Treg homeostasis. BM cells from Foxp3^{KO} and NLRP3^{KO} or NLRP3^{WT} mice were mixed and transferred IV to irradiated Rag$^{-/-}$ mice as shown (a). Tissues were harvested after 7-9 weeks. Cells were extracted, stimulated ex vivo, stained, and analyzed via FACS. Total Treg cell number in the thymus (Thy) (b), spleen (c), small intestine (SI) (d), large intestine (LI) (e), and lung (f) are shown.

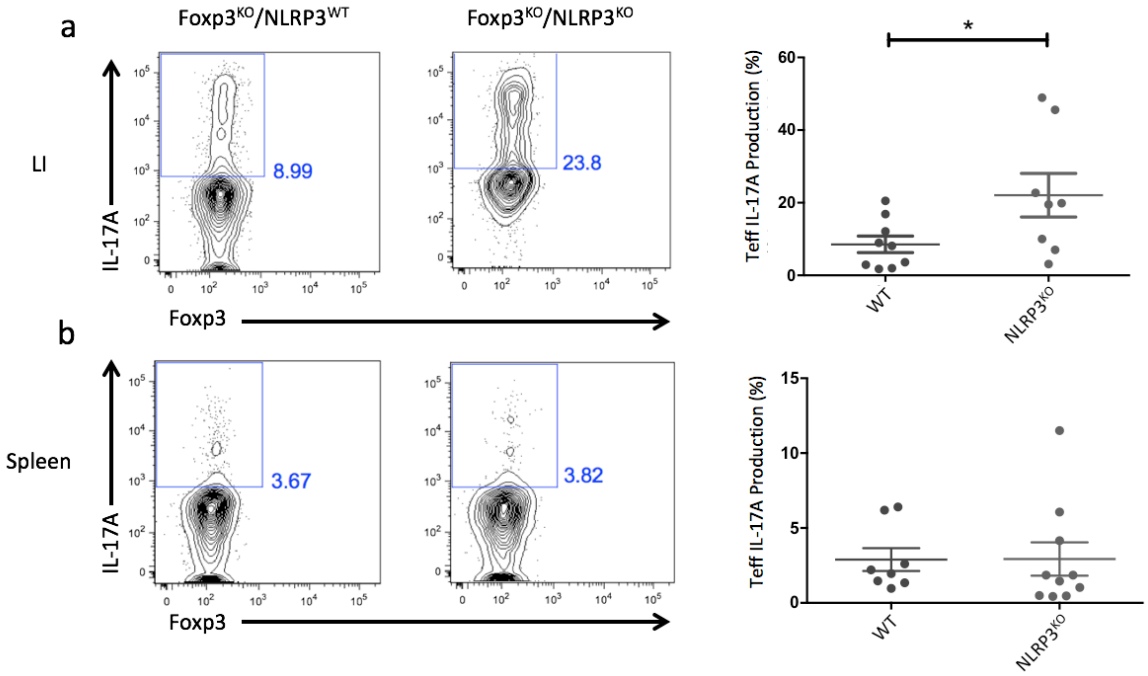

Figure 6. Evaluation of Treg-specific NLRP3-deficiency on immune phenotype. Foxp3^{KO}/NLRP3^{WT} and Foxp3^{KO}/NLRP3^{KO} BMCs were sacrificed 7-9 weeks after injection. Cells were extracted, stimulated ex vivo, stained, and analyzed via FACS. Teff production of IL-17A from the large intestine (a) and spleen (b) is shown.

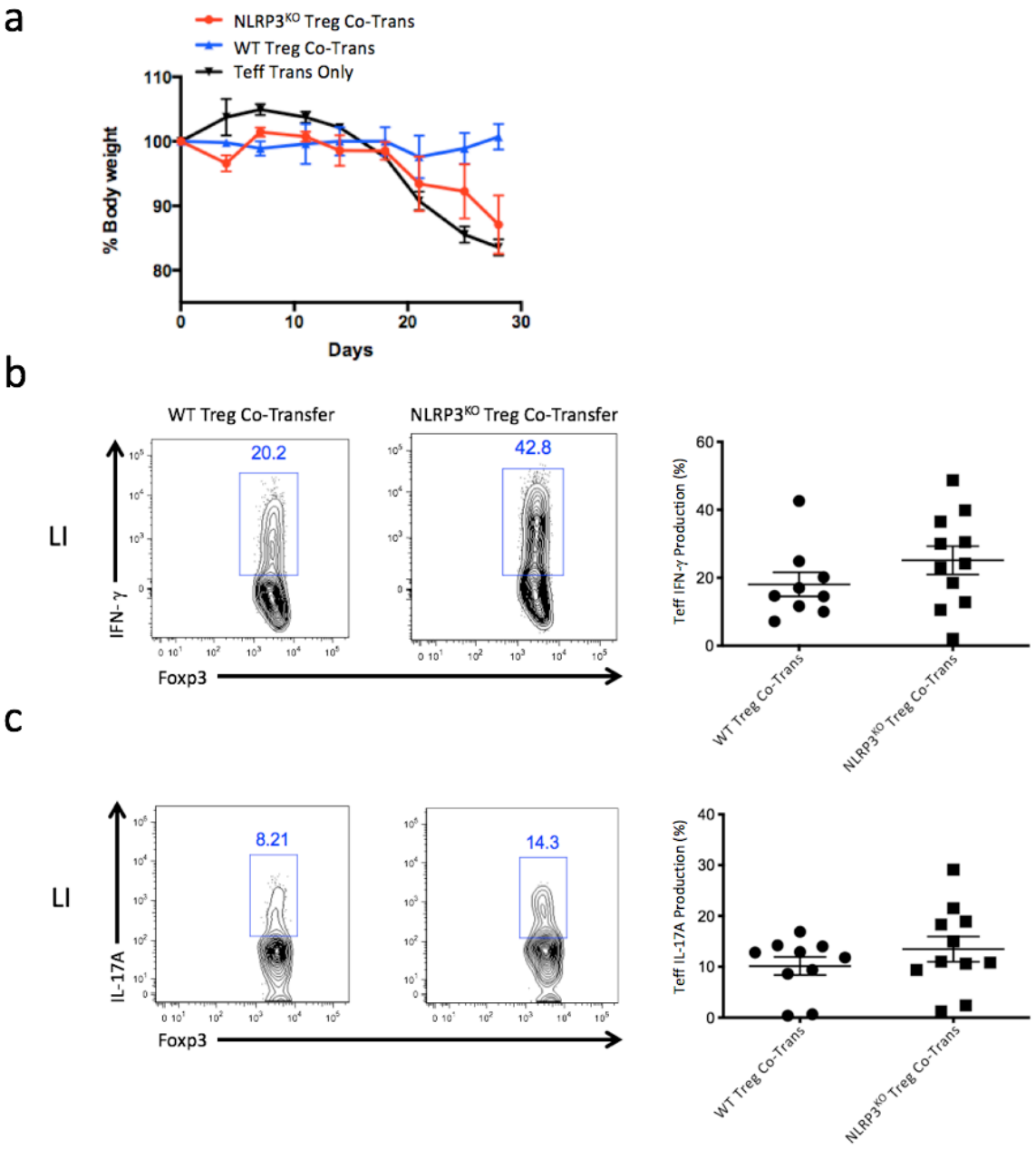

Figure 7. Use of the adoptive T cell transfer colitis modelto evaluate the efficacy of NLRP3^{KO} Treg cells in preventing colitis. Teff cells were transferred i.p. to Rag$^{-/-}$ recipients in isolation or with co-transfer of either WT Treg cells or NLRP3^{KO} Treg cells. Body weight was monitored (a). Mice were sacrificed upon severe weight loss, and tissues were harvested for FACS analysis. Teff production of IFN-γ (b) and IL-17A (c) in the large intestine is shown.

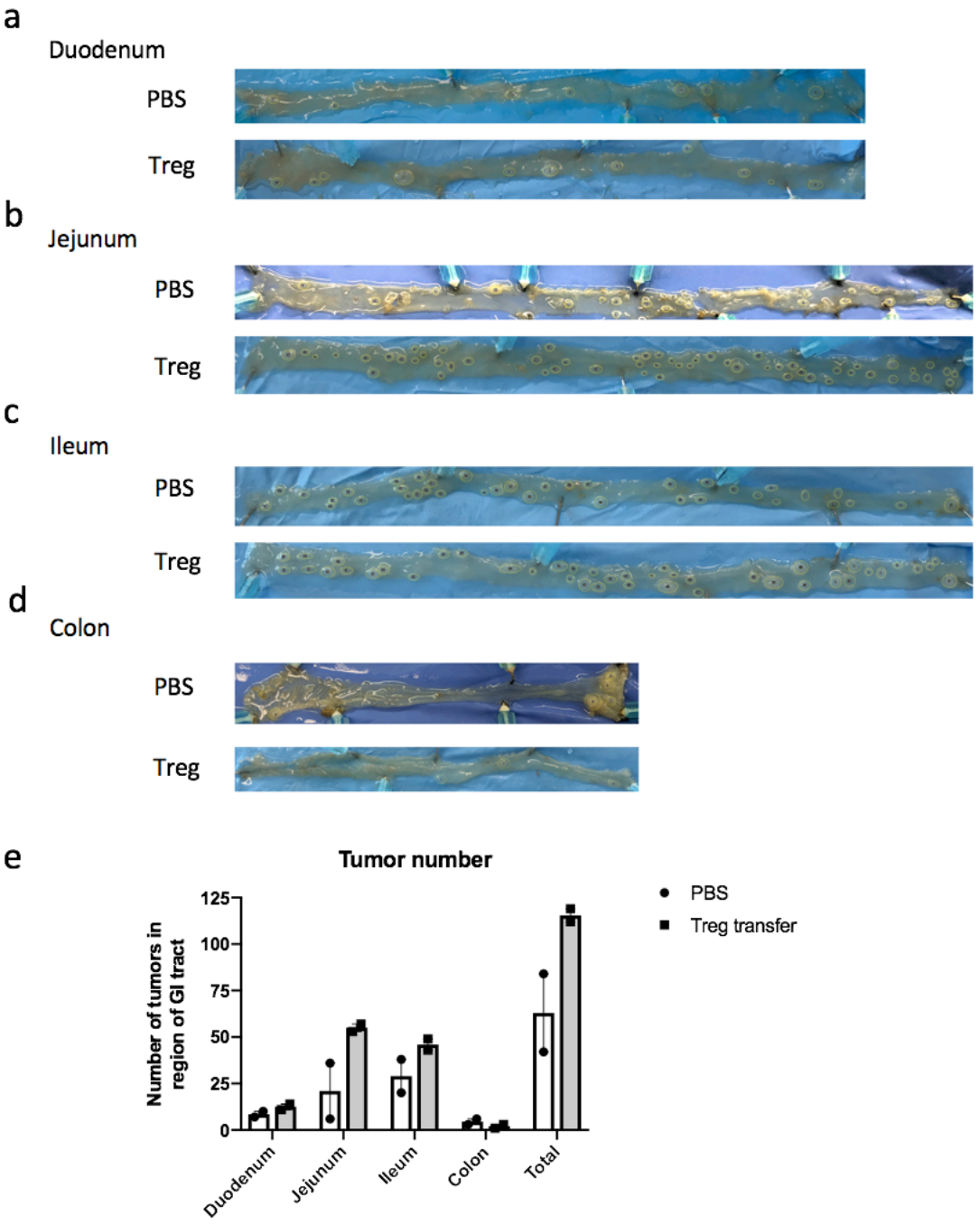

Figure 8. Quantification of tumor number in Vil1^{cre}Apc$^{fl/+}$ mice after WT Treg injection. Vil1^{cre}Apc$^{fl/+}$ mice were injected with either PBS or WT Treg cells at 3 months of age. 3 weeks later, mice were sacrificed and tumors in the duodenum (a), jejunum (b), ileum (c), and colon (d) were counted (e).

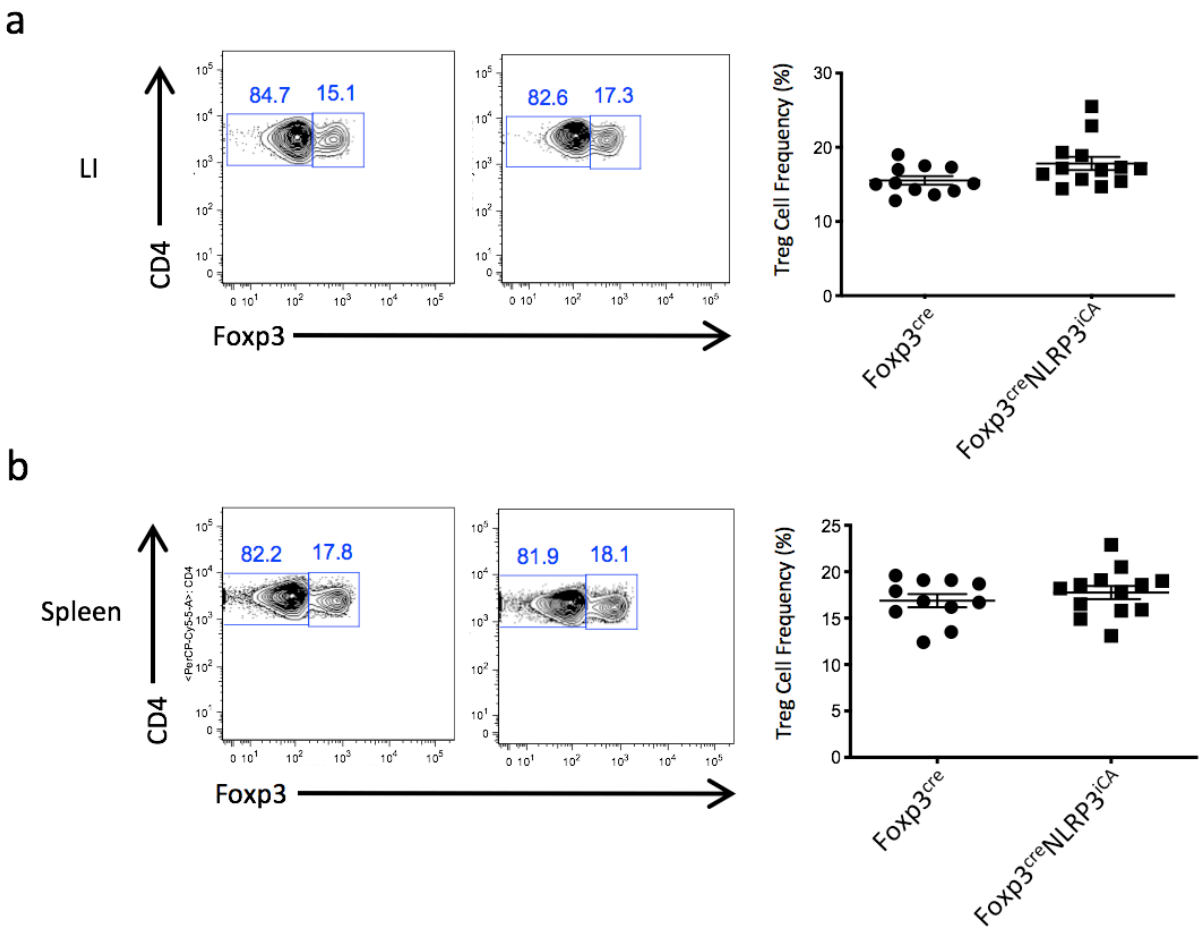

Figure 9. Treg cell frequency in Foxp3^{cre}NLRP3^{iCA} mice at steady-state. At 3-4 months of age, Foxp3^{cre} and Foxp3^{cre}NLRP3^{iCA} mice were sacrificed and Treg cell frequencies were calculated. Data from the large intestine (LI) (a) and spleen (b) are shown.

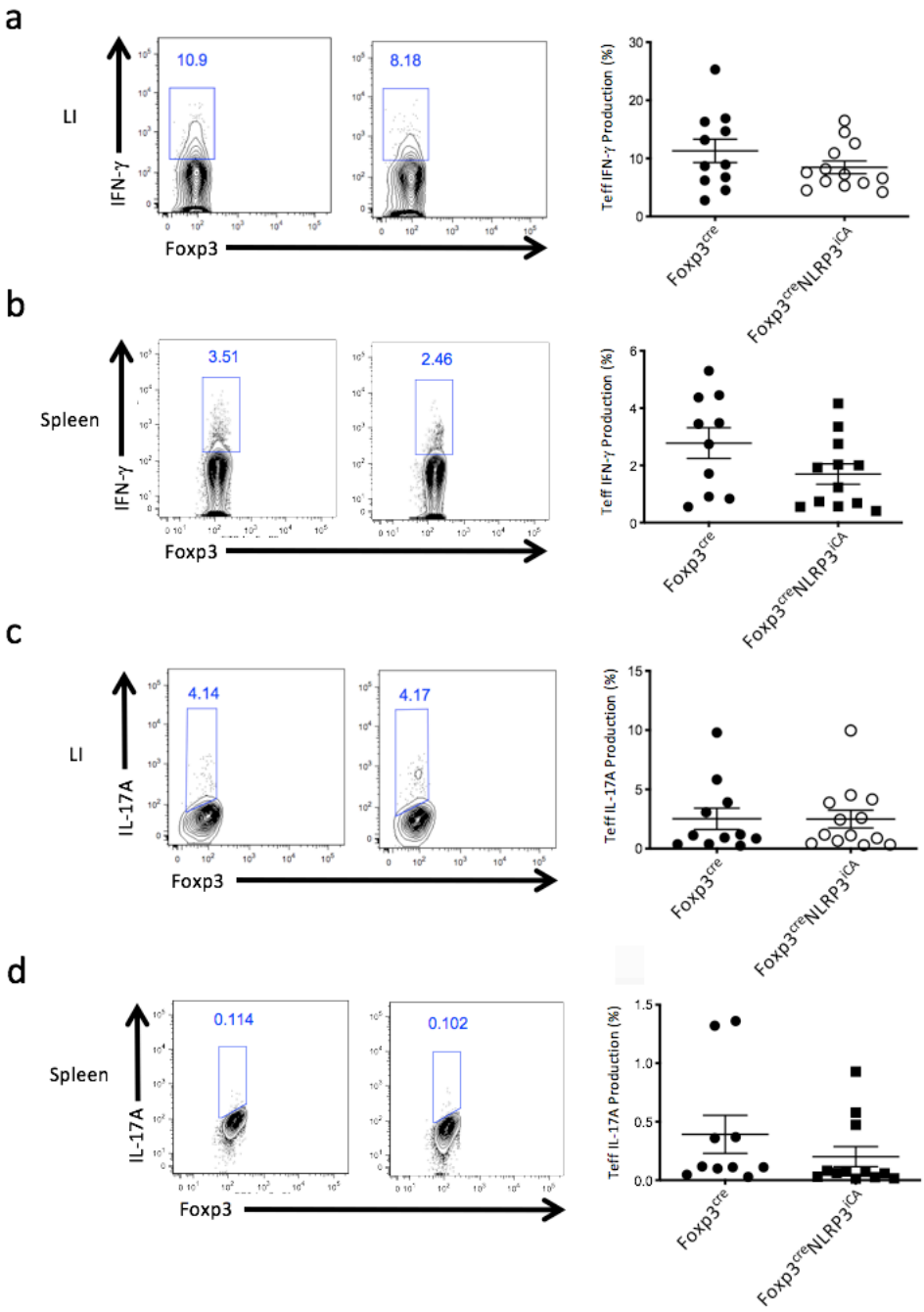

Figure 10. Teff cytokine production in Foxp3^{cre}NLRP3^{iCA} mice at steady-state. At 3-4 months of age, Foxp3^{cre} and Foxp3^{cre}NLRP3^{iCA} mice were sacrificed and Teff production of IFN-γ and IL-17A were quantified. Teff IFN-γ production from the large intestine (LI) (a) and spleen (b) are shown. Teff IL-17A production from the large intestine (c) and spleen (d) are shown.

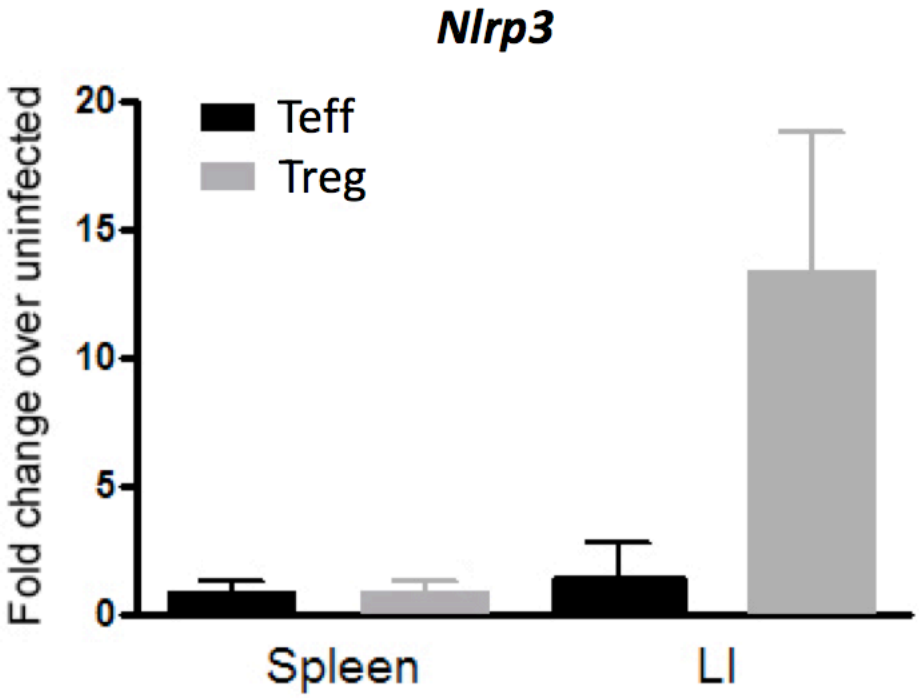

Figure 11. *Nlrp3* gene expression in Teff and Treg cells sorted and isolated from the spleen and large intestine (LI) of WT mice ten days following *C. rodentium* infection.

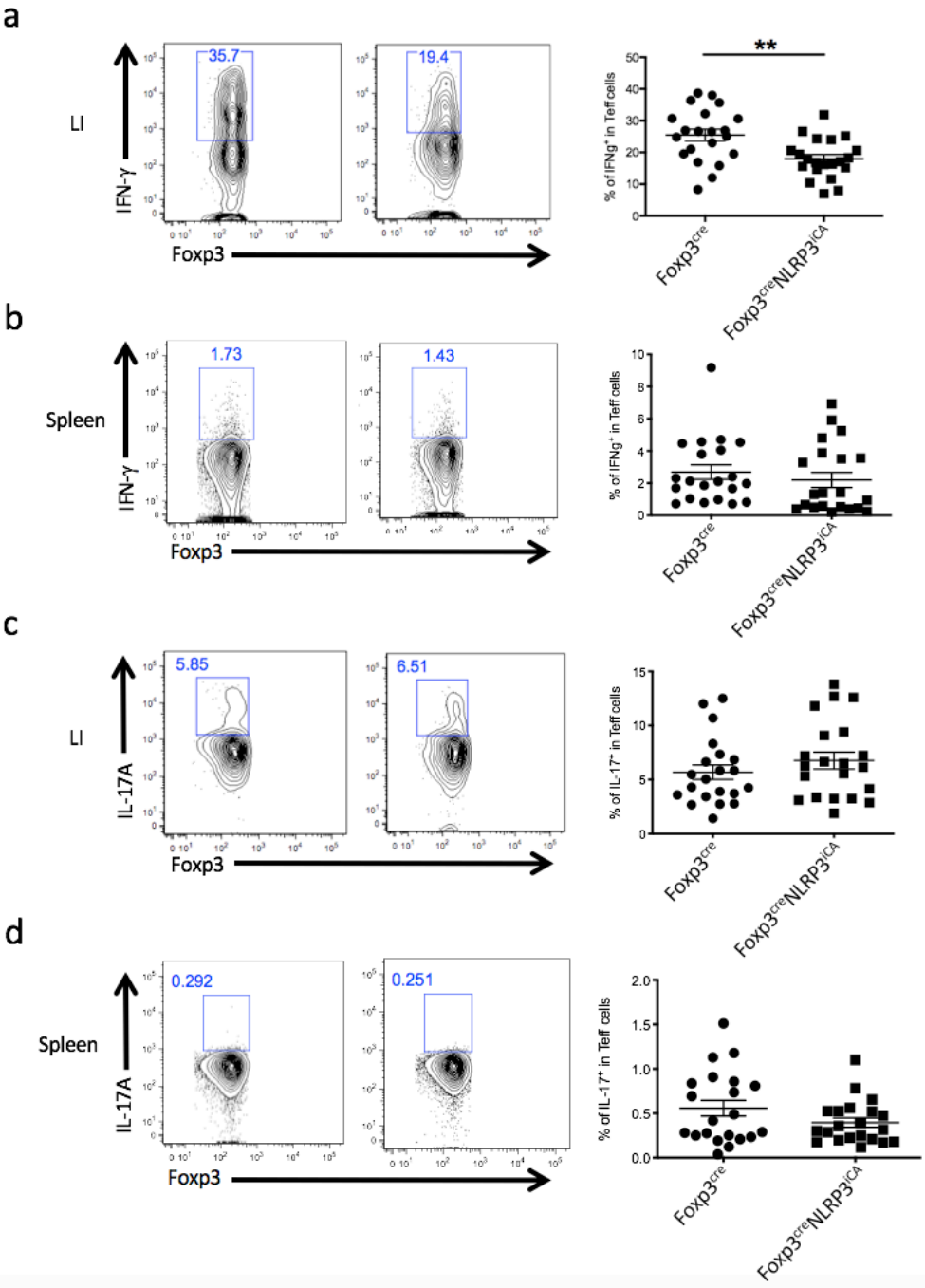

Figure 12. Teff cytokine production in Foxp3^{cre}NLRP3^{iCA} mice after *C. rodentium* infection. Ten days after *C. rodentium* infection, Foxp3^{cre} and Foxp3^{cre}NLRP3^{iCA} mice were sacrificed and Teff production of IFN-γ and IL-17A were quantified. Teff IFN-γ production from the large intestine (LI) (a) and spleen (b) are shown. Teff IL-17A production from the large intestine (c) and spleen (d) are shown.

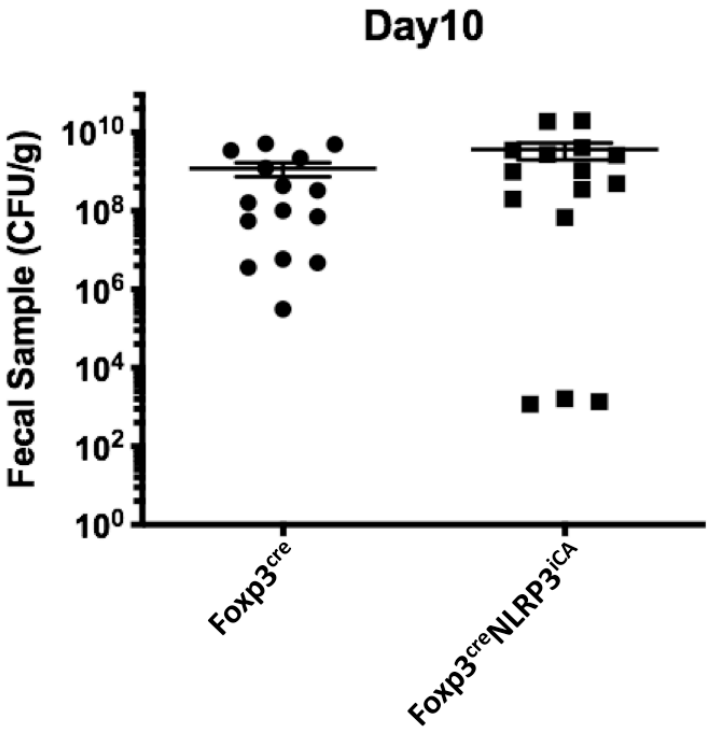

Figure 13. Evaluation of *C. rodentium* burden in Foxp3^{cre}NLRP3^{iCA} mice ten days after *C. rodentium* infection. Bacterial burden, as measured by CFUs gathered from fecal samples, was quantified and normalized by fecal weight.

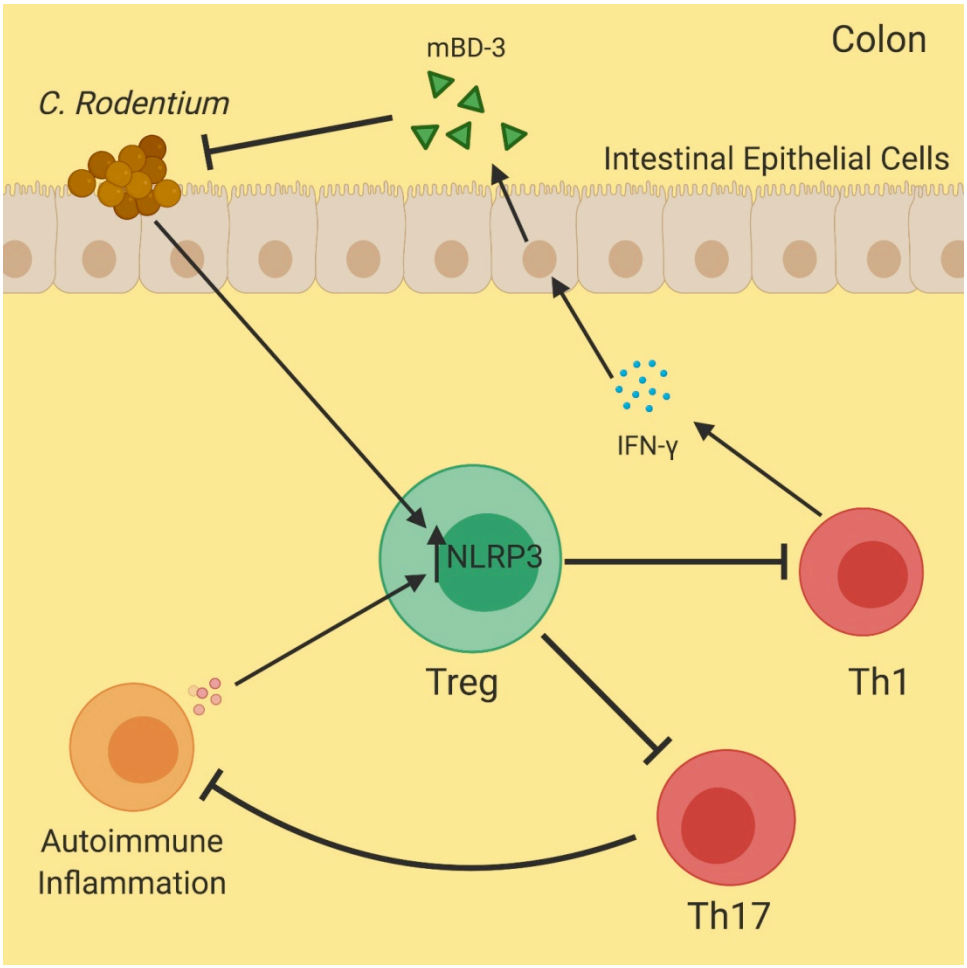

Figure 14. Proposed model of NLRP3 function in colonic Treg cells. Inflammation from a variety of sources leads to NLRP3 induction in Treg cells. NLRP3 induction endows colonic Treg cells with suppressor function to suppress T cell inflammation. Suppression of Th1 during *C. rodentium* infection can decrease IFN-γ, a cytokine which has been linked to antimicrobial peptide mBD-3 production by intestinal epithelial cells. Suppression of Th17 may protect mice from autoimmune inflammation. Created with BioRender.com.

.

REFERENCES

Agostini, L., Martinon, F., Burns, K., McDermott, M.F., Hawkins, P.N., and Tschopp, J. (2004). NALP3 forms an IL-1 beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity *20*, 319-325.

Aksentijevich, I., Nowak, M., Mallah, M., Chae, J.J., Watford, W.T., Hofmann, S.R., Stein, L., Russo, R., Goldsmith, D., Dent, P., Rosenberg, H.F., Austin, F., Remmers, E.F., Balow, J.E., Jr., Rosenzweig, S., Komarow, H., Shoham, N.G., Wood, G., Jones, J., Mangra, N., Carrero, H., Adams, B.S., Moore, T.L., Schikler, K., Hoffman, H., Lovell, D.J., Lipnick, R., Barron, K., O'Shea, J.J., Kastner, D.L., and Goldbach-Mansky, R. (2002). De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. Arthritis Rheum *46*, 3340-3348.

Arbore, G., West, E.E., Spolski, R., Robertson, A.A.B., Klos, A., Rheinheimer, C., Dutow, P., Woodruff, T.M., Yu, Z.X., O'Neill, L.A., Coll, R.C., Sher, A., Leonard, W.J., Kohl, J., Monk, P., Cooper, M.A., Arno, M., Afzali, B., Lachmann, H.J., Cope, A.P., Mayer-Barber, K.D., and Kemper, C. (2016). T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4(+) T cells. Science *352*, aad1210.

Bauer, C., Duewell, P., Lehr, H.A., Endres, S., and Schnurr, M. (2012). Protective and aggravating effects of Nlrp3 inflammasome activation in IBD models: influence of genetic and environmental factors. Dig Dis *30 Suppl 1*, 82-90.

Bauer, C., Duewell, P., Mayer, C., Lehr, H.A., Fitzgerald, K.A., Dauer, M., Tschopp, J., Endres, S., Latz, E., and Schnurr, M. (2010). Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. Gut *59*, 1192-1199.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., Hornung, V., and Latz, E. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol *183*, 787-791.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet *27*, 20-21.

Bent, R., Moll, L., Grabbe, S., and Bros, M. (2018). Interleukin-1 Beta-A Friend or Foe in Malignancies? Int J Mol Sci *19*.

Boden, E.K., and Snapper, S.B. (2008). Regulatory T cells in inflammatory bowel disease. Curr Opin Gastroenterol *24*, 733-741.

Bouladoux, N., Harrison, O.J., and Belkaid, Y. (2017). The Mouse Model of Infection with Citrobacter rodentium. Curr Protoc Immunol *119*, 19 15 11-19 15 25.

Bruchard, M., Rebe, C., Derangere, V., Togbe, D., Ryffel, B., Boidot, R., Humblin, E., Hamman, A., Chalmin, F., Berger, H., Chevriaux, A., Limagne, E., Apetoh, L., Vegran, F., and Ghiringhelli, F. (2015). The receptor NLRP3 is a transcriptional regulator of TH2 differentiation. Nat Immunol *16*, 859-870.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet *27*, 68-73.

Brydges, S.D., Mueller, J.L., McGeough, M.D., Pena, C.A., Misaghi, A., Gandhi, C., Putnam, C.D., Boyle, D.L., Firestein, G.S., Horner, A.A., Soroosh, P., Watford, W.T., O'Shea, J.J., Kastner, D.L., and Hoffman, H.M. (2009). Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. Immunity *30*, 875-887.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J.L., Cerletti, M., Jang, Y., Sefik, E., Tan, T.G., Wagers, A.J., Benoist, C., and Mathis, D. (2013). A special population of regulatory T cells potentiates muscle repair. Cell *155*, 1282-1295.

Campbell, D.J., and Koch, M.A. (2011). Phenotypical and functional specialization of FOXP3+ regulatory T cells. Nat Rev Immunol *11*, 119-130.

Chae, W.J., Gibson, T.F., Zelterman, D., Hao, L., Henegariu, O., and Bothwell, A.L. (2010). Ablation of IL-17A abrogates progression of spontaneous intestinal tumorigenesis. Proc Natl Acad Sci U S A *107*, 5540-5544.

Cheung, A.F., Carter, A.M., Kostova, K.K., Woodruff, J.F., Crowley, D., Bronson, R.T., Haigis, K.M., and Jacks, T. (2010). Complete deletion of Apc results in severe polyposis in mice. Oncogene *29*, 1857-1864.

Cipolletta, D., Feuerer, M., Li, A., Kamei, N., Lee, J., Shoelson, S.E., Benoist, C., and Mathis, D. (2012). PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. Nature *486*, 549-553.

Coccia, M., Harrison, O.J., Schiering, C., Asquith, M.J., Becher, B., Powrie, F., and Maloy, K.J. (2012). IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. J Exp Med *209*, 1595-1609.

De Robertis, M., Massi, E., Poeta, M.L., Carotti, S., Morini, S., Cecchetelli, L., Signori, E., and Fazio, V.M. (2011). The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. J Carcinog *10*, 9.

Dupaul-Chicoine, J., Yeretssian, G., Doiron, K., Bergstrom, K.S., McIntire, C.R., LeBlanc, P.M., Meunier, C., Turbide, C., Gros, P., Beauchemin, N., Vallance, B.A., and Saleh, M. (2010). Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. Immunity *32*, 367-378.

Erdman, S.E., and Poutahidis, T. (2010). Cancer inflammation and regulatory T cells. Int J Cancer *127*, 768-779.

Erdman, S.E., Sohn, J.J., Rao, V.P., Nambiar, P.R., Ge, Z., Fox, J.G., and Schauer, D.B. (2005). CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice. Cancer Res *65*, 3998-4004.

Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S., and Mathis, D. (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med *15*, 930-939.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330-336.

Godfrey, V.L., Wilkinson, J.E., Rinchik, E.M., and Russell, L.B. (1991). Fatal lymphoreticular disease in the scurfy (sf) mouse requires T cells that mature in a sf thymic environment: potential model for thymic education. Proc Natl Acad Sci U S A *88*, 5528-5532.

Guo, L., Wei, G., Zhu, J., Liao, W., Leonard, W.J., Zhao, K., and Paul, W. (2009). IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. Proc Natl Acad Sci U S A *106*, 13463-13468.

Hegazy, A.N., and Powrie, F. (2015). MICROBIOME. Microbiota RORgulates intestinal suppressor T cells. Science *349*, 929-930.

Hoffman, H.M., Mueller, J.L., Broide, D.H., Wanderer, A.A., and Kolodner, R.D. (2001). Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet *29*, 301-305.

Hyun, Y.S., Han, D.S., Lee, A.R., Eun, C.S., Youn, J., and Kim, H.Y. (2012). Role of IL-17A in the development of colitis-associated cancer. Carcinogenesis *33*, 931-936.

Ishigame, H., Kakuta, S., Nagai, T., Kadoki, M., Nambu, A., Komiyama, Y., Fujikado, N., Tanahashi, Y., Akitsu, A., Kotaki, H., Sudo, K., Nakae, S., Sasakawa, C., and Iwakura, Y. (2009). Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity *30*, 108-119.

Izcue, A., Coombes, J.L., and Powrie, F. (2009). Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol *27*, 313-338.

Izcue, A., Hue, S., Buonocore, S., Arancibia-Carcamo, C.V., Ahern, P.P., Iwakura, Y., Maloy, K.J., and Powrie, F. (2008). Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. Immunity *28*, 559-570.

Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol *30*, 531-564.

Kanneganti, T.D. (2015). The inflammasome: firing up innate immunity. Immunol Rev *265*, 1-5.

Kelley, N., Jeltema, D., Duan, Y., and He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. Int J Mol Sci *20*.

Kim, J.M., Rasmussen, J.P., and Rudensky, A.Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol *8*, 191-197.

Kolodin, D., van Panhuys, N., Li, C., Magnuson, A.M., Cipolletta, D., Miller, C.M., Wagers, A., Germain, R.N., Benoist, C., and Mathis, D. (2015). Antigen- and cytokine-driven accumulation of regulatory T cells in visceral adipose tissue of lean mice. Cell Metab *21*, 543-557.

Kuswanto, W., Burzyn, D., Panduro, M., Wang, K.K., Jang, Y.C., Wagers, A.J., Benoist, C., and Mathis, D. (2016). Poor Repair of Skeletal Muscle in Aging Mice Reflects a Defect in Local, Interleukin-33-Dependent Accumulation of Regulatory T Cells. Immunity *44*, 355-367.

Ligumsky, M., Simon, P.L., Karmeli, F., and Rachmilewitz, D. (1990). Role of interleukin 1 in inflammatory bowel disease--enhanced production during active disease. Gut *31*, 686-689.

Mao, L., Kitani, A., Strober, W., and Fuss, I.J. (2018). The Role of NLRP3 and IL-1beta in the Pathogenesis of Inflammatory Bowel Disease. Front Immunol *9*, 2566.

Martin, B.N., Wang, C., Zhang, C.J., Kang, Z., Gulen, M.F., Zepp, J.A., Zhao, J., Bian, G., Do, J.S., Min, B., Pavicic, P.G., Jr., El-Sanadi, C., Fox, P.L., Akitsu, A., Iwakura, Y., Sarkar, A., Wewers, M.D., Kaiser, W.J., Mocarski, E.S., Rothenberg, M.E., Hise, A.G., Dubyak, G.R., Ransohoff, R.M., and Li, X. (2016). T cell-intrinsic ASC critically promotes T(H)17-mediated experimental autoimmune encephalomyelitis. Nat Immunol *17*, 583-592.

Mottet, C., Uhlig, H.H., and Powrie, F. (2003). Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol *170*, 3939-3943.

Ohnmacht, C., Park, J.H., Cording, S., Wing, J.B., Atarashi, K., Obata, Y., Gaboriau-Routhiau, V., Marques, R., Dulauroy, S., Fedoseeva, M., Busslinger, M., Cerf-Bensussan, N., Boneca, I.G., Voehringer, D., Hase, K., Honda, K., Sakaguchi, S., and Eberl, G. (2015). MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat(+) T cells. Science *349*, 989-993.

Panduro, M., Benoist, C., and Mathis, D. (2016). Tissue Tregs. Annu Rev Immunol *34*, 609-633.

Park, S.H., Ham, S., Lee, A., Moller, A., and Kim, T.S. (2019). NLRP3 negatively regulates Treg differentiation through Kpna2-mediated nuclear translocation. J Biol Chem *294*, 17951-17961.

Pizarro, T.T., Michie, M.H., Bentz, M., Woraratanadharm, J., Smith, M.F., Jr., Foley, E., Moskaluk, C.A., Bickston, S.J., and Cominelli, F. (1999). IL-18, a novel immunoregulatory cytokine, is up-regulated in Crohn's disease: expression and localization in intestinal mucosal cells. J Immunol *162*, 6829-6835.

Schiering, C., Krausgruber, T., Chomka, A., Frohlich, A., Adelmann, K., Wohlfert, E.A., Pott, J., Griseri, T., Bollrath, J., Hegazy, A.N., Harrison, O.J., Owens, B.M.J., Lohning, M., Belkaid, Y., Fallon, P.G., and Powrie, F. (2014). The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature *513*, 564-568.

Schoultz, I., Verma, D., Halfvarsson, J., Torkvist, L., Fredrikson, M., Sjoqvist, U., Lordal, M., Tysk, C., Lerm, M., Soderkvist, P., and Soderholm, J.D. (2009). Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men. Am J Gastroenterol *104*, 1180-1188.

Sefik, E., Geva-Zatorsky, N., Oh, S., Konnikova, L., Zemmour, D., McGuire, A.M., Burzyn, D., Ortiz-Lopez, A., Lobera, M., Yang, J., Ghosh, S., Earl, A., Snapper, S.B., Jupp, R., Kasper, D., Mathis, D., and Benoist, C. (2015). MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. Science *349*, 993-997.

Shiomi, H., Masuda, A., Nishiumi, S., Nishida, M., Takagawa, T., Shiomi, Y., Kutsumi, H., Blumberg, R.S., Azuma, T., and Yoshida, M. (2010). Gamma interferon produced by antigen-specific CD4+ T cells regulates the mucosal immune responses to Citrobacter rodentium infection. Infect Immun *78*, 2653-2666.

Siegmund, B. (2010). Interleukin-18 in intestinal inflammation: friend and foe? Immunity *32*, 300-302.

Simmons, C.P., Goncalves, N.S., Ghaem-Maghami, M., Bajaj-Elliott, M., Clare, S., Neves, B., Frankel, G., Dougan, G., and MacDonald, T.T. (2002). Impaired resistance and enhanced pathology during infection with a noninvasive, attaching-effacing enteric bacterial pathogen, Citrobacter rodentium, in mice lacking IL-12 or IFN-gamma. J Immunol *168*, 1804-1812.

Srinivasula, S.M., Poyet, J.L., Razmara, M., Datta, P., Zhang, Z., and Alnemri, E.S. (2002). The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. J Biol Chem *277*, 21119-21122.

Swanson, K.V., Deng, M., and Ting, J.P. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol *19*, 477-489.

Tsutsumi, N., Kimura, T., Arita, K., Ariyoshi, M., Ohnishi, H., Yamamoto, T., Zuo, X., Maenaka, K., Park, E.Y., Kondo, N., Shirakawa, M., Tochio, H., and Kato, Z. (2014). The structural basis for receptor recognition of human interleukin-18. Nat Commun *5*, 5340.

Verma, D., Lerm, M., Blomgran Julinder, R., Eriksson, P., Soderkvist, P., and Sarndahl, E. (2008). Gene polymorphisms in the NALP3 inflammasome are associated with interleukin-1 production and severe inflammation: relation to common inflammatory diseases? Arthritis Rheum *58*, 888-894.

Villalta, S.A., Rinaldi, C., Deng, B., Liu, G., Fedor, B., and Tidball, J.G. (2011). Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype. Hum Mol Genet *20*, 790-805.

Villalta, S.A., Rosenthal, W., Martinez, L., Kaur, A., Sparwasser, T., Tidball, J.G., Margeta, M., Spencer, M.J., and Bluestone, J.A. (2014). Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. Sci Transl Med *6*, 258ra142.

Villani, A.C., Lemire, M., Fortin, G., Louis, E., Silverberg, M.S., Collette, C., Baba, N., Libioulle, C., Belaiche, J., Bitton, A., Gaudet, D., Cohen, A., Langelier, D., Fortin, P.R., Wither, J.E., Sarfati, M., Rutgeerts, P., Rioux, J.D., Vermeire, S., Hudson, T.J., and Franchimont, D. (2009). Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. Nat Genet *41*, 71-76.

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F.D., Byrne, G., McEuen, M., Proll, S., Appleby, M., and Brunkow, M.E. (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet *27*, 18-20.

Yasuda, K., Nakanishi, K., and Tsutsui, H. (2019). Interleukin-18 in Health and Disease. Int J Mol Sci *20*.

Zaki, M.H., Boyd, K.L., Vogel, P., Kastan, M.B., Lamkanfi, M., and Kanneganti, T.D. (2010). The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. Immunity *32*, 379-391.