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Non-conventional Regulation of Treg Development and Function

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Non-conventional Regulation of Treg Development and Function

by

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Dedication

My doctoral dissertation is dedicated to my husband, Richard Anthony Marin.

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IBD is a serious, lifelong illness that is both debilitating and financially costly. There are few effective pharmaceuticals for IBD, and many patients ultimately require surgical treatment. Hence, the development of new therapeutics is an utmost priority. Tregs are vital in maintaining immunological tolerance and preventing inappropriate inflammatory responses. Despite having no obvious functional defects, Tregs are unable to regulate inflammation in human IBD patients. The role of the gut homing molecule CCR9 in IBD is poorly understood. CCR9 is a potential drug target that is currently being investigated due to the potential to disrupt migration of pathogenic T cells to the gut. However clinical trials indicate a protracted therapeutic response. Recent studies have indicated an immunomodulatory role for chemokines in other autoimmune diseases. Metabolism is another factor whose influence on T cell responses is rapidly becoming apparently. Caloric intake, oxygen status, and the presence of specific metabolites all regulate T cell responses. We demonstrate here that CCR9 is preferentially expressed by Teffs and Tregs, and that CCR9 signaling inhibits Tregs. Additionally, CCR9 deficiency is protective in DSS colitis but not adoptive transfer colitis, and depletion of antigen-specific Tregs in a DSS model of colitis restores susceptibility to disease. Lastly, we demonstrate that exogenous glucose can alter T cell responses in a dose-dependent

manner both *in vitro* and *in vivo*. These findings present new regulatory mechanisms utilized by the intestinal immune system to control $CD4^+$ T cell fate and function. Given further research, these findings may present a new platform for the development of IBD therapeutics.

TABLE OF CONTENTS

List of Figures

List of Abbreviations

ALDH1	Aldehyde dehydrogenase family 1
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphophate
BMDC	Bone marrow-derived dendritic cell
cAMP	Cyclic adenosine monophosphate
CCL	CC chemokine ligand
CD	Cluster of differentiation
CDAI	Crohn's disease activity index
CCR	CC chemokine receptor
CLA	Conjugated linoleic acid
CNS	Central nervous system
COX	Cycoloxygenase
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lympocyte-associated protein 4
CXCL	C-X-C chemokine ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid

DTH	Delayed-type hypersensitivity
DSS	Dextran sodium sulfate
EAE	Experimental autoimmune encephalitis
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FFAR	Free fatty acid receptor
FKHR	Forkhead in rhabdomyosarcoma
FoxP3	Forkhead box P3
FSC	Forward scatter
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPR	Guanine nucleotide binding-coupled receptor
GSK3β	Glycogen synthase kinase 3 beta
GVHD	Graft-versus-host disease
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
HEV	High endothelial venule
HIF-1	Hypoxia-induced factor 1
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IEL	Intraepithelial lymphocyte

Ig	Immunoglobulin
IFN	Interferon
IL-	Interleukin-
iNKT	Invariant natural killer T
iTregs	Induced Tregs
JNK	Jun N-terminal kinase
KO	Knockout
LO	Lipoxygenase
LP	Lamina propria
LPL	Lamina propria lymphocyte
mAb	Monoclonal antibody
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule-1
MAPK	Mitogen-activated protein kinase
miR	microRNA
MLN	Mesenteric lymph node
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NFATc	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Nucleotide-binding oligomerization domain-containing protein
Nrp1	Neuropilin-1

NSAID	Non-steroidal anti-inflammatory drug
nTregs	Natural Tregs
OVA	Ovalbumin
PBS	Phospate-buffered saline
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1 receptor
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PML	Progressive multifocal leukoencephalopathy
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
RA	Retinoic acid
RAG	Recombinase activating gene
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RIPA	Radioimmunoprecipitation assay
RORγt	RAR-related orphan receptor-γt
RXR	Retinoid X receptor
SCFA	Short chain fatty acid
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
SSC	Side scatter
STAT	Signal transducer and activator of transcription

TCR	T cell receptor
TLR	Toll-like receptor
TECK	Thymus-expressed chemokine
TGF	Transforming growth factor
Th-	T helper
TNBS	Trinitrobenzenesulfonic acid
TNF	Tissue necrosis factor
Tg	Transgenic
Treg	Regulatory T cell
Tr1	T regulatory 1
TRIF	TIR-domain-containing adapter-inducing interferon-β
VAT	Visceral adipose tissue
VCAM-1	Vascular cell adhesion molecule 1
WT	Wild type

INTRODUCTION

Chapter 1: Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a lifelong, episodic inflammatory disease of the digestive tract. Symptoms can be both debilitating and embarrassing to the patient and include both intestinal and extra-intestinal manifestations. Defining symptoms of IBD include abdominal pain, blood or mucus in stool, fecal incontinence, urgency, and tenesmus[1–3]. Extra-intestinal manifestations can include skin lesions, uveitis, arthritis, and primary schlerosing cholangitis[2,4]. Diagnosis is made via colonoscopy, which may be supported by laboratory findings, and exclusion of other possible diagnoses[1,2]. There are two main varieties of IBD, Crohn's disease (CD) and ulcerative colitis (UC), which are differentiated by histological characteristics and distribution of inflamed lesions[2]. CD may occur anywhere in the digestive tract, but most commonly manifests in the ileum or large intestine[2,3]. Lesions in CD can be discontinuous (skip lesions) and inflammation is transmural[2,3]. CD patients are at increased risk for complications, including fissures, fistulae, and strictures[2,4]. Inflammation in UC occurs only in the large intestine with the exception of occasional 'backwash ileitis'[1–3]. Lesions are continuous and may affect anywhere from a few centimeters to the entire colon, with compulsory rectal disease[1,2]. Inflammation is confined to the mucosa, with abnormal crypt architecture as a major histological finding[2,3].

THE HIGH COST OF IBD

Inflammatory bowel disease is a major public health burden. Population studies indicate increasing incidence of IBD in industrialized nations[4]. The economic cost of IBD is high, both in terms of direct cost of care to patients and hospital systems and indirectly

due to of loss of productivity[5,6]. Per-patient medical care costs are estimated at anywhere from $3,000 to $11,000 per year, representing higher per capita expenses than diabetes and coronary artery disease[6]. The bulk of IBD-related expenses are attributed to hospitalization and surgery[6]. Meanwhile, indirect costs are incurred in the form of sick leave, unemployment, and disability payments[4,5]. In addition, IBD patients are at increased risk for comorbid conditions. UC patients have an elevated risk for colon cancer and require regular screenings starting 8-15 years post-diagnosis, depending on the extent of inflammation[1,2]. Inflammatory mediators in IBD patients increase the risk of cardiovascular disease[1,7]. Additionally, IBD patients have increased incidence of mood disorders, particularly anxiety and depression[7].

There are few effective treatments for inflammatory bowel disease. Currently approved therapeutics are limited to aminosalicyclates, corticosteroids, purine analogs, methotrexate, cyclosporine, anti-tumor necrosis factor-α (TNFα) antibodies, and anti-α_4 antibodies[2–4,8–10]. Many of these are either ineffective at controlling disease long-term due to loss of efficacy and the potential for serious adverse effects[8,9]. Steroids are often effective at treating IBD symptoms, but are associated with numerous significant acute and long-term side effects[1,4,5,8]. Hence, corticosteroids are not recommended for maintenance therapy and steroid sparing is a major goal in the treatment of IBD. Treatment with immunosuppressive drugs increases the risk for developing serious infections and cancer[2,8,9]. A particular concern with biological agents is the loss of efficacy due to development of drug-specific antibodies[8–10]. Additionally, adherence to treatment regimens is low, with non-adherence reported in 35-45% IBD patients[5]. Risk factors for non-adherence include recent diagnosis, inactive disease, adverse effects, complex dosing regimens, and rectal administration[5]. Lack of adherence is associated with adverse outcomes, including relapse, hospitalization, increased severity, and colorectal cancer[2,5]. For the above reasons, many IBD patients require surgical

intervention[1,2,9]. Hence, the development of new therapeutics for the treatment of IBD is of utmost importance.

PATHOGENESIS

Pathogenesis of IBD is complex and dependent on multiple factors. Genetic susceptibility, host immune responses, the microbiome, and environmental triggers all play a role the development of IBD. Twin studies indicate that both UC and CD are heritable, with higher concordance in monozygotic twins than dizygotic twins[11,12]. Non-twin family studies also indicate a genetic component, as individuals with a first-degree relative with IBD are more likely to be develop the disease than the general population[10,11]. Mutations in several genes have been associated with IBD, most notably the intracellular pattern recognition receptor (PRR) *NOD2/CARD15*[3,10–12]. Additional genetic risk factors for IBD which have been observed in clinical and veterinary settings include polymorphisms in toll-like receptor (TLR) 4, TLR5, and *ATG16L1*[12–14]. Additional susceptibility loci, such as the IL-10 gene, have been identified in animal models[3].

Monozygotic twin concordance rates are lower than 50%, indicating that an environmental trigger is required to induce disease in genetically predisposed individuals[10–12]. A major environmental factor in the development of IBD is the microbiota. Colonization by gut microbes is required for the development of intestinal inflammation in a number of animal models[3,12]. Patients with IBD produce antibodies specific for commensal antigens, such as CBir1 flagellin[12,15,16]. In addition, patients display alterations in microbiome composition characterized by decreased species diversity, increased *Proteobacteria*, increased *Enterobacteriaceae*, decreased *Clostridia*, and decreased *Firmicutes*[12]. In animal models, such alterations in the microbiota in genetically susceptible mice have been linked with the development of transmissible colitis[17,18]. Additionally, extensive antibiotic treatment during childhood is associated

with increased incidence of IBD[12,19,20]. Additional environment triggers have also been identified, including smoking, use of non-steroidal anti-inflammatory drugs (NSAIDs), intestinal infections, and appendectomy[2,4,10].

Pathological inflammation in IBD is largely driven by innate immune defects which result in inappropriate $CD4^+$ T cell responses to the microbiota[3,12,15,21–23]. Inflammation in CD is primarily dependent on interferonγ (IFNγ)-producing T helper (Th) 1 and IL-17-producing Th17 cells[3,10,23]. Neutralization of IFNγ, knockout (KO) of Tbet, or KO of Th1-associated pathways are sufficient to prevent disease in animal models[23]. The role of Th17 cells is more complex. Adoptive transfer of Th17 cells results in enhanced intestinal inflammation, but protective roles have also been demonstrated in some models[23]. This protective effect may be due to Th17 production of IL-22 or IL-17-dependent induction of IgA (immunoglobulin A) -producing B cells[23,24]. In UC, a Th2-like response is associated with disease development[3,10,23]. Major immunological features of UC include upregulation of IL (Interleukin) -5 and IL-13, natural killer T (NKT) cell involvement, and production of IgG1 and IgG4 antibodies[3,10,23].

Chapter 2: Tregs in IBD

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that regulate inflammatory responses. Tregs are essential in the induction of oral tolerance and the prevention of autoimmunity. Tregs may develop during antigen selection in the thymus (natural Tregs or nTregs) or from naïve $CD4^+$ T cells in the periphery (induced Tregs or iTregs)[23,25]. nTregs are selected in the thymus on the basis of high T cell receptor (TCR) affinity to self antigen[25]. iTregs are induced by transforming growth factor β (TGF-β), with retinoic acid (RA) enhancing differentiation[23,25]. Development of iTregs is also strongly affected by the composition of the microbiota[12,23,25]. IL-2 is required for Treg expansion and survival[25]. Tregs express the transcription factor Forkhead box P3 (FoxP3) as well as high levels of CD25, an IL-2 receptor[25].

Tregs employ multiple suppressive mechanisms to inhibit pro-inflammatory cytokine production and T effector (Teff) cell proliferation[26]. Tregs can influence APC function through cytotoxic T-lymphocyte-associated protein 4 (CTLA4) signaling, resulting in reduced capacity for antigen presentation and production of anti-inflammatory cytokines[25,26]. Tregs also suppress inflammation by producing their own anti-inflammatory cytokines, including TGF-β, IL-10, and IL-35[25,26]. Additionally, Tregs modulate inflammation by producing immunosuppressive small molecules, such as cyclic AMP (cAMP)[25,26]. Another mechanism of Treg-mediated suppression is the induction of effector cell apoptosis via growth factor starvation, particularly IL-2, which is required for survival by proliferating T cells and whose receptor is highly expressed on Tregs[25,26]. Tregs can also manipulate mammalian target of rapamycin (mTOR) signaling by regulating nutrient availability, inhibiting Teff function[27]. Tregs are also capable of regulating immune response through cytotoxic killing of effector T cells[25]. In combination, the various methods of Treg-mediated suppression result in fewer effector

cells due to apoptosis or failure to proliferate, as well as downregulated production of pro-inflammatory cytokines[26].

TREGS IN EXPERIMENTAL MODELS

The role of Tregs in IBD has been extensively investigated in animal models. Loss of function mutations in FoxP3 or TGF-β results in severe and lethal multi-organ autoimmunity, including intestinal inflammation[25,28]. IL-10 deficiency leads to the development of spontaneous colitis[25,28]. Treg-specific IL-10 production is required to prevent intestinal inflammation in animal models of IBD, though production of IL-10 by other cell types also contributes[28–30]. In addition, IL-35 deficiency in Tregs renders them unable to rescue colitis in an adoptive transfer model[25,28].

Functionally intact Tregs are protective or curative in multiple animal models of IBD. In a $CD45RB^{high}$ adoptive transfer model of colitis, co-transfer of the $CD45RB^{low}$ fraction or of isolated $CD4^+$ $CD25^+$ T cells both prevents and treats the development of inflammation[3,25,30]. Treg transfer also prevents colitis in a FoxP3-deficient $CD4^+$ T cell adoptive transfer model[29]. The protective effects of transferred Tregs in the $CD45RB^{high}$ transfer model are negated if mice are treated with anti-TGF-β antibody[3,30]. Treg-specific TGF-β KO also impairs their ability to prevent colitis[30]. Adenosine derived from Tregs also suppresses intestinal inflammation, and treatment with adenosine agonists rescues colitis[30]. Likewise, Treg depletion in chemical models of colitis (for example, Trinitrobenzenesulfonic acid (TNBS)- or dextran sodium sulfate (DSS) -induced colitis) results in more severe pathology[25].

Effector T cell responsiveness is also important for Treg-mediated suppression of intestinal inflammation. In a $CD45RB^{high}$ adoptive transfer model, Tregs were unable to suppress colitis if effector T cells lacked the ability to respond to TGF-β[30]. Knockdown of Smad7, an inhibitor of TGF-β signaling, was found to be protective in both oxazolone and TNBS colitis models[31]. Additionally, effector T cells lacking the adenosine receptor

A2A were resistant to suppression by Tregs, and transferred wild-type (WT) Tregs were unable to prevent colitis caused by A2A KO $CD4^+$ T cells[25]. Effector T cells must also be susceptible to Treg induced apoptosis. KO of the pro-apoptotic protein Bim conferred resistance to Treg-mediated suppression[30].

TREGS IN HUMAN IBD PATIENTS

Tregs are vital to the suppression of pathological inflammation and the prevention of autoimmunity. Hence, effort has been made to characterize Treg populations in human IBD patients. Patients with CD demonstrate enriched Treg populations in the blood, mesenteric lymph node (MLN), and lamina propria (LP)[32]. While $FoxP3^+$ $CD4^+$ T cells are increased in both inflamed and unaffected mucosa of CD patients compared to healthy controls, there is a greater increase in Tregs in the inflamed tissue[32]. Likewise, Tregs are upregulated in the MLN and LP of UC patients, with increased numbers of Tregs in inflamed tissue compared with unaffected tissue[33]. In addition, peripheral blood Treg numbers are increased in CD patients in remission compared to CD patients with active disease[32]. In both CD and UC patients treated with infliximab, clinical response correlated with an increase in peripheral blood Tregs and decreased numbers of Tregs in the LP[34]. It is thought that the alterations of blood and peripheral Tregs during infliximab treatment may be due to migration to the site of inflammation; however the exact reasons are unclear[30].

Tregs in IBD patients do not exhibit defects in suppressive function in *ex vivo* suppression assays. Tregs from the MLN of CD patients can adequately suppress effector cell proliferation *ex vivo*[32]. Similarly, MLN Tregs from UC patients effectively suppress effector T cell proliferation and production of pro-inflammatory cytokines *ex vivo*[33]. Interestingly, LP Tregs from both IBD patients and healthy controls demonstrated weaker suppressive function than Tregs isolated from the periphery[30].

Despite the increased number of Tregs in intestinal lesions and lack of functional defects *ex vivo*, Tregs are unable to control intestinal inflammation in human IBD patients. The mechanism of impaired Treg suppression in IBD is currently unknown. Effector T cell resistance to Treg-mediated suppression is a possible explanation, though it is unlikely considering the use of autologous effector T cells in suppression assays[32,33]. Still, some studies have shown that effector T cells from IBD patients upregulate Smad7, leading to inhibition of TGF-β signaling[3,25,31]. Hence, a likely explanation for the inability of Tregs to control inflammation in IBD is that some aspect of the intestinal environment in IBD interferes with Treg suppressor function[25,28]. The mechanism by which this occurs is currently unknown, though some have posited that defective conversion of TGF-β to its bioactive form is at fault[30].

Chapter 3: Gut homing molecules

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Homing molecules such as chemokines and integrins determine tissue trophism and aid in the migration of immune cells to the site of infection or insult. Migration to the small intestine is largely regulated by the chemokine receptor CCR9,[35,36] while β_7 integrins and guanine nucleotide binding coupled receptor (GPR) 15 control trafficking to the colon[37–39]. Chemokines and integrins have been shown to play a vital role in both the induction and regulation of inflammation in the gut. Furthermore, a small body of recent research suggests that chemokine signaling can directly modulate cellular and immunological functions. However, the role of gut-homing molecules in the regulation of IBD is not well understood. Previous studies have indicated pathogenic, protective, or neutral roles for gut homing molecules in IBD depending on the animal model, method of neutralization, and timing of treatment. In addition, recent research in cancer and experimental autoimmune encephalitis (EAE) models has shown that binding of homing receptor ligand-receptor pairs can influence non-migratory functions, including T cell differentiation and apoptosis[40–44].

Disrupting migration by preventing the ligation of homing molecules with their receptors is a new therapeutic strategy for the control of tissue-specific inflammatory diseases such as IBD[45]. Optimally, homing inhibitors induce tissue-specific immunosuppression by preventing localization of inflammatory cells to the target organ

without disrupting immune function in other organs[45]. This improves disease outcome while minimizing the risks associated with systemic immunosuppression, such as infection and cancer. Several homing inhibitors have been approved for the treatment of IBD, and more are currently being investigated in clinical trials.

GUT-TROPHIC HOMING MOLECULES

The integrin $\alpha_4\beta_7$ is expressed by lymphocytes and dendritic cells (DCs). Its ligand, MAdCAM-1 is expressed by the high endothelial venules (HEVs) of the gut lymphatic tissue and the post-capillary venules of both the large bowel (LB) and small bowel (SB) LP[46,47]. Mucosal Addressin Cell Adhesion Molecule 1 (MAdCAM-1) is essential for the development of gut-associated lymphoid tissue (GALT), and Peyer's patches (PPs) fail to develop in MAdCAM-1 KO mice[46].

Chemokine (C-C motif) receptor 9 (CCR9), also known as thymus-expressed chemokine (TECK), is likewise expressed by lymphocytes and DCs. CCR9 binds non-promiscuously to its ligand, chemokine (C-C motif) ligand 25 (CCL25), which is expressed on thymic and small intestinal epithelial cells[47]. Despite normal thymic expression of CCL25 and upregulation of CCR9 on $CD4^+$ $CD8^+$ double-positive T cells, CCR9 deficiency has negligible effects on thymic lymphocyte development[35,48,49]. CCL25 in the gut is limited to the small bowel, with expression highest in the duodenum and lowest in the terminal ileum[47]. As such, CCR9 is thought to direct migration to the small intestine, but not the colon[9]. Despite this, there is evidence that CCR9 and CCL25 play a role in regulating migration to the large bowel, most likely by enhancing lymphocyte adhesion to vascular cell adhesion molecule 1 (VCAM-1) and E-cadherin[46,50,51].

A third homing molecule which promotes gut trophism is the integrin $\alpha_E\beta_7$, or CD103[46]. The ligand for CD103 is E-cadherin, though there is evidence for a second, as-of-yet unknown ligand[52]. Frequency of CD103 expression is extremely low on peripheral blood lymphocytes and highest on tissue-resident lymphocytes of the gut epithelium[46]. In general, CD103 is associated with a tolerogenic phenotype and is primarily expressed on DCs, nTregs, and intraepithelial lymphocytes (IELs)[46]. Of note, CD103 is expressed on a subset of mucosal DCs that primes lymphocytes to become tissue-resident lymphocytes and upregulate gut homing molecules[46].

GPR15 was first discovered as a co-receptor for HIV, and its role in lymphocyte trafficking has only recently been discovered[38]. GPR15 directs migration to the large intestine, but not the small intestine, and is primarily expressed on activated T cells, including Tregs[38,39,53]. In humans, GPR15 expression is primarily observed on Th2 cells, compared to mice, which have a high Treg expression of GPR15[38,39,53].

REGULATION OF GUT TROPHISM

The vitamin A metabolite RA is essential for inducing leukocytes to develop a mucosal phenotype. RA is highly unstable and converted from vitamin A *in vivo* by aldehyde dehydrogenase family 1 a1 and a2 (ALDH1a1 and ALDH1a2), which are produced primarily by $CD103^+$ mucosal DCs[54,55]. RA signals through nuclear retinoic acid receptors (RARs), particularly RARα[55]. Once activated, RARs bind to promoter elements in DNA, subsequently inducing a mucosal phenotype in multiple cell types by upregulating the transcription of CCR9 and $\alpha_4\beta_7$ integrin[54,55]. In the bone marrow, RA induces differentiation of $\alpha_4\beta_7^+$ DC progenitors that migrate to the gut and develop into $CCR9^+$ and $CD103^+$ DCs[56]. Interestingly, despite previous findings that T cells

upregulate CD103 in the presence of RA, one study reported that inhibiting RARα signaling induces CD103 in T cells[57]. In addition to gut trophism, RA induces the development of tolerogenic phenotypes such as IgA producing B cells and Tregs[47,54,55]. This is important for maintaining homeostasis and preventing excessive inflammation in response to innocuous antigen in the gut. However, despite its tolerogenic properties, RA can also be used as a potent mucosal adjuvant in vaccines, where it enhances the recruitment of effector T cells to the gut[55].

In addition to RA signaling, CCR9 upregulation on T cells requires brief TCR stimulation resulting in inhibition of nuclear factor of activated T cells (NFATc) 1 and activation of NFATc2[58]. Both NFATc1 and NFATc2 bind to RARα and retinoid x receptor (RXR) α, altering their binding affinity to the CCR9 promotor and acting as either a transcriptional repressor or enhancer, respectively[58]. Likewise, the transcription factors FoxP3 and GATA3 bind the GPR15 promoter, enhancing gene transcription[39,53]. The differential binding affinity of FoxP3 and GATA3 to the mouse and human GPR15 enhancer region leads to differential expression of GPR15 between species, with mice upregulating GPR15 on Tregs and humans preferentially expressing GPR15 on Th2[39,53].

In vivo, mucosal DCs expressing CD103 are vital to the differentiation of tissue resident cells from naïve lymphocytes via RA-dependent upregulation of CCR9 and $\alpha_4\beta_7$[59]. *In vitro*, BMDCs generated in the presence of RA fail to express CD103 but upregulate the expression of CCR9, ALDH1a2, and TGF-β[54]. Development of functional mucosal-like DCs from BMDCs *in vitro* is dependent on myeloid differentiation primary response gene 88 (MyD88) and signal transducer and activator of transcription (STAT) 3 signaling and suppressed by suppressor of cytokine signaling (SOCS) 3[54]. In addition,

bone marrow dendritic cells (BMDCs) treated with RA induce greater numbers of Tregs and IgA-producing B cells than do control BMDCs via a TGF-β-dependent mechanism[54]. In another system, monocyte-derived DCs isolated from peripheral blood and treated with RA were able to induce the expression of $\alpha_4\beta_7$ and CCR9, further emphasizing the importance of RA in the development of a mucosal phenoytpe[60].

The gut microbiome is also involved in regulating the expression of gut-trophic chemokines and their receptors. Experiments in germ-free pigs reconstituted with either a single species of bacteria or the normal adult flora indicate that CCR9 and CCL25 are upregulated by *Escherichia coli*, but downregulated by *Lactobacillus fermentum*[61]. GPR15 is downregulated in antibiotic-treated mice[38] and upregulated by short-chain fatty acids, a microbial metabolite[62]. TLR3 signaling has also been shown to upregulate GPR15 on $CD4^+$ T cells in a TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent manner[63]. Likewise, microbial stimuli can regulate vitamin A metabolism in mucosal DCs via TLR- and MyD88-dependent upregulation of retinaldehyde dehydrogenase (RALDH)[55].

Cytokines are another important factor in regulating the expression of gut homing molecules. Pro-inflammatory cytokines have been shown to induce expression of homing molecule ligands in tissue. CCL25, the ligand for CCR9, is upregulated in small intestinal tissue in response to TNF-α treatment[36]. Likewise, MAdCAM-1 expression is upregulated by TNF-α, IL-1β, and lymphotoxin[46]. Anti-inflammatory cytokines can also modulate the expression of homing molecules. Neutralization of TGF-β inhibits lymphocyte expression of $\alpha_4\beta_7$[60]. In addition, TGF-β signaling via the Smad7 pathway is

thought to modulate the transition of migrating cells from $\alpha_4\beta_7$-expressing LPLs to CD103-expressing IELs, and Smad7 KO mice lack T cell expression of CD103[46].

THE ROLE OF GUT HOMING MOLECULES IN HOMEOSTASIS

Intestinal chemokines are essential for the normal development of the gut immune system, as well as maintenance of homeostasis. RA-mediated expression of gut-trophic chemokines on Treg cells is required for tolerance to innocuous oral antigen and prevention of pathological inflammation[50,55,64]. This essential role of homing molecules in maintaining tolerance is important to consider in the development of new IBD therapeutics designed to disrupt migration to the intestine.

CCR9 and the β_7 integrins are required for development of normal gut immune function, including the development of gut-associated lymphoid tissues[46]. Mice lacking CCR9 demonstrate impaired development of small intestine cryptopatches and reduced numbers of IELs[35,49]. In CCR9 KO mice, numbers of TCR$\gamma\delta^+$ T cells are increased in the large bowel and periphery, but decreased in the small bowel[49]. GPR15 deficiency results in impaired migration of Tregs and $CD44^+$ memory $CD4^+$ T cells to the large bowel[38,39,53]. Likewise, KO of the integrin β_7 subunit in mice results in fewer LPLs and IELs and impaired accumulation of lymphocytes in the PPs[46].

CD103 in particular is critical in the maintenance of homeostasis and in the gut through the action of $CD103^+$ DCs, which play a crucial role in inducing gut specificity by inducing lymphocytes to upregulate CCR9 and $\alpha_4\beta_7$ upon antigen presentation[54,59] $CD103^+$ DCs in the intestinal mucosa express ALDH1a1 and ALDH1a2, which allow the conversion of vitamin A into RA. In addition to upregulating gut homing molecules, $CD103^+$ DCs preferentially convert naïve T and B lymphocytes into tolerogenic $FoxP3^+$

iTregs and IgA-producing B cells, respectively, upon antigen presentation. In addition, $CD4^+$ T cells cultured with $CD103^+$ DCs express less IFN-γ than do T cells cultured with $CD103^-$ DCs[59]. Mucosal DCs upregulate the microRNA miR-10a, which is under-expressed in IBD patients[65,66]. Known IBD-associated miR-10a targets include nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and the shared IL12/IL24 p40 subunit[65,66]. In particular, targeting of IL-12/23 p40 by miR-10a limits the inflammatory response by preventing Th1 and Th17 effector cell differentiation[66].

CCR9 seems to play an especially important role in homeostasis and the function of Treg cells. KO of CCR9 in TNFΔARE mice impeded Treg migration to the LP, resulting in more severe ileitis[50]. Further study determined that CCR9 is preferentially upregulated on $CD4^+/FoxP3^+/CD25^+$ Tregs compared to non-Treg $CD4^+/FoxP3^-/CD25^-$ cells[50]. Furthermore, treatment of TNFΔARE mice with an anti-CCR9 monoclonal antibody resulted in the depletion of Treg cells and enhanced intestinal inflammation[50]. Likewise, GPR15 is upregulated on Tregs in mice[38]. GPR15 KOs demonstrate more severe inflammation during *Citrobacter rodentium* infection, with Treg expression of GPR15 preventing immunopathology in this model[38,53].

Migratory capacity via homing molecules is required for adequate development of oral tolerance. In a delayed-type hypersensitivity (DTH) model, $CCR9^{-/-}$ mice that failed to develop tolerance exhibited more severe antigen-specific inflammatory responses than did WT mice after priming with oral ovalbumin (OVA)[64]. Specifically, splenocytes from WT mice primed with OVA proliferated less, produced more IL-10, and produced less IL-2 than those of $CCR9^{-/-}$ mice[64]. Likewise, OVA-primed mice treated with antibody specific for MAdCAM1 failed to develop antigen-specific tolerance compared to mice

which received control antibodies in a DTH model[64]. Splenocytes from control-treated mice proliferated less and produced more IL-10, less IFN-γ, and less IL-2 than did α-MAdCAM1-treated mice, whose inflammatory markers were similar to those of unprimed mice [64]. Vitamin A deficiency produced similar effects to CCR9 deficiency and MAdCAM1 depletion, presumably due to failure to upregulate chemokine receptors on lymphocytes[64]. In addition, induction of oral tolerance by feeding of myelin oligodendrocyte glycoprotein (MOG) peptide in a MOG-dependent EAE model reduced EAE severity in WT, but not $CCR9^{-/-}$ mice, compared with phosphate-buffered saline (PBS)-fed controls[64].

THE ROLE OF GUT HOMING MOLECULES IN INTESTINAL INFLAMMATION

There is evidence that gut-trophic chemokines and integrins play a role in the intestinal inflammatory response. Migratory function appears to be required for control of intestinal infection. RA, which induces a mucosal phenotype by upregulating gut homing molecules, serves as a powerful adjuvant for mucosal vaccines and can drive T cell activation and proliferation[55,67]. Use of RA as a mucosal adjuvant results in increased activation of mucosal DCs, increased homing to mucosal sites, more systemic central memory $CD8^+$ T cells, and increased numbers of effector memory $CD8^+$ T cells in the MLN and IEL[67]. This translated into enhanced protection and more efficient T cell responses during viral challenge[67]. In an antigen-specific culture model using porcine peripheral blood cells, RA has been shown to enhance CCR9 and $\alpha_4\beta_7$ expression on lymphocytes along with production of antigen-specific IgA and IgG[60]. During *Toxoplasma gondii* infection, vitamin A deficiency impairs Th1 responses and parasite clearance[55]. The Th17 response in the small intestine is also impaired in the absence of

RA. Additionally, RA enhances inflammatory responses to gluten in a mouse model of Celiac disease by upregulating IL-12 and IL-23, which drive T1 and Th17 differentiation, respectively[55].

Some homing molecules are associated with particular T effector subsets. GPR15 expression is upregulated on $CD44^+$ effector memory cells in mice and Th2 cells in UC patients[39,53]. $\alpha4\beta7$ expression on $CD4^+$ T cells appears to be correlated with a Th1 phenotype, as activation under Th1-polarizing conditions and production of IFN-γ are both correlated with increased expression of $\alpha4\beta7$[46]. Its ligand MAdCAM-1 is overexpressed during chronic inflammatory diseases[46]. Aberrant tissue expression of MAdCAM-1 on the eyes, skin, and joints during inflammatory bowel disease is thought to contribute to the development of extra-intestinal manifestations[46].

In addition, the presence of inflammatory mediators has been shown to upregulate tissue ligands of gut-trophic homing molecules, driving the accumulation of inflammatory lymphocytes and serving as a positive feedback loop. Epithelial cells in the small bowel upregulate CCL25 in response to stimulation by TNF-α, leading to accumulation of inflammatory IELs and LPLs in the microvasculature[36].

THE ROLE OF GUT-HOMING MOLECULES IN IBD

Gut homing molecules appear to play a complicated role in the development and maintenance of IBD. Studies illustrate both pathogenic and protective roles of gut-homing molecules in IBD. KO or neutralization of gut-homing molecules can enhance, ameliorate, or have no effect the severity of intestinal inflammation. The precise role of leuckocyte adhesion molecules on IBD pathogenesis appears to be dependent on the disease phase, method of neutralization, and experimental model. Hence, the impact of

leukocyte adhesion molecules on intestinal inflammation is likely highly dependent on both the cell type on which they are expressed and the factors present in the intestinal microenvironment.

Experiments in some models indicate a pathogenic role for chemokines and integrins during IBD. In a Th17 transfer model of colitis, Recombinase activating gene $(Rag)^{-/-}$ mice that received WT Th17 cells treated with RA developed more severe disease than did mice that received $CCR9^{-/-}$ Th17 cells treated with RA, $Itg\beta7^{-/-}$ Th17 cells treated with RA, and WT Th17 cells that were not treated with RA[68]. These data indicate essential roles for T cell expression of CCR9 and α4β7 in driving Th17-dependent intestinal inflammation[68]. Treatment with neutralizing antibody specific for either α4β7 or α4 integrin improved disease symptoms in cotton-top tamarins and inhibited development of ileitis in SAMP1/Yit mice[46]. Likewise, KO of the α4 integrin subunit or GPR15 in donor $CD4^+$ T cells in a $CD45RB^{high}$ adoptive transfer model of colitis resulted in ameliorated disease[39,69].

There is evidence that gut homing molecules drive inflammation in human IBD patients. Peripheral blood leukocytes from both UC and CD patients demonstrated enhanced CCR9 expression compared to those in healthy controls[70]. Human IBD patients demonstrated upregulation of CCR9 on a pro-inflammatory human leukocyte antigen (HLA)-DR^{hi} $CD14^+$ subset of monocytes during active disease [71]. UC patients and mice with oxazolone-induced colitis also displayed enhanced expression of CCR9 on invariant NKT (iNKT) cells and enriched colonic iNKT populations, though the functional contribution of these cells is unknown[72]. In addition, $CCR9^+$ small bowel lymphocytes from human CD patients exhibited a more inflammatory phenotype, producing more IL-

17 and IFN-γ than did small bowel lymphocytes from non-IBD controls[51]. In human UC patients, GPR15 is upregulated on Th2 effector cells[39,53]. In addition, clinical trials for therapeutic agents that block homing molecule-receptor interactions have had modest success.

In other models, leukocyte adhesion molecules have demonstrated a protective role. In a $CD4^+$ $CD45RB^{high}$ adoptive transfer mouse model, CD103 was dispensable for induction of colitis but required for protection by the $CD45RB^{lo}$ subset[59]. In addition, CD103 expression on $CD11c^+$ $CD4^-$ cells, but not Tregs, was required for protection from colitis[59]. In a TNFΔARE mouse model, transfer of $CD103^+$ $CD8^+$ T cells attenuated ileal inflammation in a TGF-β-dependent manner[46]. In an αCD40 model of colitis, in which inflammation is mediated by innate cells, GPR15 expression on Treg cells was required for protection[38].

The effect of leukocyte adhesion models on the severity of IBD appears to depend on the method and timing of neutralization, even within the same model. Blockade of CCR9 or CCL25 during induction of small bowel inflammation in a SAMP1/YitFc mouse model prevents disease[73]. However, treatment with anti-CCR9 or anti-CCL25 was found to have no effect once inflammation was established[73]. In a TNFΔARE mouse model, KO of CCR9 did not impact disease progression, while administration of CCX282 prevented the development of ileitis[50]. In a $CD45RB^{high}$ adoptive transfer model of colitis, transferred WT and CCR9 KO CD4+ T cells were equally able to induce inflammation[74]. However, in the same model, KO of CCL25 in $Rag^{-/-}$ recipient mice led to exacerbated colitis when WT $CD4^+$ T cells were transferred[74]. Upon further investigation, $CCL25^{-/-}$ $Rag^{-/-}$ mice demonstrated enriched neutrophil populations and

altered DC phenotypes in both DSS and adoptive transfer colitis models, indicating that the migratory function of innate cells plays a vital role in the regulation of IBD[74,75]. Unfortunately, this study did not examine innate leukocyte phenotypes in CCL25$^{-/-}$ Rag $^{-/-}$ mice under homeostatic conditions[74].

CHEMOKINES AS THERAPEUTIC TARGETS IN IBD

Effective treatment of IBD is extremely challenging. Only a handful of drugs are approved for use as IBD therapeutics, and many have severe side effects, complex dosing regimens, and/or inconvenient methods of delivery[5,9]. In addition, treatment adherence for IBD patients is suboptimal, which increases the risk for severe inflammation and gastrointestinal cancer[5]. Patients who do not tolerate or become refractory to specific treatments have few options before relying on surgical interventions[9]. Hence, there is a great need for the development of new therapies for IBD. Chemokines and their receptors are considered attractive targets for IBD therapy due the hypothetical ability to disrupt migration to the gut, and therefore intestinal inflammation, with minimal effect on the rest of the immune system[9]. Several chemokine-specific interventions have recently been approved or are currently in clinical trials, including monoclonal antibodies, small molecule inhibitors, and leukapheresis therapy[9].

Natalizumab is a humanized monoclonal antibody specific for the α_4 integrin subunit that functions by blocking the interaction of $\alpha_4\beta_7$ and $\alpha_4\beta_1$ with their ligands, MAdCAM1 and VCAM-1, respectively[8,9]. It is currently being investigated as a therapeutic in both IBD and multiple sclerosis (MS) with some success[8,9]. Clinical trials indicate that natalizumab is more effective at inducing remission in CD patients than placebo, but requires co-treatment with immunosuppressants or TNF-α blockers for

greatest efficacy [8,9]. Natalizumab appears to be more effective at maintenance of remission than induction[8]. However, there are serious safety risks to natalizumab treatment, including melanoma, liver injury, and opportunistic CNS infections such as progressive multifocal leukoencephalopathy (PML)[9,76]. Targeted neutralization of the $\alpha_4\beta_7$ ligand MAdCAM-1, whose expression is limited to the gut, has been suggested as a possible method of reducing these safety risks[8].

Vedolizumab is another humanized monoclonal antibody being investigated as an IBD therapeutic. Vedolizumab targets total $\alpha_4\beta_7$ integrin, rather than the α_4 subunit[9]. This makes it more specific than natalizumab, which theoretically will result in an improved safety profile, though it may also reduce efficacy [9]. A small study showed that CD patients receiving vedolizumab experienced higher rates of remission and reduced disease severity compared to patients receiving placebo[77]. Vedolizumab appears to have a delayed clinical response, as in a larger study CD patients who received vedolizumab showed a small, but not significant, improvement at 6 weeks[78]. However, CD patients receiving vedolizumab had significantly higher rates of clinical remission, glucocorticoid-free remission, and CDAI-100 responses than did patients in the placebo group at 52 weeks, the endpoint of the study[9,78]. Vedolizumab was effective in treating UC, with patients who received vedolizumab demonstrating statistically significant endoscopic improvement at 6 weeks versus patients who received placebo[9,79]. While more effective than placebo for inducing remission, response rates are still fairly low and meta-analysis suggests that vedolizumab may be more effective at preventing relapse than inducing remission[79]. Also, Vedolizumab was associated with an increase in headache, fever, nasopharyngitis, and serious infections; however to date it has not been

associated with an increased risk of PML[77–79]. In addition, loss of efficacy due to the development of human anti-human antibodies against vedolizumab remains a concern for long-term treatment, though combination treatment with immunosuppressants may mitigate this risk[77,79].

Another monoclonal antibody currently undergoing clinical trials is etrolizumab, which targets the β7 integrin subunit[80]. Phase II trials involving 124 patients indicated that etrolizumab was more effective than placebo at inducing remission by the 10-week endpoint of the study. In addition, etrolizumab was well-tolerated, with adverse event rates similar to patients treated with placebo and no reported opportunistic infections[80]. CCX282-B, a small-molecule CCR9 antagonist currently being evaluated for the treatment of CD, has reached clinical trials[81,82]. CCX282-B is biologically available when administered orally, making it more convenient than monoclonal antibodies for ongoing treatment and therefore extremely appealing due to ease of use and decreased cost of treatment[9,81,82]. *In vitro* testing indicates that CCX282-B is highly specific and inhibits CCR9-dependent chemotaxis[81]. In a randomized controlled trial, patients with moderate-to-severe CD who received CCX282-B demonstrated decreased CDAI scores, higher remission rates, and decreased corticosteroid administration compared to those in patients that received placebo[82]. CCX282-B appears to be well tolerated, and the percentage of CCX282-B-treated patients reporting adverse events, including severe adverse events, was similar to that of patients receiving placebo[9,82]. However, the usefulness of CCX282-B as an IBD therapeutic is limited by a protracted clinical response, requiring 12 weeks to a year to observe significant improvement depending on dose[82].

Leukapheresis therapy, in which patient whole blood is passed through a column to deplete target cell populations before being returned to the patient, has seen limited investigation as a therapy for IBD. One such experimental therapy utilizes a CCL25 leukapheresis column to deplete $CCR9^+$ cells[70]. A pilot study of a single patient with moderate ulcerative colitis resulted in rapid clinical and endoscopic improvement after one treatment and stable clinical remission at ten weeks post-treatment[70]. Other types of leukyocyte apheresis devices are also being investigated, including a cellulose acetate column to deplete granulocytes and monocytes and a polyester fiber filter[8].

THE FUTURE OF CHEMOKINE RESEARCH: REGULATION OF CELLULAR AND IMMUNOLOGICAL FUNCTION BY HOMING MOLECULES

While the migratory function of chemokines and integrins has been well-characterized, little research has been conducted with regard to other possible functions of homing molecules. A small, but growing body of literature suggests that chemokine signaling plays a role in non-migratory functions, including $CD4^+$ T cell polarization and apoptosis. However, much of this research has not investigated gut-trophic chemokines or been done in cancer models. Hence, we know little about the functional roles of chemokines and integrins specific to the intestines, particularly in the context of IBD. Investigation of non-migratory functions of gut homing molecules would provide vital data regarding how therapeutic interventions targeting chemokines and their receptors may affect $CD4^+$ T cell subsets and long-term prognosis in IBD patients.

Several chemokine receptors and ligands have been shown to affect $CD4^+$ T cell polarization and disease outcome in an EAE model. The chemokine (C-X-C motif) ligand 12 (CXCL12) upregulates IL-10 and IL-4 and suppresses production of IL-12, IL-23,

IFN-γ, and TNF-α[41]. Neutralization of CXCL12 results in more severe EAE, whereas treatment with a stable CXCL12 Ig construct improves disease outcome[41]. CXCL11 signaling through chemokine (C-X-C motif) receptor 3 (CXCR3) is likewise anti-inflammatory, and treatment with CXCL11-Ig suppresses EAE in a $CD4^+$ T cell, IL-10 dependent manner[40]. CXCL11 induces IL-10 producing T regulatory 1 (Tr1) cells and suppresses Th1 and Th17 development through a STAT-3 and -6-dependent, Gα1-indepndent pathway[40]. In contrast, CXCL10 signaling through CXCR3 drives an inflammatory $CD4^+$ T cell phenotype with enhanced IFN-γ and IL-17[40]. CCR5 is also inflammatory, and neutralization of CCR5 or its ligand CCL3 ameliorates EAE[42]. The impact of gut-trophic chemokine signaling on $CD4^+$ T cell phenotype has not yet been investigated.

Research findings from studies in ovarian and prostate cancer cells point to an anti-apoptotic role for CCR9 signaling[43,44]. These studies indicate that CCL25 reduces susceptibility to apoptosis in response to treatment with cisplastin and etoposide[43,44]. CCR9-dependent resistance to apoptosis appears to be mediated by PI3K and AKT via a signaling cascade that includes glycogen synthase kinase 3β (GSK-3β) and forkhead in rhabdomyosarcoma (FKHR)[43,44]. Antibody neutralization of CCR9 restores apoptotic function in response to chemotherapeutic drugs both *in vitro* and *in vivo*[43]. It is currently unknown if anti-apoptotic signaling through CCR9 also occurs in healthy cells. If the pathway is preserved, CCR9 signaling may have significant effects on Treg suppressive function and inflammatory T cell proliferation.

Conclusions

Gut trophism in lymphocytes and leukocytes is primarily controlled via RA-dependent upregulation of CCR9, CD103, and $\alpha_4\beta_7$. Additional factors, such as cytokines or the microbiota composition, also influence homing functions by controlling tissue expression of ligands CCL25, MAdCAM1, and E-cadherin. These proteins are essential to the development of both oral tolerance and the protective immune response against intestinal pathogens. The role of gut-specific homing molecules in IBD appears to be highly nuanced and dependent on disease phase, experimental model, and cell type. Further research involving the use of cell-type-specific KOs such as a flox-Cre system may help further elucidate the role of chemokine and integrin expression in specific cell types on the development and maintenance of IBD. In addition, possible non-migratory functions for gut homing molecules need be examined.

Despite early promise of tissue-specific homing inhibitors in the treatment of IBD, there are several concerns regarding their use. Slow clinical response rates limit the clinical usefulness of these therapies for patients with active disease. However, chemotaxis inhibitors may still be useful as maintenance therapy or in combination with other pharmaceuticals, similar to the way purine analogs (azathioprine and 6-mercaptopurine) are currently utilized in treatment of IBD. In addition, the integral role of leukocyte adhesion molecules in inducing and maintaining tolerance means that the effect on patient Treg populations must be carefully examined to ensure that inflammation is not exacerbated in patients receiving such treatments.

Chapter 4: T cell metabolism

Immunometabolism is an exciting and rapidly expanding field. The metabolic pathways utilized by $CD4^+$ T cells are irrevocably linked to T cell fate and function[27,83,84]. Energy production in the cell, or cellular respiration, relies on the conversion of glucose to adenosine triphosphate (ATP). In addition, biosynthetic pathways are crucial for lymphocyte proliferation and cytokine production. There are two distinct mechanisms of respiration: glycolysis and oxidative phosphorylation. Glycolysis is inefficient, producing only 2 molecules of ATP per molecule of glucose, but produces ATP quickly and generates biosynthetic building blocks as byproducts[84–86]. Oxidative phosphorylation is extremely energy-efficient, producing 36 molecules of ATP per glucose, but occurs too slowly and produces too few biological intermediates to serve the needs of rapidly proliferating cells[83–86]. These processes are tightly regulated by a network of cytokines, costimulatory molecules, and metabolic signaling pathways.

Metabolite availability and host nutritional status has a profound impact on immune function[83,87]. Obesity has been linked to chronic inflammation and Treg dysfunction, whereas nutrient deprivation is associate with immunosuppression[83,87]. Metabolic hormones and signaling pathways also have profound influence T cell function[83,84,87,88]. Likewise, both host and commensal metabolites have been shown modulate immunological function, including the differentiation of T helper subsets[12,27,83–85,88]. Given this information, it is no surprise that alterations in T cell metabolism have been observed in a number of diseases, including asthma, systemic lupus erythematosis (SLE), and graft-versus-host disease (GVHD)[83,86–88]. Hence, metabolic processes are being investigated as potential targets for immunotherapy[86].

METABOLIC SIGNALING PATHWAYS

Metabolism is controlled by a vast array of interconnected signaling pathways. These pathways respond to a variety of signals to tightly regulate cellular metabolic processes, including respiration and biosynthesis. Many metabolic signaling pathways have profound effects on immune function[27,83,85,86,88].

One major regulator of both cell metabolism and T cell function is the mammalian target of rapamycin (mTOR). mTOR interacts with a number of scaffolding proteins to form two distinct signaling complexes, mTORC1 and mTORC2, both of which include mTOR and mLST8[27]. The mTORC1 also complex consists of Rheb, Raptor, and PRAS40[27]. The scaffolding proteins in the mTORC2 include Rictor, Sin1, and Protor[27]. Signaling through mTOR is a complex and tightly regulated process influenced by input from a variety of stimuli, which costimulatory molecules, growth factors, nutrient availability, hypoxia, and oxidative stress[27,83–85,88]. The primary activator of mTOR is PI3K/Akt[27,88]. Activation of mTOR upregulates glycolysis, protein translation, and lipid biosynthesis while inhibiting autophagy[27,85,88]. A number of nutrient transporters are also upregulated by mTOR, including the glucose transporter GLUT1[83,85,87].

Another important regulator of metabolism is AMP-activated protein kinase (AMPK). AMPK senses cellular energy levels primarily by sensing high intracellular concentrations of AMP[27,85]. AMPK is activated during states of stress, such as glucose deprivation and hypoxia, as well as during TCR stimulation[85,88]. Once activated, AMPK promotes ATP generation by oxidative phosphorylation of glucose and lipids while downregulating glycolysis and glucose transport[83,85,88]. AMPK also promotes autophagy to provide both fuel and material for biosynthesis[83]. Additionally, activation of AMPK inhibits proliferation by arresting the cell cycle at the gap 1 phase and preventing progression to the synthesis phase[85]. AMPK is also an important inhibitor of mTOR

signaling, and the mTOR/AMPK axis serves as a metabolic switch for glycolysis and oxidative phosphorylation[27,83,85,88].

Hypoxia-induced factor 1 (HIF-1) is another important metabolic signaling molecule. HIF-1 plays a vital role in oxygen sensing, and is stable under hypoxic conditions but rapidly degraded under normoxic conditions[83,88]. HIF-1 is also activated by a variety of stimuli during normoxia, particularly inflammation, by upstream signaling through STAT3 and mTOR[83,88]. Downstream, HIF-1 primarily induces glycosis by upregulating transcription of glucose transporters and glycolytic enzymes[83,86,88]. Other roles for HIF-1 include cell survival, angiogenesis, and enhancement of innate immune cell antimicrobial function[88]. These functions make HIF-1 a target of interest to both oncologists and immunologists[88].

The nuclear hormone receptors in the peroxisome proliferator-activated receptor (PPAR) family, which occur in α, β/δ, and γ isoforms, are another major regulator of metabolism[88]. PPAR receptors are activated by intracellular fatty acids and can both act as a transcription factor by binding to specific DNA sequences to and suppress gene expression by binding to other transcription factors[88]. PPARs primarily regulate lipid metabolism, including lipid transport and β-oxidation, though they also regulate a number of immunological pathways[88]. PPARs are also a key regulator of development and function in adipose tissue, including adipocytes and VAT immune cells[88].

THE ROLE OF METABOLISM IN $CD4^+$ T CELL FATE AND FUNCTION

$CD4^+$ T cells utilize different metabolic pathway according to T helper subtype and activation status. The metabolic pathways utilized by $CD4^+$ T cells are largely dependent on the energy and biosynthetic requirements of the cell. $CD4^+$ T cell populations with high energetic and biosynthetic needs and thus utilize glycolysis to produce ATP rapidly[27,83,85]. Meanwhile, $CD4^+$ T cells with lower energetic and biosynthetic needs rely on the more energy-efficient pathway of oxidative

phosphorylation for ATP production[27,83,85]. It is important to note that both modes of energy production must be actively maintained, and without the appropriate signals growth factor withdrawal and apoptosis occur[85].

Resting or quiescent T cells, such as naïve and memory cells, have low energetic needs and produce little new biomass[27,83,85,87]. Energy is primarily utilized for cytoskeletal rearrangements as the cells move through the periphery[83]. Because of this, ATP is primarily synthesized via the highly efficient method oxidative phosphorylation[83,85]. Raw materials for protein synthesis are derived primarily from authophagy, rather than *de novo* synthesis[27]. Homeostasis in these cells is maintained via IL-7 signaling through the IL-7 receptor[27,83,85]. In the absence of IL-7, quiescent T cells suffer from growth factor starvation and eventually apoptose[83,85].

Upon activation, $CD4^+$ T cells proliferate explosively, vastly increasing their energy requirements and rapidly synthesizing new biomass[27,83,85–88]. Hence, Teff metabolism favors glycolysis, which prioritizes speed of ATP production and *de novo* synthesis of biomolecules over energy efficiency[27,83,88]. Once activated, $CD4^+$ T cells rapidly upregulate glycolytic machinery, translocate GLUT1 to the cell surface, and transition from IL-7 dependency to IL-2 dependency[85–87]. TCR signaling along with costimulation through CD28 is required for T cell activation[27,85–88]. Tregs are metabolically unique compared to other activated $CD4^+$ T cells. Though Tregs require IL-2, actively proliferate, and synthesize proteins; Tregs have low expression of GLUT1 and respiration occurs primarily via oxidative phosphorylation rather than glycolysis[27,83,86–88]. Treg preference for oxidative phosphorylation is thought to be due to the need to maintain a stable pool of antigen-specific Tregs, similar to the maintenance of memory $CD4^+$ T cells[86,88].

Differentiation and function of the various T helper subsets are also influenced by metabolism[27,83,87]. $CD4^+$ T helper subset differentiation is largely dependent on the status of mTOR at the time of TCR engagement[27,88]. KO of mTOR or inhibition of mTOR via

rapamycin results in impaired Teff responses and enhanced generation of Tregs[27,83,87,88]. Treatment with metformin, which upregulates AMPK and thus inhibits mTOR signaling, also enhances Treg generation[87,88]. Conversely, AMPK deficiency results in constitutive activation of mTOR, fewer Tregs, and more severe inflammation[88]. Naïve T cells which lack mTORC1 activity due to Rheb deficiency fail to develop into Th1 or Th17 Teffs, but retain Th2 function[27,83,87,88]. Conversely, naïve T cells deficient in Rictor or mTORC2 signaling differentiate into Th1 and Th17 cells, but not Th2 cells[27,83,87,88]. HIF-1 also plays a role in $CD4^+$ T cell differentiation by fine-tuning the balance between Th17 and Treg signals[83,86–88]. HIF-1 is crucial to the generation of Th17 cells and binds to the signature Th17 transcription factor RAR-related orphan receptor-γ (RORγt) to enhance transcription of Th17 genes[86–88]. HIF-1 appears to destabilize FoxP3 and HIF-1 deficiency leads to an increase in Tregs[83,86–88].

NUTRITIONAL STATUS AND IMMUNE REGULATION

Overall nutritional status plays an important role in immune function. Obesity results in a state of chronic, low-grade inflammation[83,87]. Obese patients exhibit higher levels of IL-6, TNF-α, and c-reactive protein (CRP); along with metabolic abnormalities such as insulin resistance[83,87]. TNF-α in particular has been associated with insulin resistance, and treatment with exogenous TNF-α can reduce insulin sensitivity[83]. Both metabolic dysregulation and excessive inflammation are ameliorated upon weight loss[83]. IL-17 is also upregulated in obese subjects, though its role appears to be more complex. In mice, IL-17 appears to regulate weight gain but also promotes insulin resistance[83]. Leptin, a hormone secreted by adipocytes, also regulates immune function by promoting an inflammatory Th1 response[83]. In Tregs, leptin activates mTOR, resulting in anergy and failure to proliferate[83]. Constitutive expression of GLUT1, and thus high intracellular glucose concentration, also results in a highly inflammatory state[83]. Conversely,

malnutrition, impaired glycolysis, and leptin deficiency all result in immunosuppression[83,87].

A number of fatty acids have been shown to regulate immune function. Fatty acid signaling through PPAR family receptors has a profound anti-inflammatory effect, enhancing Tregs and IL-10 production while decreasing Th1 and Th17 polarization and function[88]. Cojugated linoleic acid (CLA), a microbial metabolite, is one PPAR ligand which has demonstrated immunomodulatory properties[12]. CLA has been shown to downregulate cyclooxygenase (COX) and lipoxygenase (LO) as well as a number of iκlammatory signaling pathways, including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), $p38^{MAPK}$,and jun N-terminal kinase (JNK)[12]. In addition, short-chain fatty acid (SCFA) signaling through free fatty acid receptor (FFAR) 2 regulates histone deacetylase (HDAC) 6 and HDAC9 expression, leading to enhanced Treg populations and IL-10 production[12].

Intracellular nutrient availability can also regulate T cell function. High levels of amino acids are required for mTOR activation and optimal Teff function[27,88]. Low levels of essential amino acids lead to impaired effector T cell responses and Treg generation[27,88]. In addition, specific amino acids have been shown to modulate the immune response. Glutamine catabolism is required for proper activation, differentiation, and function of effector T cells[83,84,86,88]. In part, glutamine is required for the transport of another amino acid, leucine, which plays a vital role in the activation of mTOR[27,84]. Cysteine is also required for Teff function, and availability is controlled by APCs to limit inflammatory responses[27,85]. The breakdown of tryptophan by the enzyme indoleamine 2,3-dioxygenase (IDO) results in enhanced Treg responses and impaired Th17 function[85,88]. Likewise, breakdown of arginine by arginase I has been shown to generate anti-inflammatory responses[88]. Glucose is also required for optimal Teff function, as it is essential to glycolysis[27,83,86,87]. Glucose deprivation in the form of treatment with glucose analogs blocks mTOR activation and impairs Teff function[27,83,84,86,88].

TARGETING METABOLISM IN IMMUNOTHERAPY

Improper or aberrant $CD4^+$ T cell responses are implicated in the pathogenesis of many infectious diseases, autoimmune disorders, and cancers. Inordinate Teff responses result in uncontrolled inflammation and immunopathology. Conversely, excessive Treg function leads to immunosuppression, failure to clear infection, and unchecked progression of cancer. Skew toward the wrong Teff subset can also result in failed pathogen clearance and chronic infection. Metabolic pathways play a key role in fine-tuning the differentiation and function of the various T helper subsets. Exhausted and anergic Teffs, which contribute to the progression of many chronic infections and cancers, demonstrate metabolic defects[27,83]. Additionally, there is evidence that T cell metabolism is dysregulated during disease. T cells from patients with certain immune disorders display an altered metabolic phenotype compared with those from healthy controls[83]. Due to the influence of metabolism on immunological function, along with the presence of metabolic abnormalities in dysfunctional T cells, there has been keen interest in utilizing metabolic pathways to manipulate $CD4^+$ T cell differentiation and function[86].

Targeting HIF-1 in Th17-mediated autoimmunity is one possible method of modulating immune function by influencing metabolic factors. MS is one such illness. Blocking HIF-1 function in an EAE model by either T cell-specific KO or pharmacological inhibition leads to less severe disease[88]. Inhibition of mTOR upstream of HIF-1 similarly reduced EAE severity[88]. HIF-1 has also been proposed as a therapeutic target for IBD and is upregulated in inflamed intestinal tissue from IBD patients[88]. However, the exact role of HIF-1 in colitis is not currently clear[88]. While Th17 cells are pathogenic in IBD, there is some evidence that expression of HIF-1 by epithelial cells may play an anti-inflammatory role[88]. Hence, more investigation is required to properly evaluate HIF-1 as a therapeutic target in IBD[88]. Lastly, HIF-1 in both tumor cells and T cells has been investigated as a therapeutic target for anticancer drugs. HIF-1 in tumor

cells promotes a number of oncogenic processes, including metastasis, angiogenesis, and resistance to apoptosis[88]. In addition, HIF-1 promotes Th17 conversion, which in turn supports tumor growth[88]. However, it is likely that synergistic therapies inhibiting Treg function would be required with this approach[88].

Improving tolerance by targeted upregulation of AMPK is another therapeutic strategy being investigated[88]. 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and metformin, two activators of AMPK, have been investigated in the treatment of several inflammatory disorders[88]. Treatment with AICAR in a TNBS model of colitis resulted in improved pathology and decreased Th1 and Th17 responses[88]. Administration of metformin resulted in Treg generation in an asthma model[86–88]. Upregulation of AMPK also improves disease outcome in an EAE model by upregulating Tregs and inhibiting Teffs[88]. Treatment with metformin or AICAR in this model resulted in less severe disease, decreased CNS infiltrate, decreased Th1 and Th17 responses, and increased IL-10[88]. Conversely, AMPK deficiency in an EAE model leads to more severe disease[88]. Hence, AMPK agonists appear promising for the treatment of human autoimmune disease, particularly since metformin is already approved for use in humans.

PPARs are another promising target for metabolism-related immunotherapy. PPARγ agonists are currently being investigated as therapies for several autoimmune disorders[88]. Early studies in mouse models of asthma indicate that PPARγ agonist treatment resulted in protection from disease and decreased pro-inflammatory cytokine production[88]. In a DSS colitis model, PPARγ expression by Tregs was required for protection, and global PPARγ deficiency increased disease severity and production of pro-inflammatory cytokines[88]. PPARγ agonist treatment was also effective in clinical trials involving human ulcerative colitis patients[88]. Probiotic bacteria that produce PPAR ligands such as CLA have also been investigated as IBD therapeutics[12]. In both a DSS mouse model and human CD patients, treatment with CLA or CLA-producing bacteria resulted in less severe disease and reduced production of pro-inflammatory cytokines[12].

PPAR function has been extensively investigated in EAE models. Administration of PPARα, PPARβ/δ, or PPARγ agonists resulted in downregulation of Th17 responses, increased IL-10, and decreased EAE severity[88]. Conversely, KO of PPARβ/δ resulted in enhanced disease[88]. In clinical trials, human MS patients treated with a PPARγ agonist demonstrate less atrophy in the brain, though clinical scores were not found to be improved[88].

Teffs demonstrate abnormal metabolic profiles in several autoimmune disorders. Teffs from patients with SLE, rheumatoid arthritis, and GVHD all have decreased glycolytic activity and increased energy production through oxidative phosphorylation[83,84,86,87]. Mitochrondrial mass is increased in these alloreactive Teffs, whereas lactate production is decreased[83,86,87]. Interestingly, despite primarily utilizing oxidative phosphorylation for energy production, these Teffs also have increased GLUT1 expression[86]. The mechanism of dysregulated metabolism in alloreactive Teffs is unclear. It is hypothesized that signaling through inhibitory receptors such as programmed cell death protein 1 (PD-1) and CTLA-4 inhibits c-Myc, a signaling protein that regulates survival, proliferation, and glycolysis[86,87]. Despite this, therapeutics that inhibit oxidative phosphorylation have shown promising results in animal models of GVHD[86,87].

T cell exhaustion and anergy, in which T cells become unresponsive to antigen, are a major issue in the resolution of chronic infections and cancer[84]. These cells fail to proliferate in response to antigen stimulation and have impaired cytokine production[27,83,84]. Exhausted and anergic T cells also have impaired metabolic function, including decreased IL-2 production, decreased glycolysis, and increased oxidative phosphorylation[27,83,84]. Anergy is thought to result from insufficient costimulation through CD28 during antigen presentation, resulting in failure to activate mTOR[27,83]. Inhibition of metabolic pathways or lack of metabolic substrates such as glucose also results in anergy[83,84]. In exhausted T cells, there is evidence that mTOR is downregulated due to CTLA-4 and PD-1 signaling[83]. Hence, in these T cells, glycolytic and biosynthetic

pathways are not sufficiently upregulated[83,87]. It is thought that restoration of glycolytic metabolism in these cells would restore T cell effector function, enabling pathogen clearance and promoting anti-tumor immunity[27,83].

RESULTS

Chapter 5: CCR9 expression on CD4$^+$ T cell subsets

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INTRODUCTION

Lympocyte migration is a tightly regulated process mediated by ligand-receptor pairs. Homing receptors, including chemokines and integrins, play an important role in lymphocyte migration and tissue specificity. CCR9 is a gut-trophic chemokine receptor expressed on DCs and lymphocytes. Its ligand, CCL25, is expressed in the small bowel (SB) and thymus. CCR9 is thought to play a role in the development of IBD, and several therapeutics targeting CCR9 are currently in clinical trials. It is known that CCR9 and other gut homing receptors can be induced by RA[54,55,89]. Cytokines and the microbiota are also capable of regulating the expression of CCR9 and its ligand CCL25[36,61]. It is well-documented that chemotactic molecules are often preferentially expressed on specific T helper subsets[38–40,53]. A previous report indicated that CCR9 was preferentially expressed by Tregs[50]. However, this report only examined the differences between Tregs and non-Tregs, and differences between Tregs and Teffs (as opposed to naïve CD4$^+$ T cells) were not compared. Hence, we sought to characterize the expression of patterns of CCR9 on Tregs, Teffs, and naïve CD4$^+$ T cells in order to more comprehensively

examine the phenotype of $CCR9^+$ $CD4^+$ T cells. In addition, we investigated a possible mechanism for differential regulation of CCR9 in T helper subsets.

MATERIALS AND METHODS

Mice. CBir1 flagellin-specific TCR transgenic mice (CBir1 Tg) were maintained in the animal facilities of the University of Texas Medical Branch. $CCR9^{-/-}$ mice were kindly provided by Dr. Joshua Farber of NIAID, NIH, and crossed with CBir1 transgenic (Tg) mice. Tail snips were collected 3 weeks of age to confirm genotype. Littermates were selected and cohoused until they were 6 to 8 weeks old, when experiments were performed. TCRβx$\delta^{-/-}$ mice were purchased from Jackson Laboratories. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Antibodies and reagents. All flow cytometry antibodies, CCL25, and TGF-β were obtained from BioLegend. CD4 magnetic beads were purchased from BD Biosciences.

Isolation of $CD4^+$ and APCs. $CD4^+$ T cells were isolated by using mouse anti-CD4 magnetic beads as previously described [90]. The mice were euthanized by CO_2 inhalation followed by cervical dislocation. Mouse splenocytes were incubated with CD4 magnetic beads for 30 minutes at 4 C. Afterward, cells were placed on a magnetic column three times, for durations of 8, 4, and 4 minutes. After each incubation, the supernatant was aspirated, collected, and replaced by fresh buffer. The $CD4^-$ fraction collected from the CD4+ isolation procedure was irradiated for use as antigen presenting cells (APCs).

Necropsy and LPL isolation. The spleen, MLN, and small and large intestines were extracted and fat and connective tissue carefully removed. The large and small intestines were cleaned via scraping and cut into pieces, then were washed by shaking in PBS and straining through a sieve several times. Tissue was then incubated with magnetic stirrers in PBS with 1% fetal bovine serum (FBS) and 1:1000 ethylenediaminetetraacetic acid (EDTA) at 37 C for 40 minutes to remove the epithelium. Remaining tissue was collected, washed in PBS several times, then dried and chopped finely. The tissue was then incubated with collagenase twice for 30 minutes at 37 C. Supernatant was collected and and fresh collagenase was added after the first incubation. After the second incubation, all supernatant was strained through a filter washed via centrifugation. The pellet was then layered into a 75%/40% percoll gradient, centrifuged, and the interface layer was collected.

Preparation of spleen and MLN leukocytes. Single-cell suspensions were prepared from the spleen, MLN, and thymus by removing fat and connective tissue and smashing between glass slides. Leuckocyte supsensions were then washed via centrifugation and resuspended. Additionally, splenic erythrocytes were then lysed with Tris-ammonia and the remaining leukocytes washed twice via centrifugation.

Flow cytometry. Intracellular and surface staining were performed as previously described[90]. For experiments where intracellular cytokines were measured, cells were restimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin, with Golgi Stop being added for the last 3 hours of restimulation. Cells were washed via centrifugation and surfaced stain added for 20 minutes at room temperature. Cells were then washed and permeablized for 30 minutes at 4 C using a FoxP3

permeabilization/fixation (perm/fix) kit manufactured by eBiosciences. After permeablization cells were washed, intracellular stain was added, and cells were incubated for 30 minutes at 4 C before being washed a final time. Data were collected using the LSRII/Fortessa and compensation performed using FACSDiva software. Further analysis was carried out using FlowJo. All flow cytometry plots shown are first gated on lymphocytes using FSC and SSC, then gated on CD4 positive, live dye negative cells.

T cell culture and polarization. Whole or naïve CBir1 Tg $CD4^+$ T cells ($1x10^5$) were isolated as described above and cultured in 24-well plates with irradiated APCs ($1x10^5$) and 1 µg CBir1 peptide in RPMI with 10% FBS for 5 days. WT APCs were used for all tissue culture experiments. Tregs were induced by addition of 10 ng/mL TGF-β to the media. RA, where indicated, was used at a 1 µM concentration.

Statistical analysis. Samples were analyzed in Prism (GraphPad) via Student's T test. The results were considered significant at a P value of less than 0.05.

RESULTS

CCR9 is upregulated on $FoxP3^+$ and activated T cells *in vivo*

We first determined CCR9 expression in effector T cells compared with Tregs. We sacrificed CBir1 mice and collected the spleen, MLN, SB, and LB and analyzed CCR9 expression on the $FoxP3^+$ and $FoxP3^-$ fractions using flow cytometry. We found that expression of CCR9 on $CD4^+$ T cells was enriched on the $FoxP3^+$ fraction compared with the $FoxP3^-$ fraction in the spleen, MLN, SB and LB (**Fig 1**). This indicates that CCR9 is expressed more frequently on Tregs than non-Tregs.

We then examined the expression of CD44 and CD69 on $FoxP3^+$ and $FoxP3^-$ $CD4^+$ T cells from naïve CBir1 tg mice. CD44 and CD69 are markers of activation and

are generally thought to be present on memory and recently activated $CD4^+$ T cells, respectively. We harvested the spleen, MLN, SB, and LB and prepared them for flow cytometry as described. We found that in the spleen and MLN, both CD44 and CD69 were upregulated on the $FoxP3^+$ population compared to the $FoxP3^-$ population. In the SB and LB, CD69 was also upregulated on the $FoxP3^+$ population compared with the $FoxP3^-$ population. There was no difference in CD44 expression between $FoxP3^+$ and $FoxP3^-$ $CD4^+$ T cells in the SB and LB (**Fig 2**). These data indicated that a greater percent of $FoxP3^+$ cells had an activated phenotype when compared with $FoxP3^-$ cells. This information posed a question as to whether or not the increase in CCR9 expression on Treg cells was due to a large number of $CCR9^-$ naïve T cells in the pool of $FoxP3^-$ cells.

To answer this question, we examined the expression of CCR9 in conjunction with both FoxP3 expression and activation phenotype. Again, we sacrificed naïve CBir1 tg mice and harvested the spleen, MLN, SB and LB. To examine CCR9 expression dependent on Treg status and CD44 expression, $CD4^+$ lymphocytes were gated on the following populations: $FoxP3^+CD44^+$, $FoxP3^+CD44^-$, $FoxP3^-CD44^+$, and $FoxP3^-CD44^-$. The percent of each of these populations expressing CCR9 was then determined. In the spleen, we found that CCR9 was downregulated on the $CD44^-$ populations compared to the $CD44^+$ populations for both $FoxP3^+$ and $FoxP3^-$ cells, though there was no difference in CCR9 expression in the $FoxP3^+$ and $FoxP3^-$ populations. In the MLN, CCR9 was downregulated in the $FoxP3^-CD44^-$ population, with no significant difference in any of the other groups. In the intestines, however, CCR9 expression is significantly downregulated on both $CD44^+$ and $CD44^-$ cells in the FoxP3- population (**Fig 3A**). Similarly, we gated cells from each orgain into into $FoxP3^+CD69^+$, $FoxP3^+CD69^-$, $FoxP3^-CD69^+$ and $FoxP3^-CD69^-$ populations and analyzed CCR9 expression. We found that in the spleen and MLN, CCR9 expression was downregulated in $CD69^-$ cells but was independent of FoxP3 status. In the SB and LB, CCR9 expression was higher on Tregs and reduced on both CD69+ and CD69- FoxP3- T cells (**Fig 3B**). These data indicate

CCR9 is enhance on Tregs compared to Teffs in the intestines. CCR9 is also enhanced on activated T cells compared to naïve T cells in lymphoid organs. It is notable that even naïve T cells demonstrate expression of CCR9, albeit at low levels.

RA induces CCR9 upregulation on Tregs *in vitro*

Having observed upregulation of CCR9 on Tregs compared to non-Tregs, we set out to confirm these findings *in vitro.* We cultured isolated splenic CBir1 tg $CD4^+$ T cells under neutral conditions, with TGF-β, or with TGF-β and RA. RA has previously been reported to upregulate expression of gut-trophic homing molecules and enhances Treg generation[54,55,89]. We found that in consistence with previous reports[58], RA induced T cell expression of CCR9. Interestingly, $FoxP3^+$ T cells expressed higher levels of CCR9 than $FoxP3^-$ cells from the same culture when treated with RA, but not when treated with TGF-β alone (**Fig 4A-B**). This suggests that CCR9 is preferentially upregulated on Treg cells via an RA-dependent, TGF-β-independent mechanism.

DISCUSSION

We demonstrate here that CCR9 is differentially expressed on various $CD4^+$ T cell subsets. We found that CCR9 was expressed on both Tregs and Teffs, with very low levels of expression on naïve $CD4^+$ T cells. The high expression of CCR9 on Tregs is in agreement with a previous report[50]. Interestingly, expression of CCR9 on Tregs compared to Teffs is organ dependent. In the spleen and MLN, the percent of $CD44^+$ and $CD69^+$ Tregs and Teffs expressing CCR9 is similar, and CCR9 is even enhanced on $CD69^+$ Teffs in the spleen. However, in the SB and LB, percent expression of CCR9 on $CD44^+$ and $CD69^+$ cells is much higher on Tregs than Teffs. The reason for this is unclear, though it is consistent with a report that CCR9 is more important for regulation of colitis by Tregs than for induction of colitis[74]. Further investigation is required to

determine if differential regulation of CCR9 by specific T cell subsets affects T cell function and development of intestinal inflammation. However, this suggests the use of caution in developing CCR9-specific therapeutics, so as not to deplete resident Treg populations.

Interestingly, our data also indicated that the CCR9 expression was higher in Foxp3^{+} T cells when compared to findings in Foxp3^{-} T cells after treatment with RA *in vitro*. While we expected RA to upregulate both Treg numbers and CCR9 expression on CD4^{+} T cells, our data unexpectedly indicate that the effect of RA on CCR9 expression is dependent on T helper subset. In addition, treatment with TGF-β independent of RA resulted in similar expression of CCR9 on Tregs and Teffs. These data indicate that RA upregulates expression of CCR9 in a TGF-β-independent manner.

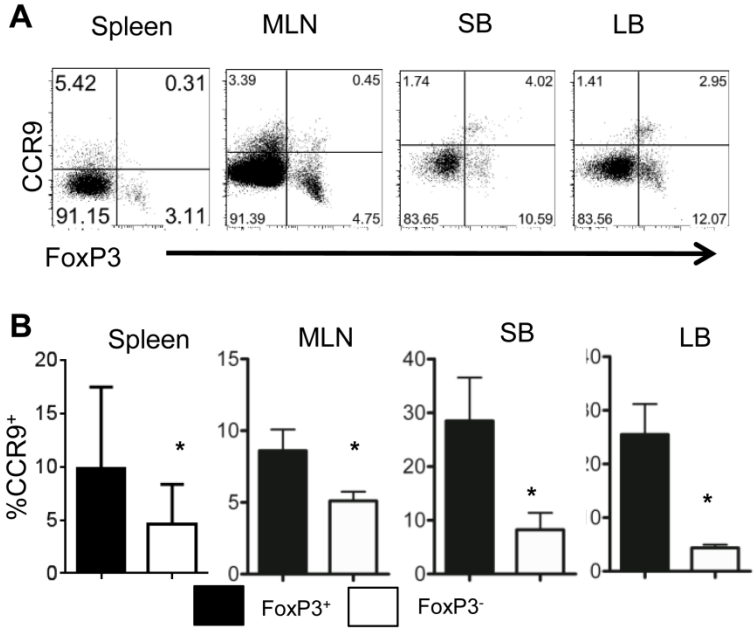
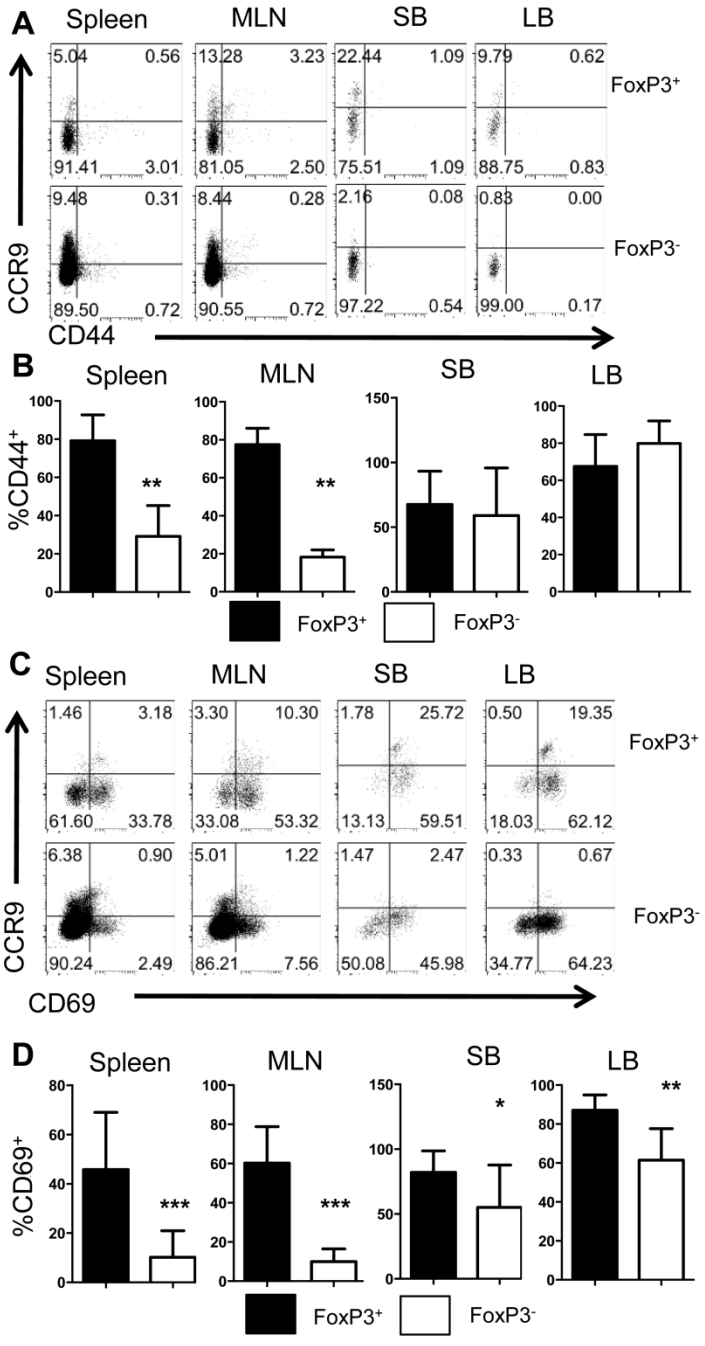


Figure 1. CCR9 Expression is enhanced on Tregs.

Spleen, MLN, SB LP and LB LP CD4^{+} T cells were harvested from healthy WT mice and prepared for flow cytometry as described. Coexpression of CCR9 and FoxP3 was examined via flow cytometry. Representative plots are shown. *(B)* Live CD4^{+} T cells were gated into FoxP3- positive and -negative fractions and analyzed for expression of CCR9. *P<0.05 compared with Foxp3^{+} cells.


A
Spleen
MLN
SB
LB
5.04
0.56
91.41
3.01
13.28
3.23
81.05
2.50
22.44
1.09
75.51
1.09
9.79
0.62
88.75
0.83
FoxP3+
9.48
0.31
89.50
0.72
8.44
0.28
90.55
0.72
2.16
0.08
97.22
0.54
0.83
0.00
99.00
0.17
FoxP3-
CCR9
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B
Spleen
MLN
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**
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FoxP3+
FoxP3-
C
Spleen
MLN
SB
LB
1.46
3.18
61.60
33.78
3.30
10.30
33.08
53.32
1.78
25.72
13.13
59.51
0.50
19.35
18.03
62.12
FoxP3+
6.38
0.90
90.24
2.49
5.01
1.22
86.21
7.56
1.47
2.47
50.08
45.98
0.33
0.67
34.77
64.23
FoxP3-
CCR9
CD69
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LB
%CD69+
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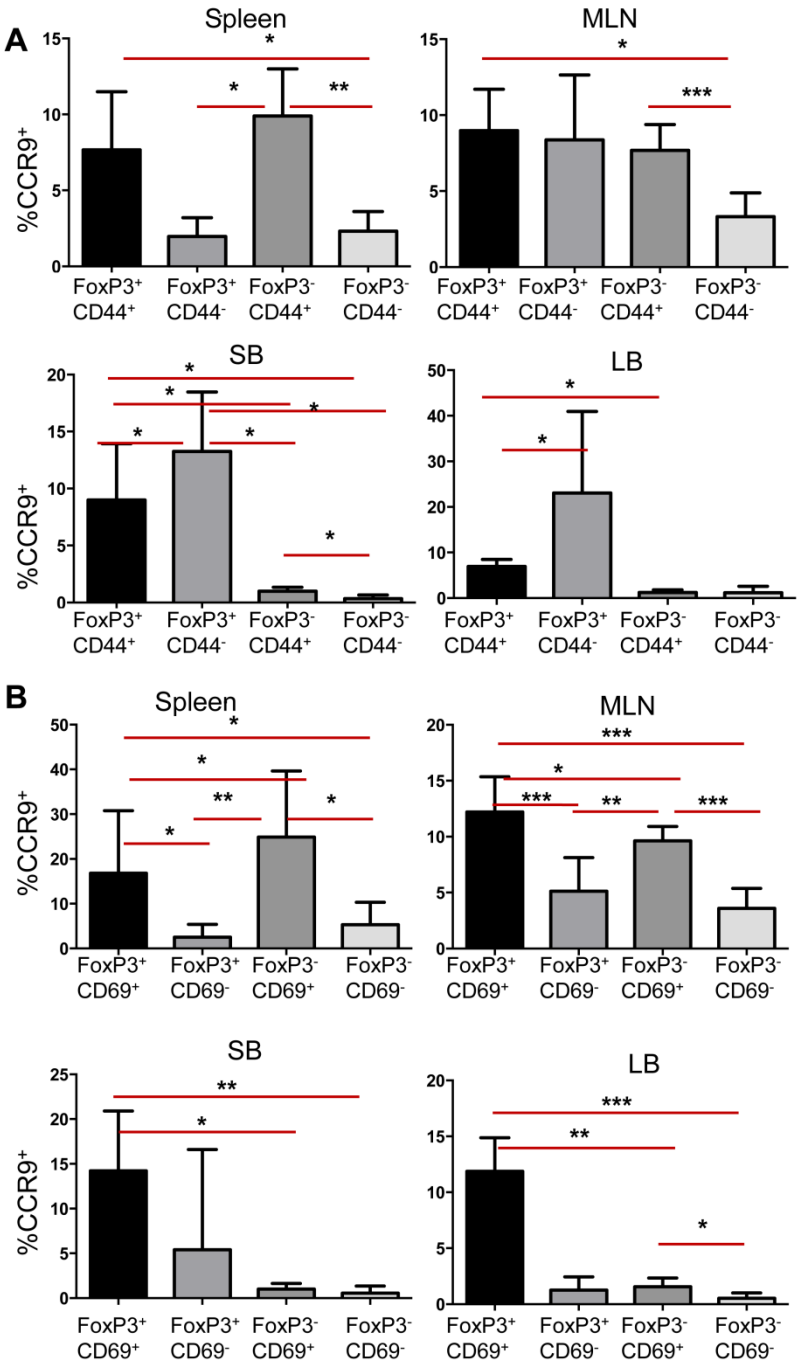
FoxP3+
FoxP3-

Fig 2. Tregs are more likely to express a memory phenotype than non-Tregs.

Spleen, MLN, SB LP and LB LP $CD4^+$ T cells were harvested from healthy WT mice and prepared for flow cytometry as described. *(A-B)* The coexpression of FoxP3 with CD44, a marker for memory T cells, was analyzed. Representative flow cyotmetry plots are shown. Mean percent expression of CD44 on $FoxP3^+$ $CD4^+$ T cells comapared with $FoxP3^-$ $CD4^+$ T cells was then statistically analyzed. *(C-D)* We also analyzed coexpression of FoxP3 with CD69, a marker for recently activated T cells. Flow cytometry plots of representative data are shown. Mean percent expression of CD69 was analyzed, comparing the $FoxP3^+$ and $FoxP3^-$ $CD4^+$ populations. Experiments are representative of 7 mice. *P<0.05; **P<0.01; ***P<0.001 compared with $FoxP3^+$ cells.


A
Spleen
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FoxP3+ CD44+
FoxP3+ CD44-
FoxP3- CD44+
FoxP3- CD44-
B
Spleen
MLN
SB
LB
%CCR9+
FoxP3+ CD69+
FoxP3+ CD69-
FoxP3- CD69+
FoxP3- CD69-

Figure 3. CCR9 is upregulated by activated T cells, with highest expression on Tregs.

Spleen, MLN, SB LP and LB LP $CD4^+$ T cells were harvested from healthy WT mice and prepared for flow cytometry as described. Cells were gated on live $CD4^+$ cells for analysis. *(A)* Cells were gated on $FoxP3^+CD44^+$, $FoxP3^+CD44^-$, $FoxP3^-CD44^+$, and $FoxP3^-CD44^-$ populations. The percent of cells expressing CCR9 in each of these populations was determined and statistical difference was compared between each group. *(B)* Cells were gated on $FoxP3^+CD69^+$, $FoxP3^+CD69^-$, $FoxP3^-CD69^+$, and $FoxP3^-CD69^-$ populations. Mean percent expression of CCR9 in each population was determined and statistical analysis was performed. Data are representative of 7 mice. Statistical significance between groups is indicated by red lines. *P<0.05; **P<0.01; ***P<0.001

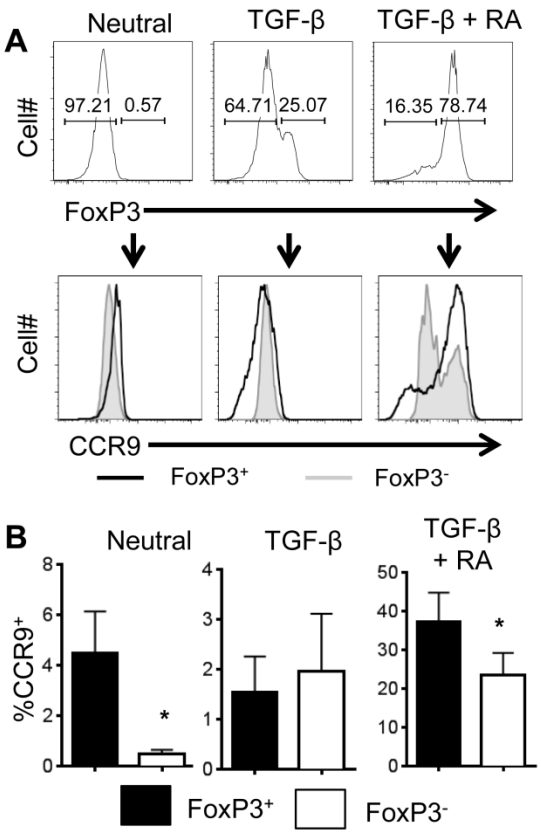


Figure 4. RA preferentially upregulates CCR9 on Tregs *in vitro*.

$CD4^+$ T cells were isolated as described and cultured under neutral conditions, with TGF-β, or with TGF-β and RA and examined via flow cytometry. Live $CD4^+$ lympocytes were gated for analysis. *(A-B)* $CD4^+$ T cells were gated into $FoxP3^+$ and $FoxP3^-$ compartments, and expression of CCR9 in these populations was analyzed. Representative flow cytometry profiles and mean percent expression data from 3 experiments totaling 9 samples are shown. *$P<0.05$ compared to $FoxP3^+$ cells.

Chapter 6: CCR9-dependent inhibition of Treg development

Originally published in *PLoS ONE*. Heather L Evans-Marin, Anthony T Cao, Suxia Yao, Feidi Chen, Chong He, Han Liu, Wei Wu, Maria G Gonzalez, Sara M Dann and Yingzi Cong. 2015, 10;(7):e0134100.Unexpected regulatory role of CCR9 in regulatory T cell development. *PLoS ONE*. Republished in modified form.

INTRODUCTION

Chemokines receptors have well-established roles in lymphocyte trafficking and migration. While it has long been known that some chemokine receptors are preferentially expressed on specific $CD4^+$ subsets, there has been little research conducted regarding whether they can modulate immune function. Recent studies conducted in an EAE model indicate that binding of chemokine receptors to specific ligands can directly influence $CD4^+$ T cell polarization and phenotype[40–42,91,92].CXCL12 ligation drives an anti-inflammatory response in both macrophages and $CD4^+$ T cells, resulting in the upregulated production of IL-10, but not of FoxP3, and decreased production of IFN-γ and TNF-α [41,91,92]. Likewise, CXCL11 ligation induces regulatory Tr1 and Th2 $CD4^+$ T cell phenotypes via mTOR, and treatment with a stable CXCL11-Ig construct results in IL-10-dependent rescue from EAE[40]. In contrast, CXCL10 ligation induces an inflammatory response characterized by increased $CD4^+$ T cell expression of IFN-γ, IL-17, T-bet, and RORγt[40].

There have been few studies regarding possible non-chemoattractant functions of CCR9. These studies have mainly been conducted in cancer models and indicate that ligation of CCR9 to CCL25 induces anti-apoptotic signaling and prevents chemotherapy-

induced cell death in cancer cells [43,44]. However, it is currently unknown if CCR9 can regulate immune function. Here, we examine the effects of CCR9 on the differentiation and function of various $CD4^+$ T helper subsets.

MATERIALS AND METHODS

Mice. CBir1 were maintained in the animal facilities of the University of Texas Medical Branch. $CCR9^{-/-}$ mice were kindly provided by Dr. Joshua Farber of NIAID, NIH, and crossed with CBir1 Tg mice. Tail snips were collected 3 weeks of age to confirm genotype. Littermates were selected and cohoused until they were 6 to 8 weeks old, when experiments were performed. TCRβx$\delta^{-/-}$ mice were purchased from Jackson Laboratories. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Antibodies and reagents. All flow cytometry antibodies, CCL25, and TGF-β were obtained from BioLegend. CD4 magnetic beads were purchased from BD Biosciences.

Isolation of $CD4^+$ and APCs. $CD4^+$ T cells were isolated by using mouse anti-CD4 magnetic beads as previously described [90]. The mice were euthanized by CO_2 inhalation followed by cervical dislocation. Mouse splenocytes were incubated with CD4 magnetic beads for 30 minutes at 4 C. Afterward, cells were placed on a magnetic column three times, for durations of 8, 4, and 4 minutes. After each incubation, the supernatant was aspirated, collected, and replaced by fresh buffer. The $CD4^-$ fraction collected from the CD4+ isolation procedure was irradiated for use as antigen presenting cells (APCs).

Necropsy and LPL isolation. The spleen, MLN, and small and large intestines were extracted and fat and connective tissue carefully removed. The large and small intestines were cleaned via scraping and cut into pieces, then were washed by shaking in PBS and straining through a sieve several times. Tissue was then incubated with magnetic stirrers in PBS with 1% FBS and 1:1000 EDTA at 37 C for 40 minutes to remove the epithelium. Remaining tissue was collected, washed in PBS several times, then dried and chopped finely. The tissue was then incubated with collagenase twice for 30 minutes at 37 C. Supernatant was collected and and fresh collagenase was added after the first incubation. After the second incubation, all supernatant was strained through a filter washed via centrifugation. The pellet was then layered into a 75%/40% percoll gradient, centrifuged, and the interface layer was collected.

Preparation spleen, MLN, and thymus leukocytes. Single-cell suspensions were prepared from the spleen, MLN, and thymus by removing fat and connective tissue and smashing between glass slides. Leuckocyte supsensions were then washed via centrifugation and resuspended. Additionally, splenic erythrocytes were then lysed with Tris-ammonia and the remaining leukocytes washed twice via centrifugation.

Flow cytometry. Intracellular and surface staining were performed as previously described [90]. For experiments where intracellular cytokines were measured, cells were restimulated for 5 hours with PMA and ionomycin, with Golgi Stop being added for the last 3 hours of restimulation. Cells were washed via centrifugation and surfaced stain added for 20 minutes at room temperature. Cells were then washed permeablized for 30 minutes at 4 C using a FoxP3 perm/fix kit manufactured by eBiosciences. After permeablization cells were washed, intracellular stain was added, and cells were

incubated for 30 minutes at 4 C before being washed a final time. Data was collected using the LSRII/Fortessa and compensation performed using FACSDiva software. Further analysis was carried out using FlowJo. All flow cytometry plots shown are first gated on lymphocytes using FSC and SSC, then gated on CD4 positive, live dye negative cells.

T cell culture and polarization. Whole or naïve CBir1 Tg $CD4^+$ T cells ($1x10^5$) were isolated as described above and cultured in 24-well plates with irradiated APCs ($1x10^5$) and 1 μg CBir1 peptide in RPMI with 10% FBS for 5 days. WT APCs were used for all tissue culture experiments. Cells were cultured with 2 ng/mL TGF-β to induce a Treg phenotype. A Th17 phenotype was induced by the addition of 10 ng/mL TGF-β, 30 ng/mL IL-6, anti-IFNγ and anti-IL-4.

Protein extraction. 2 million T cells and 2 million APCs were cultured with no treatment, TGF-β, or TGF-β and CCL25 and harvested at 30 minutes, 60 minutes, and 4 hours. Untreated, uncultured cells were used as controls. Protein was isolated by treating with RIPA buffer and protease inhibitors then sonicating twice to lyse cells, after which the supernatant was collected. Protein yields were measured using a BCA protein assay.

Western blot. Protein lysates were suspended in water to standardize concentrate. Loading and reducing buffer were added and proteins were run on a NuPage gradient gel for 1.5 hours at 200 volts. Protein was transferred to a PVDF membrane at 100 volts for 3 hours. Membrane was washed 3 times and blocked by shaking gently in milk for one hour at room temperature. Membrane was washed once and rocked in primary antibody overnight at 4 C, then for an hour at room temperature. Membrane was washed three times and HRP-conjugated secondary antibody was added and shaken for an hour at

room temperature. Chemiluminescent HRP substrate from life technologies was added to detect protein. Membranes were developed for 2 minutes for β-actin and 30 minutes for target protein.

Protein quantification. Protein quantification was performed using ImageJ software. Pixel density was determined by measuring peak size in ImageJ, and percent area was calculated by dividing the size of each peak by the total measurement for all peaks and multiplying by 100. The pixel density was then standardized by dividing the value of each peak by the value of the control peak, setting the value of the control to 1. All samples were then normalized to β-actin by dividing the value of the target protein by the value of β-actin for the corresponding lane.

Statistical analysis. Samples were analyzed in Prism (GraphPad) via Student's T test. The results were considered significant at a P value of less than 0.05.

RESULTS

CCR9 knockout mice have more Tregs

To determine whether CCR9 deficiency affects Treg cell frequency, WT mice and $CCR9^{-/-}$ mice were sacrificed and lymphocytes from the spleen, MLN, SB, and LB were harvested and stained for flow cytometry. We found an increase in $Foxp3^{+}$ Tregs in the spleen and MLN of $CCR9^{-/-}$ mice compared to that in WT mice. However, there was no significant difference in the number of intestinal Tregs in $CCR9^{-/-}$ mice compared to WT mice (**Fig 5A-B**). This is possibly due to impaired Treg migration to intestine in $CCR9^{-/-}$ mice, as there are more Tregs in the periphery. Interestingly, we observed no difference in FoxP3 expression by $CD4^{+}$ $CD8^{-}$ thymocytes in WT vs $CCR9^{-/-}$ mice (**Fig 5A-B**), suggesting that CCR9 differentially regulates development of iTreg and nTreg.

To further investigate this, we examined the expression of the nTreg markers Helios and Neuropilin 1 (Nrp1) on FoxP3^{+} cells from WT and CCR9$^{-/-}$ mice. For the purposes of statistical analysis, we designated Helios^{+} Nrp1^{+} Tregs as nTregs and Helios^{-} Nrp^{-} Tregs as iTregs. We found that nTregs were downregulated in the SB of CCR9$^{-/-}$ mice. In contrast, CCR9$^{-/-}$ mice had increased numbers of iTregs in the SB. We found no statistically significant difference between the nTreg or iTreg populations in WT mice compared to CCR9$^{-/-}$ mice in the spleen, thymus, MLN, or LB. These data suggest that CCR9 signaling downregulates Treg populations by decreasing iTreg generation in the periphery. That significant differences in nTreg and iTreg populations occur in the SB, where the CCR9 ligand CCL25 is expressed, is compelling evidence that CCR9 signaling impairs generation of iTregs.

CCR9 knockout does not affect frequency of effector CD4^{+} subsets

To determine whether CCR9 deficiency affects effector T cell frequency, WT mice and CCR9$^{-/-}$ mice were sacrificed and lymphocytes from the spleen, MLN, SB, and LB were harvested and stained for flow cytometry. IFN and IL-17 were used as surrogate markers for Th1 and Th17 CD4^{+} T cells, respectively. There was no significant difference in CD4^{+} T cell production of IFN-γ and IL-17 in WT mice compared to CCR9$^{-/-}$ mice (**Fig 6**). These data indicate that CCR9 signaling does not influence the generation or function of Th1 and Th17 Teffs.

CCL25 treatment inhibits Treg generation *in vitro*

After determining that Tregs were increased in CCR9$^{-/-}$ mice, we sought to determine if CCR9 signaling could indeed inhibit Tregs. We cultured WT and CCR9$^{-/-}$ mice under Treg conditions and examined FoxP3 expression. We found that cultured CCR9$^{-/-}$ CD4^{+} T cells expressed significantly more FoxP3 than WT CD4^{+} T cells **Fig 7A-B)**. As these T cell cultures were not treated with CCL25, increased expression of FoxP3

by CCR9$^{-/-}$ CD4^{+} T cells could be due to a number of factors. These factors include the increased number of Tregs in the seeded CD4^{+} T cell population from CCR9$^{-/-}$ mice, as well as functional reprogramming due to CCR9 signaling *in vivo* in the WT mice.

To further determine the effect of CCR9 on Treg differentiation or expanision, we investigated whether binding of CCR9 to its ligand CCL25 inhibits Treg cells. We cultured total CBir1 Tg CD4^{+} T cells under and Treg conditions in the presence or absence of CCL25. About 3% of CD4^{+} T cells were Foxp3^{+} prior to culture (**data not shown**). We observed that addition of CCL25 significantly inhibited FoxP3^{+} Treg development or expansion in WT CD4^{+} T cells under Treg conditions when compared to cultures that were not treated with CCL25. Downregulation of FoxP3^{+} cells with CCL25 treatment was not observed in CCR9$^{-/-}$ CD4^{+} T cells (**Fig 7C-D**). As WT APCs were co-cultured with both WT and CCR9$^{-/-}$ CD4^{+} T cells, this effect is not due to modulation of APC function by CCL25. These data indicate that inhibition of Treg differentiation or development is directly dependent on CCR9-CCL25 interaction. We also examined the effect of CCL25 on IL-10 production. CCL25 treatment did not affect IL-10 production by CD4^{+} T cells.

CCL25 treatment does not affect generation of effector CD4^{+} subsets

In addition to its effects on Tregs, we also examined the effect of CCR9 signaling via CCL25 ligation on effector CD4^{+} T cell subsets. To do this, we cultured isolated CBir1 tg CD4^{+} splenocytes with or without CCL25 in neutral or Th17 conditions. Under neutral culture conditions, treatment with CCL25 did not affect production of IL-2. Th1 generation, as measured via IFNγ, was also unaffected (**Fig 8A-B**). Likewise, CCL25 treatment had no effect on IL-17 production under Th17 polarizing conditions (**Fig 8C-D**). These data indicate that that CCR9 signaling affects the generation and/or expansion of Tregs, but not other CD4^{+} T cell subsets.

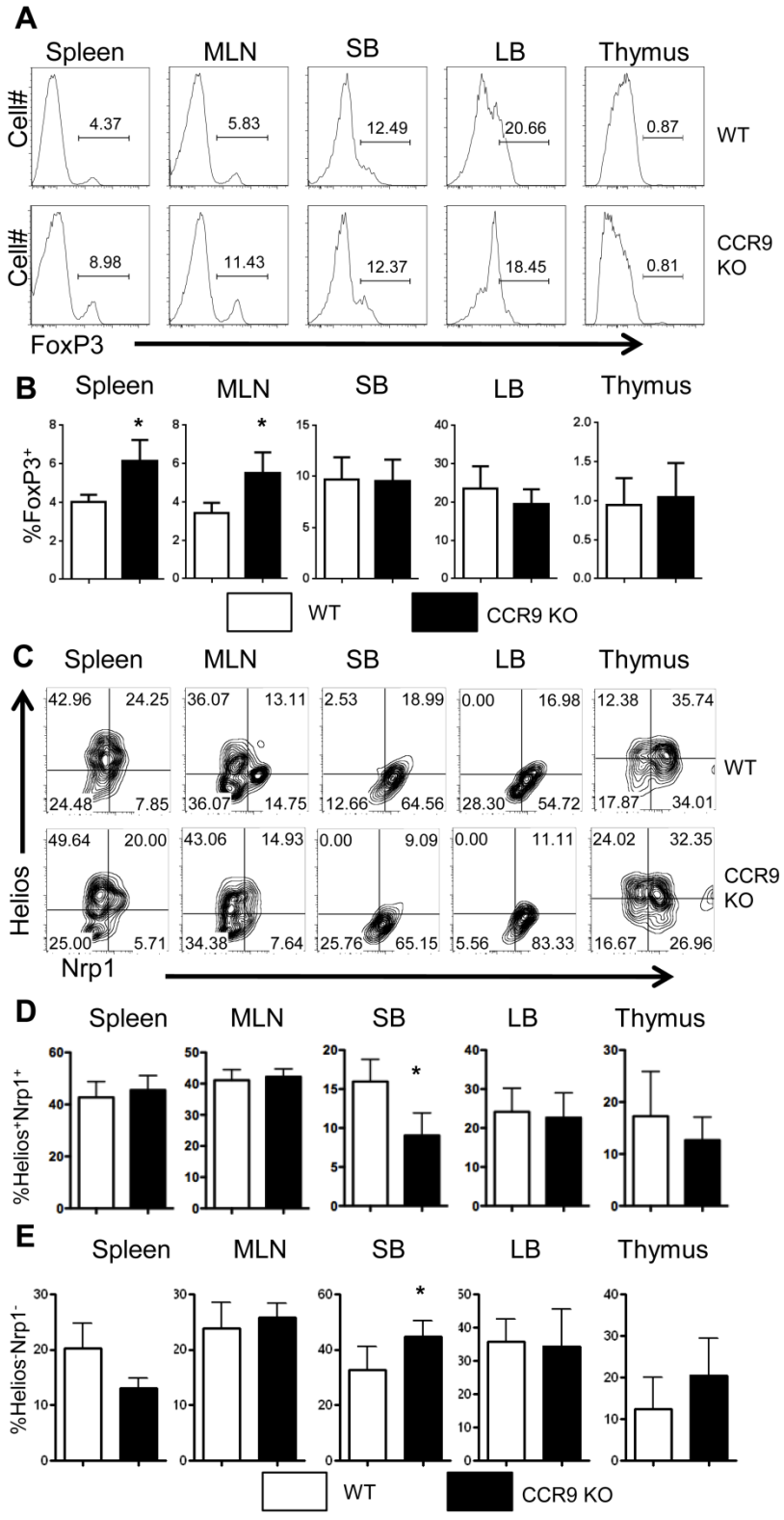
mTOR signaling influences CCR9-dependent regulation of Tregs

To determine the mechanism by which CCR9 regulates T cell function, we cultured $CD4^+$ mouse splenocytes with TGF-β, TGF-β and RA, or with no treatment. Cells were harvested at 0 minutes, 30 minutes, 60 minutes, and 4 hours. The proteins were extracted and Western blots were performed. Relative density of protein bands was calculated in ImageJ and normalized to β-actin. We found that mTOR was differentially phosphorylated in CCL25-treated samples. In untreated samples and TGF-β-treated samples, p-mTOR was increased at 4 hours compared with 0 hours, though p-mTOR was also upregulated at 60 minutes in the untreated samples. However, in samples treated with TGF-β and RA, p-mTOR was increased at 30 minutes and 60 minutes, but not at 4 hours **(Fig 9A-D)**. These data indicate that decreased mTOR phosphorylation is involved in the inhibition of Tregs by CCR9, though the other signaling pathways involved remain unclear.

DISCUSSION

CCR9 deficiency appeared to promote $Foxp3^+$ Treg cell development *in vivo*, with $CCR9^{-/-}$ mice demonstrating enriched $Foxp3^+$ Treg populations compared to WT mice. Furthermore, ligation of CCR9 with CCL25 *in vitro* resulted in fewer $Foxp3^+$ Tregs in $CD4^+$ T cell cultures treated with TGF-β, indicating that CCR9 signaling inhibits $Foxp3^+$ Treg cell development. The development of effector T cells appears to be unaffected by CCR9 signaling. Additionally, we determined that mTOR is differentially phosphorylated in CCL25-treated samples compared to samples that were not treated with CCL25. Therefore, our study strongly supports the notion that in addition to its well-established function of mediating T cell migration to certain specific tissues, signaling via chemokine receptors can also directly regulate T cell function.

We still do not understand the mechanisms mediating CCR9-CCL25 inhibition of Treg cells, though this effect appears to mTOR-dependent. The similarity in thymic expression of FoxP3 in WT and CCR9$^{-/-}$ mice, along with the decrease in nTreg markers in the SB of CCR9$^{-/-}$ mice, suggests that CCR9 signaling inhibits iTreg generation. However, Helios and Nrp1 are controversial markers for nTregs, and no definitive markers for nTregs and iTregs have been found[25]. As naïve CD4 T cells express very low levels CCR9, it is also possible that CCR9 signaling may inhibit Treg cell survival and/or proliferation. The possibility that CCR9 may inhibit Treg suppressive function, in addition to affecting Treg numbers, also warrants investigation.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Spleen
MLN
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Thymus
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WT
Cell#
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CCR9 KO
FoxP3
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42.96 24.25
24.48 7.85
36.07 13.11
36.07 14.75
2.53 18.99
12.66 64.56
0.00 16.98
28.30 54.72
12.38 35.74
17.87 34.01
WT
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25.00 5.71
43.06 14.93
34.38 7.64
0.00 9.09
25.76 65.15
0.00 11.11
5.56 83.33
24.02 32.35
16.67 26.96
CCR9 KO
Helios
Nrp1
D
Spleen
MLN
SB
LB
Thymus
%Helios+Nrp1+
*
E
Spleen
MLN
SB
LB
Thymus
%Helios-Nrp1-
*
WT
CCR9 KO

Figure 5. CCR9 deficiency results in enhanced Treg populations

Spleen, MLN, SB LP and LB LP $CD4^+$ T cells were harvested from WT and $CCR9^{-/-}$ mice and stained for flow cytometry as described. *(A-B)* Cells were gated on live $CD4^+$ lymphocytes and the percent of $FoxP3^+$ Tregs out of total $CD4^+$ cells was analyzed. Representative flow cytometry profiles and average percent FoxP3 expression data from 10 mice per group are shown. *(C-E)* FoxP3+ Tregs were analyzed for expression of the nTreg markers Helios and Nrp1. For purposes of analysis, $Helios^+Nrp1^+$ Tregs were considered nTregs and $Helios^-Nrp^-$ Tregs were considered iTregs. Representative flow cytometry plots and bar charts showing percent Helios and Nrp1 double positive and double negative Tregs are shown. Data are representative of 6 mice per group. *P<0.05 compared with WT.

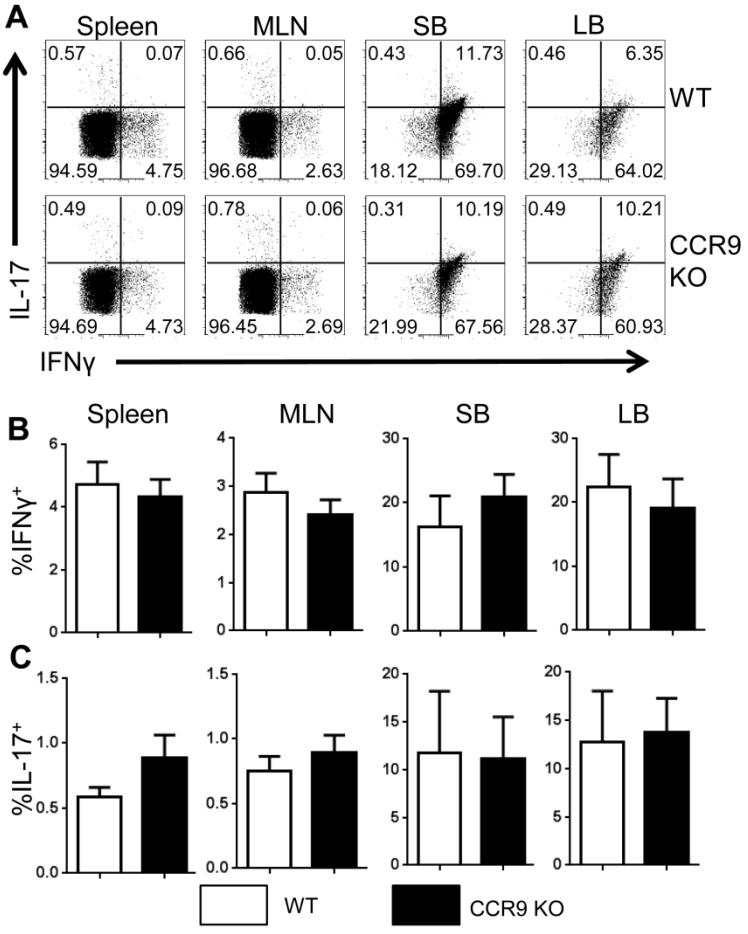

Figure 6. Deficiency in CCR9 does not affect Th1 or Th17 cell populations *in vivo*

Spleen, MLN, SB LP and LB LP $CD4^+$ T cells were harvested from naïve WT and $CCR9^{-/-}$ mice and stained for flow cytometry as described. *(A)* Cells were gated on live $CD4^+$ lymphocytes, and the percentage of $CD4^+$ cells expressing IFNγ and IL-17 was analyzed. Representative FACS plots are shown. *(B-C)* Mean percent expression data for IFNγ and IL-17 were also analyzed. Data from 3 experiments totaling 9 mice are shown.

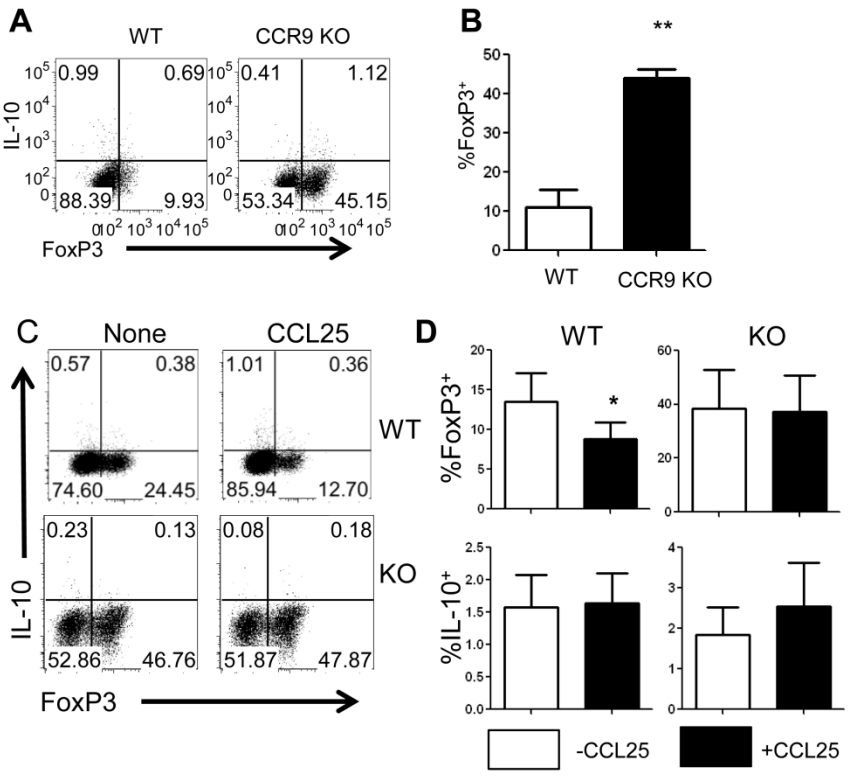

Figure 7. Ligation of CCR9 by CCL25 inhibits Treg development.

Purified WT and CCR9$^{-/-}$ CD4^{+} T cells were cultured under Treg conditions. Cells were stained for flow cytometry as described and gated on live CD4^{+} lymphocytes, which were then analyzed for the percentage of expression of FoxP3 and IL-10. *(A-B)* Percent expression of FoxP3 in WT and CCR9$^{-/-}$ was analyzed. Representative flow cytometry plots and bar charts representing mean percent expression of FoxP3 from 3 experiments are shown. **P<0.01 compared with WT. *(C-D)* Isolated CD4^{+} T cells cultured with or without CCL25 (20 ng/mL). Percent expression of FoxP3 and IL-10 was compared between cultures treated with or without CCL25. Representative FACS plots and mean percentage of expression calculated from 6 independent experiments are shown. Data are representative of 4 independent experiments are shown. *P<0.05 compared with cells cultured in the absence of CCL25.

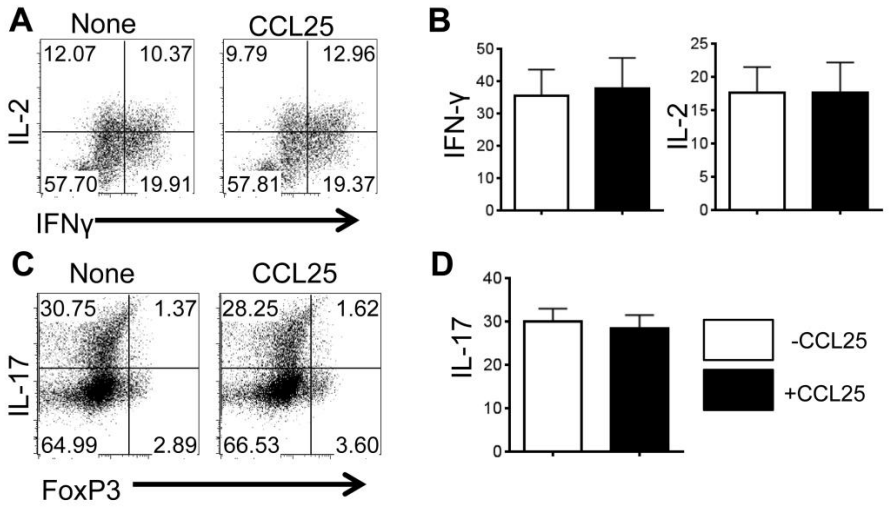

Figure 8. Ligation of CCR9 by CCL25 does not alter effector T cell development.

Purified WT and CCR9-/- CD4+ T cells were cultured under various T helper polarizing conditions with or without CCL25 (20 ng/mL). Cells were stained for flow cytometry as described and gated on live CD4+ lymphocytes. *(A-B)* Cells cultured under neutral conditions were analyzed for IFN γ and IL-2, with representative flow cytometry plots and average percent expression from 6 independent experiments shown. *(C-D)* Cells cultured under Th17 polarizing conditions were analyzed for IL-17, with representative flow cytometry plots and mean percent expression from 4 independent experiments shown.

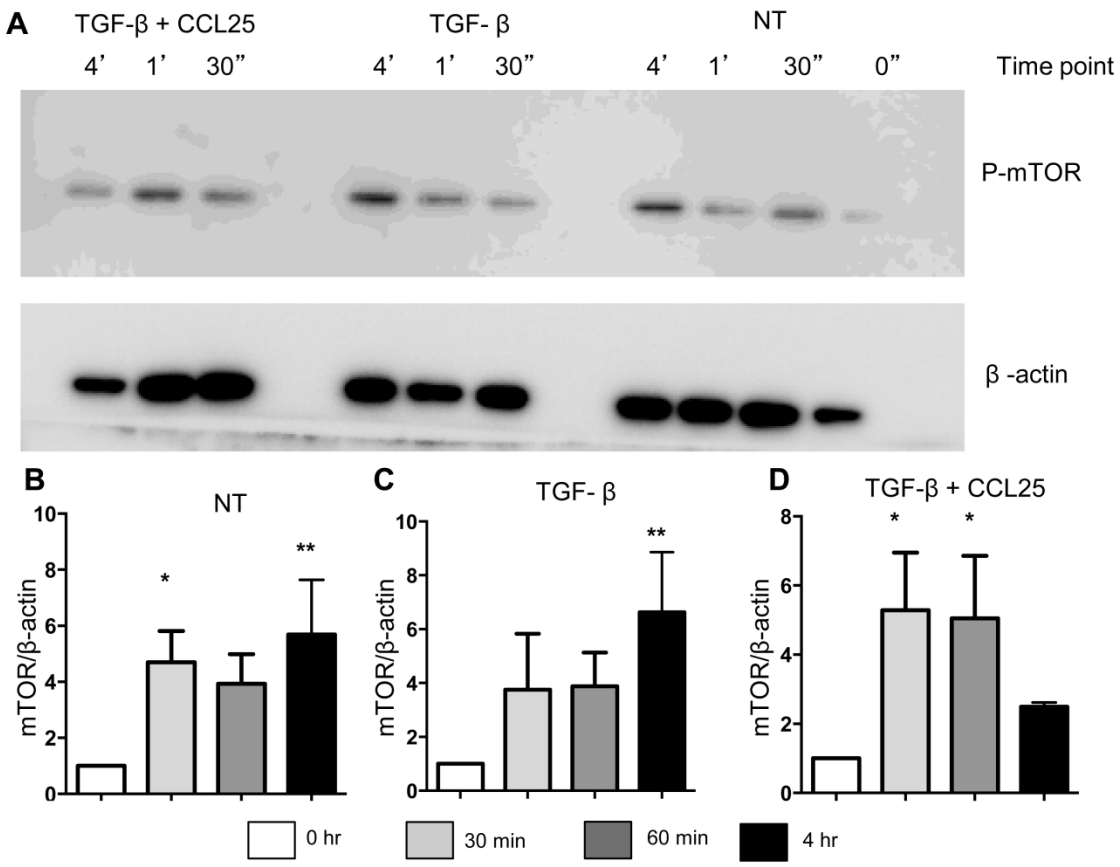

Figure 9. Regulation of $CD4^+$ cells by CCR9 signaling involves mTOR.

Isolated $CD4^+$ T cells were co-cultured with APCs and treated with TGF-β, TGF-β and CCL25, or given no treatment. Cells were collected and lysed and 20 minutes, 60 minutes, and 4 hours, with untreated cells at 0 hours serving as controls. *(A)* Protein was lysed and quantified and phosphorylated mTOR protein was detected via Western blot. β-actin was used as a loading control. Image is representative of 3 independent gels. *(B-D)* Pixel density was determined and relative expression of p-mTOR compared to β-actin was calculated. Mean relative expression from 3 independent gels is shown here. *P<0.05; **P<0.01 compared to 0 hour control.

Chapter 7: The role of CCR9 on effector and regulatory CD4$^+$ T cells during colitis

Originally published in *PLoS ONE*. Heather L Evans-Marin, Anthony T Cao, Suxia Yao, Feidi Chen, Chong He, Han Liu, Wei Wu, Maria G Gonzalez, Sara M Dann and Yingzi Cong. 2015, 10;(7):e0134100.Unexpected regulatory role of CCR9 in regulatory T cell development. *PLoS ONE*. Republished in modified form.

INTRODUCTION

Chemokine (C-C motif) receptor 9 (CCR9) is a gut-trophic chemokine receptor expressed by lymphocytes and dendritic cells[49,50,64]. CCR9 binds non-promiscuously to its ligand Chemokine (C-C motif) ligand 25 (CCL25), which is expressed in the small intestine and thymus[49,50,81,82]. During intestinal inflammation, both intestinal expression of CCL25 and recruitment of CCR9$^+$ T cells are increased in cases of experimental colitis and in patients with Crohn's disease[36,51,73]. Notably, CCR9 is upregulated on inflammatory leukocytes in human IBD patients[51,70–72]. This finding may indicate that CCL25-CCR9 interaction mediates the recruitment of T cells to inflamed sites, which could contribute to the progression of colitis.

However, the role of CCR9 in inducing and sustaining inflammation in the context of IBD is poorly understood. Conflicting data implicate CCR9 as being critical to both development of pathological inflammation and protection by Tregs [9,50,70,73,81,82,75]. CCR9 deficiency or inhibition produces different results in experimental models depending on the method of CCR9 neutralization and manner in which intestinal inflammation is induced[50,73–75,81]. Furthermore, the efficacy of CCR9 blockade is

dependent on the disease phase, with anti-CCR9 antibodies attenuating early, but not late, disease[9,73]. Here, we investigate the role of CCR9 expression on both Tregs and Teffs in experimental colitis using adoptive transfer and chemical injury models.

MATERIALS AND METHODS

Mice CBir1 flagellin specific TCR transgenic mice (CBir1 Tg) were maintained in the animal facilities of the University of Texas Medical Branch. $CCR9^{-/-}$ mice were kindly provided by Dr. Joshua Farber of NIAID, NIH, and crossed with CBir1 Tg mice. Tail snips were collected 3 weeks of age to confirm genotype. Littermates were selected and cohoused until they were 6 to 8 weeks old, when experiments were performed. $TCR\beta x\delta^{-/-}$ mice were purchased from Jackson Laboratories. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Antibodies and reagents. All flow cytometry antibodies and cytokines were obtained from BioLegend. CD4 magnetic beads were purchased from BD Biosciences. The CD25 MACS kit was manufactured by Miltenyi Biotec. TaqMan CCL25 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were obtained from Applied Biosystems. The qScript cDNA SuperMix was obtained from Quanta. SsoAdvanced Universal SYBR Green Supermix was purchased from BioRad.

Isolation of $CD4^+$ T cells. $CD4^+$ T cells were isolated by using mouse anti-CD4 magnetic beads as previously described [90]. The mice were euthanized by CO_2 inhalation followed by cervical dislocation. Mouse splenocytes were incubated with CD4 magnetic beads for 30 minutes at 4 C. Afterward, cells were placed on a magnetic column three

times, for durations of 8, 4, and 4 minutes. After each incubation, the supernatant was aspirated, collected, and replaced by fresh buffer.

Isolation of Tregs. $CD25^+$ T cells were tagged using anti-CD25 MACS kit. Cells were incubated with anti-CD25 PE conjugated antibody for 15 minutes, washed via centrifugation, and then incubated with anti-PE magnetic beads for 5 minutes. Cells were isolated MACS sorter as described by the manufacturer's protocol.

Necropsy and LPL isolation. The spleen, MLN, and small and large intestines were extracted and fat and connective tissue carefully removed. The large and small intestines were cleaned via scraping and cut into pieces, then were washed by shaking in PBS and straining through a sieve several times. Tissue was then incubated with magnetic stirrers in PBS with 1% FBS and 1:1000 EDTA at 37 C for 40 minutes to remove the epithelium. Remaining tissue was collected, washed in PBS several times, then dried and chopped finely. The tissue was then incubated with collagenase twice for 30 minutes at 37 C. Supernatant was collected and and fresh collagenase was added after the first incubation. After the second incubation, all supernatant was strained through a filter washed via centrifugation. The pellet was then layered into a 75%/40% percoll gradient, centrifuged, and the interface layer was collected.

Preparation of spleen and MLN leukocytes. Single-cell suspensions were prepared from the spleen, MLN, and thymus by removing fat and connective tissue and smashing between glass slides. Leuckocyte supsensions were then washed via centrifugation and resuspended. Additionally, splenic erythrocytes were then lysed with Tris-ammonia and the remaining leukocytes washed twice via centrifugation.

Adoptive transfer of $CD4^+$ and $CD25^+$ T cells and induction of colitis. CBir1 $CD4^+$ cells cells were transferred to TCRβxδ$^{-/-}$ mice via tail vein injection. In Treg transfer experiments, Tregs were isolated using a CD25 MACS kit as described above. All mice received equal numbers of effector cells, and Tregs were transferred in a 1:1 ratio with T effectors. Mice were weighed weekly and examined for signs of disease. Mice were sacrificed and necropsy performed at 6 weeks post-transfer, when symptoms of disease were evident.

DSS and αCD25 treatment. Mouse drinking water was supplemented with 2% DSS for 7 days to induce colitis, after which they were returned to regular water for three days. Mice also received intraperitoneal injections of either either αCD25 or PBS (vehicle) twice per week. Mice were weighed daily and examined for signs of disease. Mice were sacrificed and necropsy performed at 10 days post-transfer.

Flow cytometry. Intracellular and surface staining were performed as previously described [90]. For experiments where intracellular cytokines were measured, cells were restimulated for 5 hours with PMA and ionomycin, with Golgi Stop being added for the last 3 hours of restimulation. Cells were washed via centrifugation and surfaced stain added for 20 minutes at room temperature. Cells were then washed permeablized for 30 minutes at 4 C using a FoxP3 perm/fix kit manufactured by eBiosciences. After permeablization cells were washed, intracellular stain was added, and cells were incubated for 30 minutes at 4 C before being washed a final time. Data was collected using the LSRII/Fortessa and compensation performed using FACSDiva software. Further analysis was carried out using FlowJo. All flow cytometry plots shown are first

gated on lymphocytes using FSC and SSC, then gated on CD4 positive, live dye negative cells.

Histopathologic assessment. During necropsy, sections of the large bowel and cecum were removed and swiss rolls prepared. Tissue sections were fixed in 10% PFA overnight and embedded in paraffin. Sections were sliced to 5 μm, stained with H&E, and analyzed by a trained pathologist. The following histological features were analyzed: crypt epithelial hyperplasia, degeneration, and loss; mucosal ulceration; submucosal edema; LP and submucosal cellular infiltrate; transmural inflammation; crypt exudate; and goblet cell loss. Each component was scored separately for severity (0-absent, 1-mild, 2-moderate, 3-severe) and extent of tissue affected (0-absent, 1-25%, 2-50%, 3-75%, 4-100%), with the total score being the sums of the severity multiplied by the extent for each feature.

Quantitative real-time PCR. RNA was quantified using two-step polymerase chain reaction (PCR). RNA was isolated from the small and large bowel using TRIzol reagent. cDNA was synthesized using qScript cDNA SuperMix and BioRad C1000 thermal cycler. Pre-designed TaqMan primers were used for cDNA synthesis. Quantitative real-time PCR was performed with the SsoAdvanced Universal SYBR Green Supermix using a BioRad CFX96 real-time PCR detection system. DNA amplification was detected with a SYBR green probe. Expression data was normalized to GAPDH mRNA levels and a designated control sample was arbitrarily given a value of 1.0.

Statistical analysis. Samples were analyzed in Prism (GraphPad) via Student's T test or one- or two- way ANOVA utilizing a Newman-Keuls post-test. Paired tests were

used where appropriate. The results were considered significant at a P value of less than 0.05.

RESULTS

Effector cell CCR9 deficiency does not impair colitis development in a microbiota-specific adoptive transfer model

Considering that blockade of CCR9 attenuates early intestinal inflammation [9,73], we first sought to examine the role of CCR9 in the pathogenesis of T cell-mediated colitis by using the CBir1-specific adoptive transfer model. We have previously shown that adoptive transfer of CBir Tg $CD4^+$ T cells induces colitis in $RAG^{-/-}$ or $TCR\beta\delta^{-/-}$ mice via *in vivo* generation of Th1 and Th17 Teff cells [21,90]. Colitis was induced by adoptive transfer of $CD4^+$ T lymphocytes isolated from either WT CBir1Tg or $CCR9^{-/-}$ CBir1 Tg mice into T cell-deficient $TCR\beta\delta^{-/-}$ mice. Mice were examined weekly and sacrificed once signs of disease became evident, which usually occurs at 6 weeks post T cell transfer. Histology samples were taken from the colon and cecum. Cytokine production by lymphocytes from the spleen, MLN, and LP was measured via flow cytometry. Consistent with a previous report [74], there were no significant differences in pathology, IL-17 production, IFN-γ production, or FoxP3 expression in the spleen, MLN, or LP between WT and $CCR9^{-/-}$ $CD4^+$ T cell recipients (**Fig 1A-C**).

We then utilized quantitative real-time PCR to examine the expression of CCL25, the receptor for CCR9, in CBir1 $CD4^+$ T cell recipient $TCR\beta\delta^{-/-}$ mice compared with control $TCR\beta\delta^{-/-}$ mice. We found robust expression of CCL25 in the SB, with only minimal CCL25 expression in the LB. In addition, CCL25 was upregulated in the small bowel of colitic mice compared to control mice, but not in the large bowel (**Fig 1D**).

These data are in agreement with previous reports which found that CCL25 is primarily expressed in the small bowel and is upregulated under inflammatory conditions [36,47]. Hence, the similarity between the CCR9$^{-/-}$ and WT CD4^{+} recipient groups cannot be explained by downregulation of CCL25 in our model. Collectively, these data indicate that CCR9 deficiency does not limit the capacity of Teff cells to induce disease in a T-cell mediated model of IBD.

Treg CCR9 deficiency does not impair suppressive function in a microbiota-specific adoptive transfer model of colitis

We then sought to examine the effect of CCR9 deficiency in Tregs on their ability to suppress inflammation. Colitis was induced via adoptive transfer of CD4^{+} Teff cells isolated from CBir1 Tg mice into TCRβδ$^{-/-}$ mice as described above. The recipient mice also received an equivalent number of CD25^{+} Tregs from WT or CCR9$^{-/-}$ CBir1 Tg mice. Mice that received Teff cells but no Tregs served as positive controls. Mice were examined weekly and sacrificed once signs of disease became evident, generally at 6 weeks post transfer. We observed that mice which received WT or CCR9$^{-/-}$ Tregs had lower pathology scores than did mice that received CBir1 Teff cells alone. However, mice which received CCR9$^{-/-}$ Tregs had similar pathology scores to mice that received WT Tregs (**Fig 2A and B**). This finding indicates that CCR9$^{-/-}$ Tregs had a similar capacity to inhibit CBir1 T cell-induced colitis as that of WT Tregs. T cell production of IFN-γ and IL-17 was decreased in mice which received WT Treg or CCR9$^{-/-}$ Tregs compared to that in mice that received CBir1 Teffs alone (**Fig 2C and D**). These data indicate that loss of CCR9 in Treg cells does not impair their ability to control intestinal inflammation.

CCR9 knockout mice exhibit Treg-mediated protection against DSS colitis

To determine if increased Tregs moderate intestinal inflammation in $CCR9^{-/-}$ mice, we treated WT or $CCR9^{-/-}$ CBir1 Tg mice with 2% DSS in drinking water for 7 days followed by a 3 day recovery period. Mice were sacrificed 10 days after starting DSS treatment and the spleen, MLN, SB, and LB were harvested and prepared for flow cytometry. Mice were weighed daily to monitor colitis severity and the percent change in body mass was calculated. $CCR9^{-/-}$ CBir1 Tg mice were divided into two groups, one of which received either anti-CD25 monocolonal antibody (mAb) to deplete Tregs and one of which received the control treatment. We selected a dose of anti-CD25 mAb which partially depleted $CD25^{+}$ Tregs in $CCR9^{-/-}$ CBir1 Tg mice. When FoxP3 expression was measured via flow cytometry, Treg populations in $CCR9^{-/-}$ mice treated with anti-CD25 mAb decreased to a level similar to that of WT mice (**Fig 10A**). This partial depletion of Tregs allowed examination of the effect of enhanced Treg populations in $CCR9^{-/-}$ mice on colitigenesis. We found that WT mice lost weight more rapidly and developed more severe colitis than $CCR9^{-/-}$ mice upon DSS insult (**Fig 10B and C**). Partial depletion of Tregs in $CCR9^{-/-}$ mice via anti-CD25 resulted in more severe weight loss comparable to WT mice, and anti-CD25 treated mice failed to recover after DSS treatment was removed. In addition, $CCR9^{-/-}$ mice treated with anti-CD25 antibody upregulated IL-17A and IFNγ expression by $CD4^{+}$ T cells in the lamina propria compared to control $CCR9^{-/-}$ mice, producing a phenotype similar to WT mice (**Fig 10D**). Collectively, these data indicate that increased Tregs in $CCR9^{-/-}$ mice, rather than impaired homing function, contribute to their resistance to colitis upon DSS insults.

DISCUSSION

Our data indicate that enhanced antigen-specific Treg populations in $CCR9^{-/-}$ mice, rather than altered migratory function, confer protection to acute colitis. It has been previously demonstrated in a $CD45RB^{high}$ adoptive transfer model of colitis that

CCR9 expression on T effector cells or on Treg cells is not required for induction or regulation of colitis[74]. Consistently, when we transferred microbiota antigen-specific T cells from CBir1 Tg mice into $Rag^{-/-}$ mice, CCR9-deficient T cells were able to induce equally severe of colitis when compared to that induced by WT $CD4^{+}$ T cells. Furthermore, CCR9-deficient Treg cells demonstrated suppressive function similar to that of WT Treg cells in our model, confirming the notion that CCR9 expression is not required for Teff or Treg cell migration and function in the LB.

Previous reports examining CCR9 deficiency in a DSS model have indicated that $CCR9^{-/-}$ are more susceptible to colitis induction[74,75]. We suspect that this difference is due to our use of $CCR9^{-/-}$ mice with a CBir1 tg background, as opposed to WT $CCR9^{-/-}$ mice. Thus, it is likely protection in our mice was conferred by the presence a pre-existing pool of iTregs specific for commensal antigen that would be absent in mice with more diverse TCRs.

Current clinical trials using the CCR9 antagonist CCX282-B, which inhibits CCR9- and CCL25-dependent chemotaxis, to treat IBD have reported only minimal efficacy[36,51,73]. Notably, patients treated with CCX282-B do not demonstrate statistically significant improvement compared to the placebo group until 12 weeks after treatment begins[82]. Our data suggest that this is because inhibition of CCR9 does not impair migration to the intestines, but rather alters immune function to enhance tolerance. Hence, development of reagents inhibiting CCR9 signaling to promote Treg development, in addition to blocking effector T cell migration to inflamed sites of the intestines, warrants consideration.

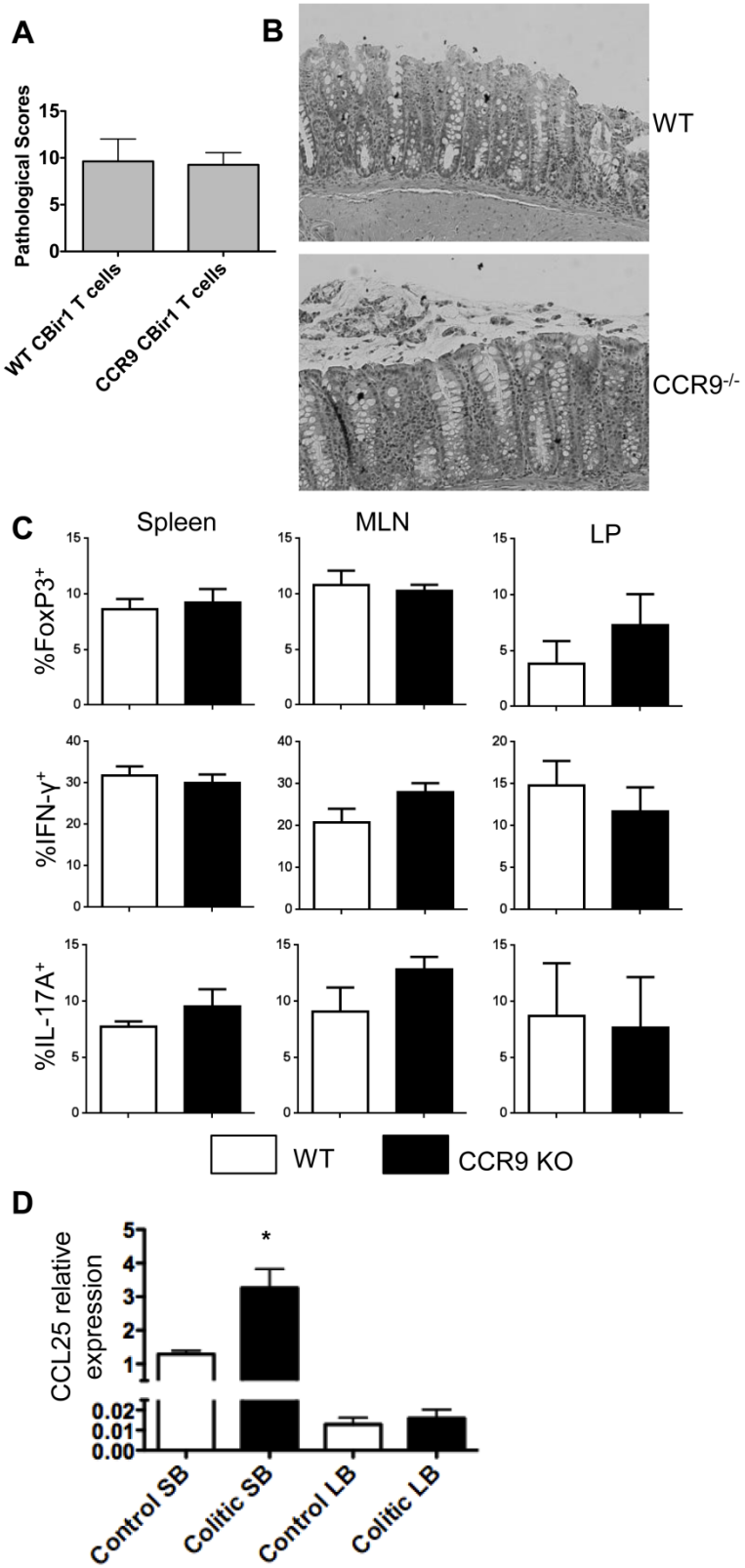
A
Pathological Scores
15
10
5
0
WT CBir1 T cells
CCR9 CBir1 T cells
B
WT
CCR9-/-
C
Spleen
MLN
LP
%FoxP3+
%IFN-γ+
%IL-17A+
WT
CCR9 KO
D
CCL25 relative expression
*
0.02
0.01
0.00
Control SB
Colitic SB
Control LB
Colitic LB

Figure 10. CCR9 deficiency in effector T cells does not affect colitis development.

Isolated $CD4^+$ T cells ($1x10^6$) from WT or $CCR9^{-/-}$ CBir1 TCR transgenic mice were adoptively transferred to $TCR\beta\delta^{-/-}$ recipient mice. Colitis development was observed after six weeks, at which point the mice were sacrificed and necropsy performed. *(A)* Pathology was scored as described *(B)* and representative H&E-stained histopathology images from one experiment with 4 mice are shown. *(C)* Isolated lymphocytes from the spleen, MLN, and large intestine (LB) LP were stained for flow cytometry. Percentages of $CD4^+$ T cells expressing IFN-γ, IL-17, and FoxP3 were determined by gating on live $CD4^+$ populations and comparing relative expression. Averaged data from 2 experiments totaling 8 mice per group are shown. *(D)* CCL25 expression levels in the SB and LB of untreated $TCR\beta\delta^{-/-}$ mice were compared with those of CBir1 T cell recipient $TCR\beta\delta^{-/-}$ mice via quantitative real-time PCR. CCL25 expression levels are normalized to the reference gene GAPDH. The relative expression of CCL25 in the small intestines (SB) in control mice was arbitrarily set to 1.0. CCL25 expression was compared between the SB of colitic mice and the control SB. Data are representative of four mice per group. *$P<0.01$ compared with the control SB.

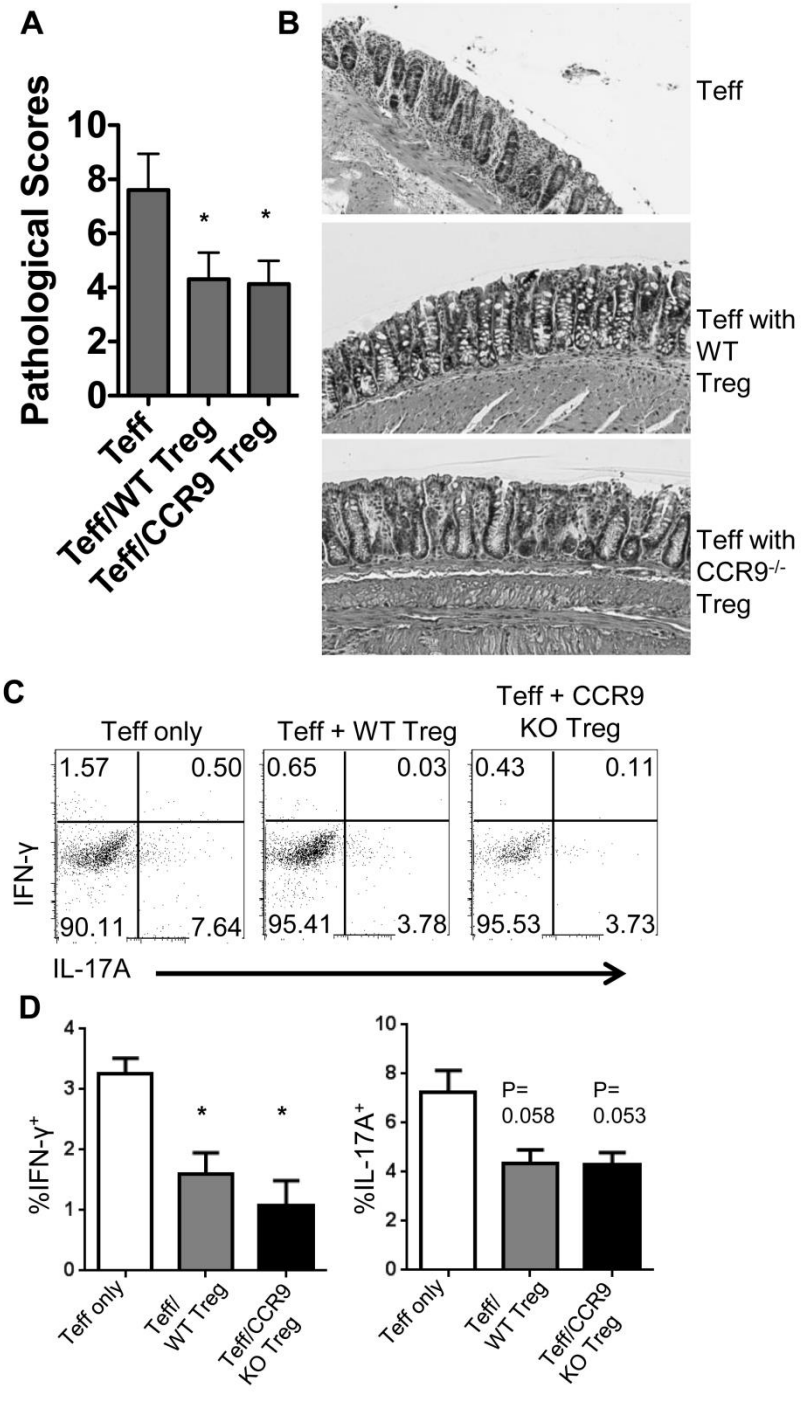
A
B
Pathological Scores
10
8
6
4
2
0
*
*
Teff
Teff/WT Treg
Teff/CCR9 Treg
Teff
Teff with WT Treg
Teff with CCR9-/- Treg
C
Teff only
Teff + WT Treg
Teff + CCR9 KO Treg
1.57
0.50
90.11
7.64
0.65
0.03
95.41
3.78
0.43
0.11
95.53
3.73
IFN-γ
IL-17A
D
%IFN-γ+
%IL-17A+
P= 0.058
P= 0.053
Teff only
Teff/ WT Treg
Teff/CCR9 KO Treg

Figure 11. CCR9 deficiency in Tregs does not affect their ability to inhbit colitis.

Purified WT CBir1 TCR transgenic $CD4^+$ Teff cells (1×10^6) were adoptively transferred to $TCR\beta\delta^{-/-}$ recipients to induce colitis. Two groups of mice also received 1×10^6 of purified $CD25^+$ $CD4^+$ Treg cells from WT or $CCR9^{-/-}$ CBir1 Tg mice in addition to the purified $CD4^+$ T cells. *(A)* Pathology was scored as described. *P<0.01 compared with mice receiving Teff alone. *(B)* Representative H&E-stained histopathology images from one representative experiment of 3-4 mice are shown. *(C-D)*Lymphocytes were isolated from the LB LP and stained for flow cytometry. Percentages of $CD4^+$ T cells in the LP expressing IFNγ and IL-17 were determined by gating on live $CD4^+$ populations and comparing relative expression. Representative FACS plotsand bar charts from one representative experiment of 3-4 mice per group are shown. Data are reflective of 2 independent experiments. *P<0.05 compared with the mice receiving Teff alone.

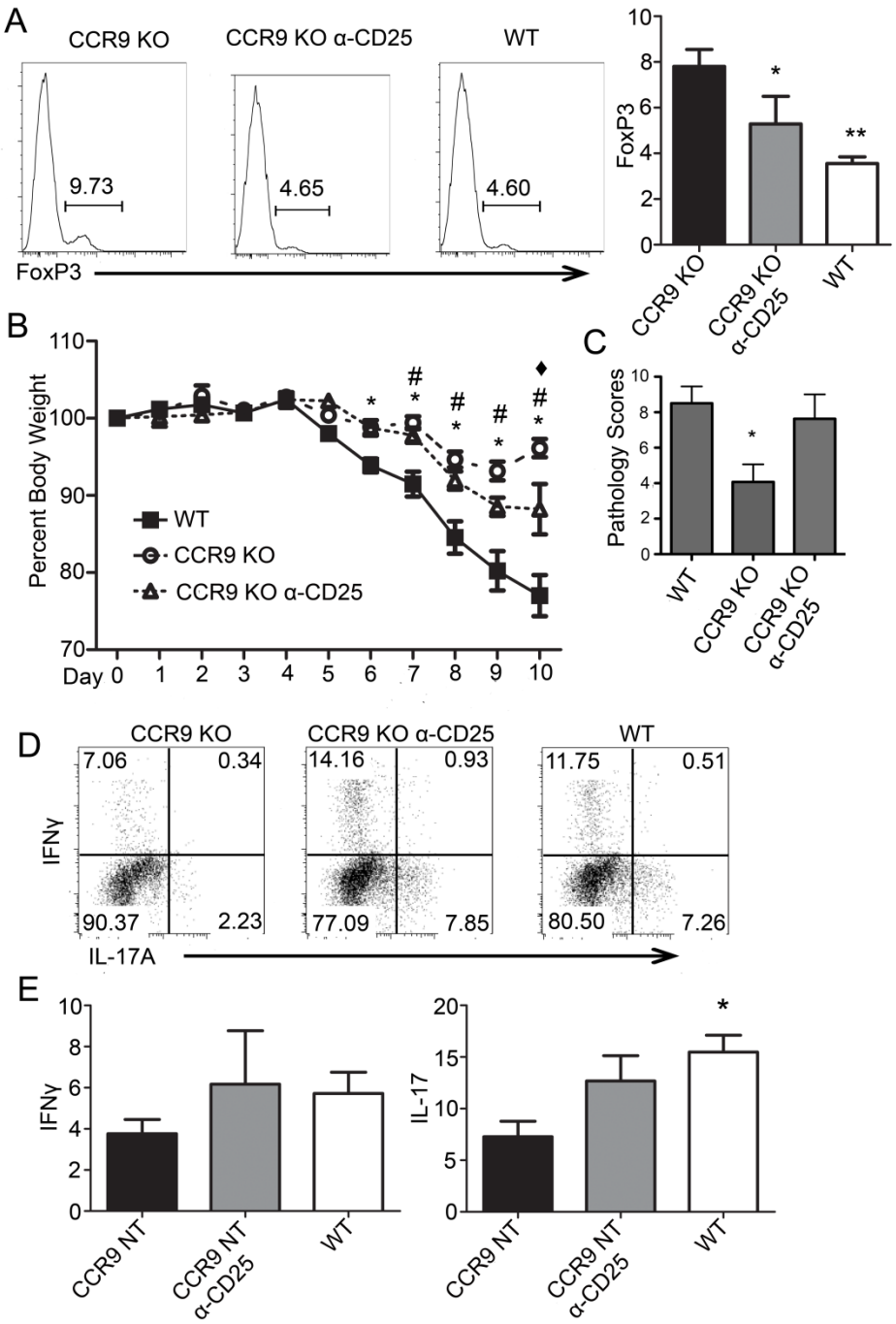
A
CCR9 KO
CCR9 KO α-CD25
WT
9.73
4.65
4.60
FoxP3
FoxP3
CCR9 KO
CCR9 KO α-CD25
WT
B
Percent Body Weight
Day 0 1 2 3 4 5 6 7 8 9 10
WT
CCR9 KO
CCR9 KO α-CD25
C
Pathology Scores
WT
CCR9 KO
CCR9 KO α-CD25
D
CCR9 KO
CCR9 KO α-CD25
WT
7.06
0.34
90.37
2.23
14.16
0.93
77.09
7.85
11.75
0.51
80.50
7.26
IFNγ
IL-17A
E
IFNγ
IL-17
CCR9 NT
CCR9 NT α-CD25
WT

Figure 12. Increased Treg populations in CCR9$^{-/-}$ mice confer resistence to DSS-induced colitis.

Colitis was induced in WT and CCR9$^{-/-}$ mice via administration of DSS in drinking water for 7 days followed by a 3-day recovery period. CCR9$^{-/-}$ mice were additionally treated with either anti-CD25 antibody or PBS control twice per week. *(A)* Splenic T cell expression of FoxP3 was analyzed via flowy cytometry to determine efficiency of Treg depletion by anti-CD25 antibody. *(B)*Mice were weighed daily and percent change in body weight for each mouse calculated. Significance between the WT and CCR9$^{-/-}$ NT (*), WT and CCR9$^{-/-}$ anti-CD25 (#), and CCR9$^{-/-}$ NT and CCR9$^{-/-}$ anti-CD25 (♦) was determined by matched two-way ANOVA. *(C)* Combined histopathology scores of 4-5 mice per group are shown. *(D-E)* Percent of lamina propria CD4^{+} T cells expressing IL-17 and IFNγ was measured via flow cytometry. Representative flow cytometry plots and mean percent expression are shown. Representative and combined data from 4-5 mice per group are shown. *P<0.05; **P<0.01 compared with WT

Chapter 8: Metabolic regulation of CD4$^+$ T cell differentiation

INTRODUCTION

Metabolic signals are intimately connected to CD4$^+$ T cell differentiation and function. A number of signaling intermediates regulate both metabolism and immunological function, including mTOR, AMPK, HIF-1, and PPARs[27,83,85,88]. Caloric intake, local oxygenation status, and the presence of specific nutrients shape T helper polarization[83,86–88,93]. Metabolic factors also influence the intensity of T cell responses. The availability of nutrients and functionality of nutrient transporters can lead to Teff hyperresponsiveness or anergy[83]. Thus, immunometabolism is currently an exploding field. The role that dietary composition plays in immune function is one question this field seeks to answer.

However, most research regarding the influence of dietary modification on immune function has been conducted in obese mice fed a high-fat diet[93]. There has been some investigation as to the roles of vitamins, specific fatty acids, and probiotics[93]. However, there is little information regarding the role of dietary carbohydrates in regulating the immune system. Additionally, much research investigating the role of dietary macronutrients on immune function focuses on visceral adipose tissue (VAT) immune cells[93]. While VAT is clearly important in metabolic regulation, the gut boasts a robust immune milieu and is the first point of contact for ingested macronutrients. Thus, we examine herein the influence of glucose supplementation on CD4$^+$ T cell polarization both *in vitro* and *in vivo*, focusing on modulation of the gut immune system.

MATERIALS AND METHODS

Mice. CBir1 were maintained in the animal facilities of the University of Texas Medical Branch. Tail snips were collected 3 weeks of age to confirm genotype. Littermates were selected and cohoused until they were 6 to 8 weeks old, when

experiments were performed. C57BL/6J mice were purchased from Jackson Laboratories. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Isolation of $CD4^+$ T cells. $CD4^+$ T cells were isolated using mouse anti-CD4 magnetic beads as previously described [90]. Splenocytes of CBir1 mice were incubated with CD4 magnetic beads for 30 minutes at 4 C. Afterward, cells were placed on a magnetic column three times, for durations of 8, 4, and 4 minutes. After each incubation, the supernatant was aspirated and collected, then replaced by fresh buffer. The $CD4^-$ fraction collected from the CD4+ isolation procedure was and irradiated for use as APCs.

Necropsy and LPL isolation. The spleen, MLN, and small and large intestines were extracted and fat and connective tissue carefully removed. The large and small intestines were cleaned via scraping and cut into pieces, then were washed by shaking in PBS and straining through a sieve several times. Tissue was then incubated with magnetic stirrers in PBS with 1% FBS and 1:1000 EDTA at 37 C for 40 minutes to remove the epithelium. Remaining tissue was collected, washed in PBS several times, then dried and chopped finely. The tissue was then incubated with collagenase twice for 30 minutes at 37 C. Supernatant was collected and and fresh collagenase was added after the first incubation. After the second incubation, all supernatant was strained through a filter washed via centrifugation. The pellet was then layered into a 75%/40% percoll gradient, centrifuged, and the interface layer was collected.

Preparation of spleen and MLN leukocytes. Single-cell suspensions were prepared from the spleen, MLN, and thymus by removing fat and connective tissue and smashing between glass slides. Leuckocyte supsensions were then washed via

centrifugation and resuspended. Additionally, splenic erythrocytes were then lysed with Tris-ammonia and the remaining leukocytes washed twice via centrifugation.

T cell culture and polarization. CBir1 Tg $CD4^+$ T cells ($1x10^5$) were isolated as described above and cultured in 24-well plates with irradiated APCs ($1x10^5$) and 1 µg CBir1 peptide in RPMI with 10% FBS for 5 days. WT APCs were used for all tissue culture experiments. Cells were cultured with 10 ng/mL TGF-β to induce a Treg phenotype. A Th17 phenotype was induced by the addition of 10 ng/mL TGF-β, 30 ng/mL IL-6, anti-IFNγ and anti-IL-4. Additionally, cells were cultured with 0, 100, 500, 1500, 2500, and 3500 mg/L of glucose.

Mouse feeding experiments. Mice were supplemented with 0.6% or 6% glucose solution in drinking water for 3 weeks. Fresh glucose solution was supplied as needed during the course of the experiment. Control mice received plain drinking water. All mice were weighed regularly to ensure that each group had comparable trends in body mass over time. At the end of 3 weeks mice were sacrificed and necropsies were performed.

Flow cytometry. Intracellular and surface staining were performed as previously described[90]. For experiments where intracellular cytokines were measured, cells were restimulated for 5 hours with PMA and ionomycin, with Golgi Stop being added for the last 3 hours of restimulation. Cells were washed via centrifugation and surfaced stain added for 20 minutes at room temperature. Cells were then washed permeablized for 30 minutes at 4 C using a FoxP3 perm/fix kit manufactured by eBiosciences. After permeablization cells were washed, intracellular stain was added, and cells were incubated for 30 minutes at 4 C before being washed a final time. Data was collected using the LSRII/Fortessa and compensation performed using FACSDiva software. Further analysis was carried out using FlowJo. All flow cytometry plots shown are first

gated on lymphocytes using FSC and SSC, then gated on CD4 positive, live dye negative cells.

Quantitative real-time PCR. RNA was quantified using two-step PCR. RNA was isolated from the small and large bowel using TRIzol reagent. cDNA was synthesized using qScript cDNA SuperMix and BioRad C1000 thermal cycler. Pre-designed TaqMan primers were used for cDNA synthesis. Quantitative real-time PCR was performed with the SsoAdvanced Universal SYBR Green Supermix using a BioRad CFX96 real-time PCR detection system. DNA amplification was detected with a SYBR green probe. Expression data was normalized to GAPDH mRNA levels and a designated control sample was arbitrarily given a value of 1.0.

Statistical analysis. Samples were analyzed in Prism (GraphPad) via Student's T test or one-way ANOVA utilizing a Newman-Keuls post-test. Paired tests were used where appropriate. The results were considered significant at a P value of less than 0.05.

RESULTS

Glucose regulates Th1 and Th17 frequency in a dose-sensitive manner

To examine the effects of glucose on $CD4^+$ T cell differentiation, we used isolated antigen-specific $CD4^+$ mouse splenocytes. Cells were cultured under neutral, Th17, or Treg conditions and supplemented with 0, 100, 500, 1500, 2500, or 3500 mg/L of exogenous glucose. These numbers were chosen because 3500 mg/L is the difference in glucose concentration between commercially available RPMI and DMEM media. We found that under Treg conditions, FoxP3 expression and thus Treg generation was enhanced at high doses of glucose. Cells treated with the 2500 and 3500 mg/L increased FoxP3 expression to a significant level compared with cells that did not receive glucose supplementation **(Fig 13A-B)**. Conversely, under Th17 conditions, IL-17 production was

significantly upregulated in cells treated with low doses (100 and 500 ng/mL) of additional glucose **(Fig 13C-D)**. No changes in IFNγ production were observed in CD4+ T cells cultured under neutral conditions **(Fig 13E-F)**. These data indicate that glucose most likely regulates T cell differentiation through molecular machinery shared by Tregs and Th17 cells.

As both Treg and Th17 cells require TGF-β for differentiation, we sought to examine whether or not treatment with glucose resulted in upregulation of the TGF-β receptor. Isolated $CD4^+$ spleen cells were cultured with or without TGF-β. Cells received either a high (2500 mg/L) or low (500 mg/L) dose of glucose, and cells that did not receive glucose served as controls. We found that treatment with glucose did not significantly alter TGF-β receptor expression, and that this was the case in both neutral and Treg conditions **(Fig 14)**. Hence, regulation of Treg and Th17 differentiation by glucose is likely TGF-β-independent. Thus, it is necessary to investigate other shared pathways on the Treg/Th17 axis.

Dietary glucose alters the intestinal immune milieu

Having demonstrated that extracellular glucose concentration regulates T cell populations *in vitro*, we set out to determine if dietary glucose supplementation could alter immune function *in vivo.* C57BL/6J mice were supplemented with 0.6 percent and 6 percent glucose solutions in their drinking water for three weeks, with mice that were given normal drinking water serving as controls. Mice were permitted to self-administer glucose solution, which was replaced as needed. Mice were weighed weekly to monitor body mass and determine if obesity was affecting experimental results. All groups of mice showed similar changes in body mass over the course of the experiment **(data not shown)**. Interestingly, the 6% solution required frequent replacement. This suggests that the mice may have enjoyed the sweet taste of the more concentrated glucose solution compared to normal drinking water and the less concentrated glucose solution.

Alternatively, the tonicity of the more highly concentrated glucose solution may have conferred a dehydrating effect, requiring increased water intake.

At three weeks, mice were sacrificed and the spleen, MLN, SB, and LB were collected and prepared for flow cytometry as described We found that FoxP3 was upregulated in the MLN and downregulated in the SB of mice given 0.6% glucose solution compared with mice given normal drinking water **(Fig 15A-B)**. IL-10 was upregulated in the MLN of mice given 0.6% and 6% glucose solution compared to control mice. IL-10 was also downregulated in the SB of mice given 6% glucose solution **(Fig 14A, C)**. There was no significant change in production of IL-17 in either of the glucose-treated groups compared with the control group **(Fig 14D-E)**. However, we observed significant upregulation of IFNγ in the MLN of mice treated with 0.6% drinking water compared to controls **(Fig 14D, F)**. No significant changes in the expression of IL-10, IL-17, FoxP3, or IFNγ were detected in the spleen. These data suggest that dietary supplementation with glucose can influence the composition of gut-specific $CD4^+$ T cells, with systemic $CD4^+$ T cells remaining unaffected. Additionally, both inflammatory and anti-inflammatory cytokines were upregulated by dietary glucose supplementation. Interestingly, the lower dose of glucose produced a more robust immunomodulatory effect than the high dose of glucose. This could possibly be due to increased caloric intake from the glucose solution independent of body mass increase.

DISCUSSION

We have demonstrated that extracellular glucose concentrations regulate $CD4^+$ function both *in vitro* and *in vivo*. *In vitro*, supplementing culture media with glucose upregulates Th17 and Treg populations in a dose-dependent manner. Th17 cells are increased when cells are treated with low concentrations of glucose, whereas Tregs are increased in the presence of high glucose concentrations. *In vivo*, FoxP3 expression is increased in the MLN but decreased in the small bowel when mouse drinking water is

supplemented by a dilute glucose solution. Additionally, IL-10 is upregulated in the MLN of mice supplemented with both high and low concentrations of glucose in drinking water, but downregulated in the SB of mice receiving the higher dose. IFNγ was also upregulated in the MLN of mice given low concentrations of glucose. Hence, we demonstrate that dietary carbohydrates can modify intestinal immune responses *in vivo*. This effect appears to be dose-dependent, with low doses of glucose having the most effect. However, since supplemented water was not restricted, the dose effect is difficult to measure accurately. In particular, the water supplemented with the higher glucose concentration was more rapidly consumed, increasing both glucose and water intake. Thus, electrolyte balance and caloric intake independent of body mass may have influenced the outcome in the high-glucose treated mice. These results provide insight as to how diet impacts T cell development. These findings have far-reaching implications for oncology, autoimmunity, and infectious disease.

The mechanism by which environmental glucose regulates $CD4^+$ T cell development is currently unknown; however, regulation appears to occur independently of TGF-β. A number of metabolic signaling pathways have been implicated in CD4+ T cell differentiation, including mTOR, HIF-1, and AMPK[27,83,87]. Of these, HIF-1 and AMPK are especially important to the differentiation and function of Th17 and Treg cells, respectively[83,86–88]. Interestingly, both HIF-1 and AMPK are activated under hypoxic conditions but result in opposing downstream effects, with HIF-1 upregulating glycolysis and AMPK inducing oxidative metabolism[83,85,88]. Indeed, previous research indicates that high concentrations of glucose inhibit HIF-1 in non-transformed cells[94]. Hence, the HIF-1/AMPK axis seems a promising mechanistic lead. Additionally, investigating the coordinated effects of hypoxia and glucose supplementation on CD4+ T cell function mimics the tumor microenvironment and has the potential to generate interesting and medically relevant results.

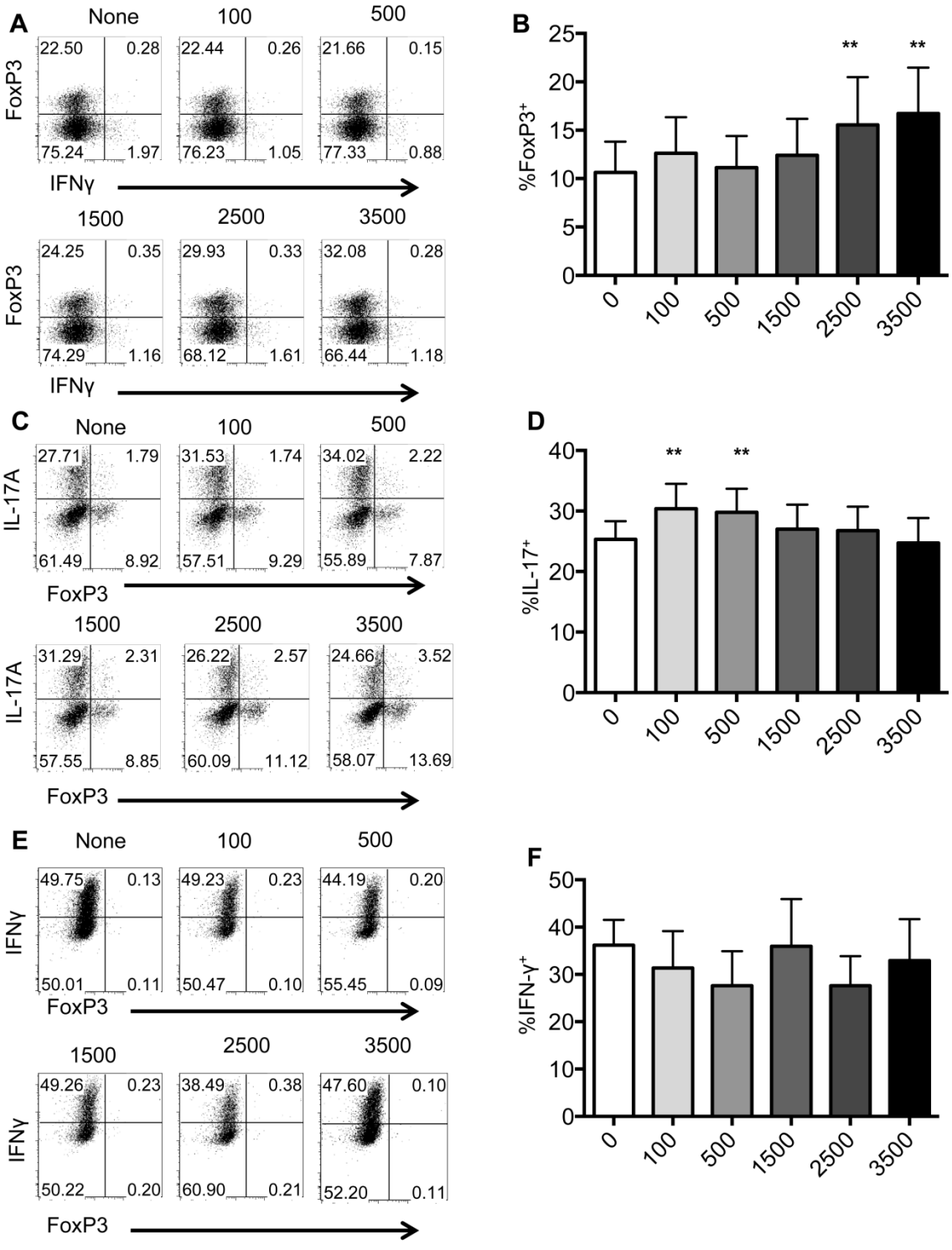
A
None
100
500
FoxP3
22.50
0.28
75.24
1.97
22.44
0.26
76.23
1.05
21.66
0.15
77.33
0.88
IFNγ
1500
2500
3500
24.25
0.35
74.29
1.16
29.93
0.33
68.12
1.61
32.08
0.28
66.44
1.18
B
%FoxP3+
25
20
15
10
5
0
**
**
0
100
500
1500
2500
3500
C
IL-17A
27.71
1.79
61.49
8.92
31.53
1.74
57.51
9.29
34.02
2.22
55.89
7.87
FoxP3
31.29
2.31
57.55
8.85
26.22
2.57
60.09
11.12
24.66
3.52
58.07
13.69
D
%IL-17+
40
30
20
10
0
E
IFNγ
49.75
0.13
50.01
0.11
49.23
0.23
50.47
0.10
44.19
0.20
55.45
0.09
49.26
0.23
50.22
0.20
38.49
0.38
60.90
0.21
47.60
0.10
52.20
0.11
F
%IFN-γ+
50
40
30
20
10
0

Figure 13. Glucose differently regulates Treg and Th17 differentiation in a dose-dependent manner.

Isolated $CD4^+$ T cells were cultured under various T helper polarizing conditions with 0, 100, 500, 1500, 2500, or 3500 mg/L of glucose. *(A-B)* Cells cultured under Treg conditions were analyzed for FoxP3 expression. Flow plots and graphs representing mean percent expression of FoxP3 from 5 independent experiments are shown. *(C-D)* Cells cultured under Th17 conditions were analyzed for mean percent expression of IL-17A. Representative flow cytometry plots depicting 4 experiments are shown, as are bar charts representing percent expression of IL-17 *(E-F)* Cells cultured under neutral condition were analyzed for percent IFNγ expression. Representative flow cytometry plots and bar charts descriptive of 5 independent experiments are shown. **P<0.01 compared with cells that received no exogenous glucose.

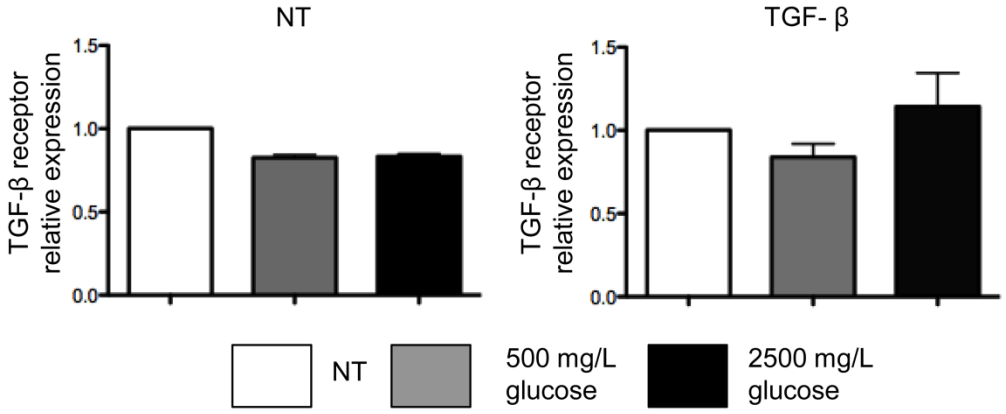

Figure 14. Upregulation of Treg and Th17 cells by glucose is TGF-β receptor independent

Isolated CD4+ T cells were cultured for were cultured with a low dose of glucose (500 mg/L), a high dose of glucose (2500 mg/L), or no glucose under neutral (NT) or Treg (TGF-β) conditions. Cells were harvested at 24 hours and RNA was harvested. Two-step quantitative real-time PCR was used to quantify mRNA transcripts for the TGF-β receptor, which were normalized to GAPDH expression.

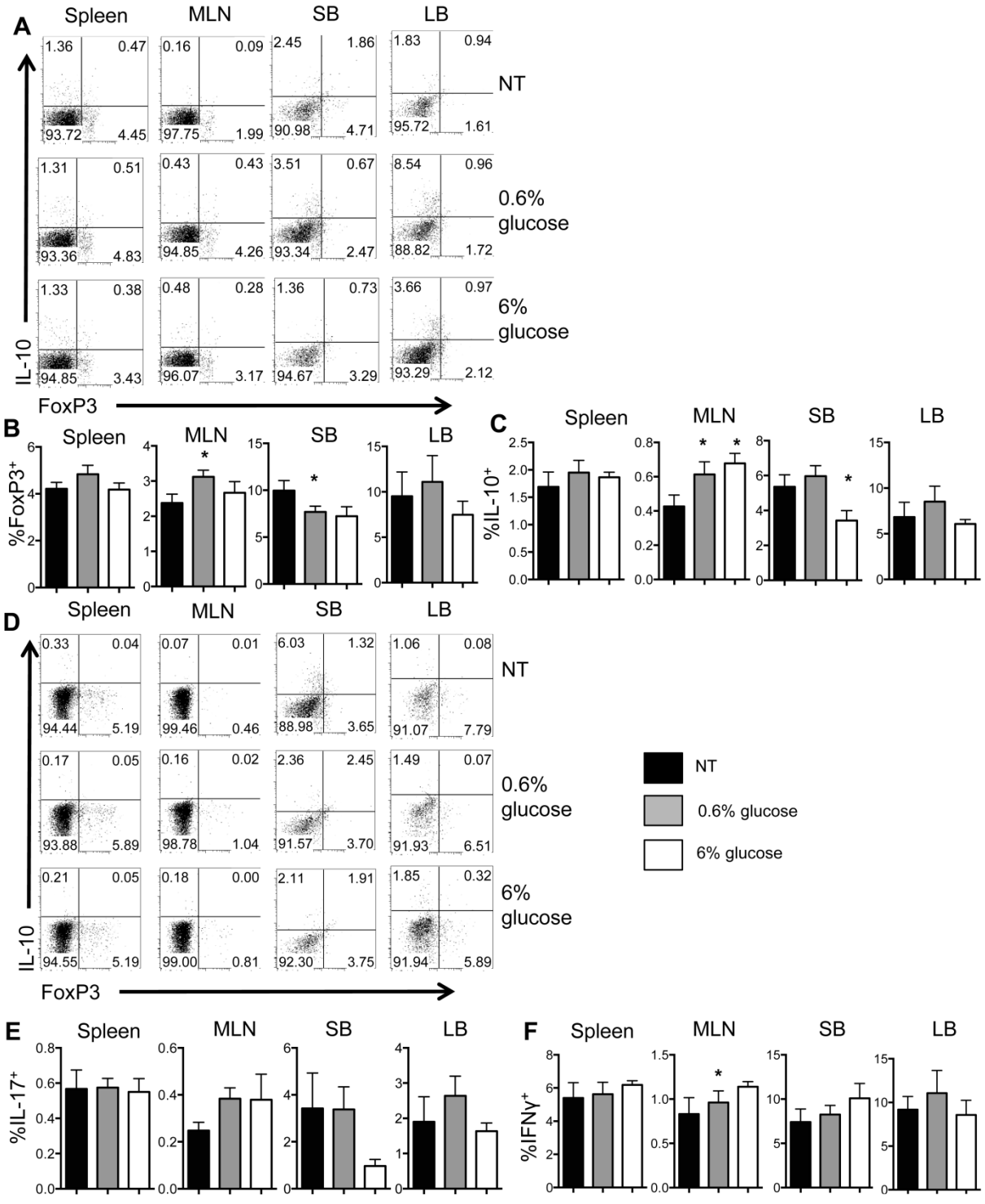
A
Spleen
MLN
SB
LB
1.36 0.47 93.72 4.45
0.16 0.09 97.75 1.99
2.45 1.86 90.98 4.71
1.83 0.94 95.72 1.61
NT
1.31 0.51 93.36 4.83
0.43 0.43 94.85 4.26
3.51 0.67 93.34 2.47
8.54 0.96 88.82 1.72
0.6% glucose
1.33 0.38 94.85 3.43
0.48 0.28 96.07 3.17
1.36 0.73 94.67 3.29
3.66 0.97 93.29 2.12
6% glucose
IL-10
FoxP3
B
Spleen
MLN
SB
LB
%FoxP3+
C
Spleen
MLN
SB
LB
%IL-10+
D
Spleen
MLN
SB
LB
0.33 0.04 94.44 5.19
0.07 0.01 99.46 0.46
6.03 1.32 88.98 3.65
1.06 0.08 91.07 7.79
NT
0.17 0.05 93.88 5.89
0.16 0.02 98.78 1.04
2.36 2.45 91.57 3.70
1.49 0.07 91.93 6.51
0.6% glucose
0.21 0.05 94.55 5.19
0.18 0.00 99.00 0.81
2.11 1.91 92.30 3.75
1.85 0.32 91.94 5.89
6% glucose
IL-10
FoxP3
NT
0.6% glucose
6% glucose
E
Spleen
MLN
SB
LB
%IL-17+
F
Spleen
MLN
SB
LB
%IFNγ+

Figure 15. Dietary glucose regulates immune responses in the MLN and intestines.

Mice received either plain drinking water (NT), 0.6% glucose solution, or 6% glucose solution for 3 weeks. Water and glucose solution were replenished on demand. Mice were weighed weekly to monitor for increasing body mass between groups *(not shown)*. At 3 weeks, mice were sacrificed and the spleen, MLN, SB, and LB were harvested and prepared for flow cytometry as described. *(A-C)* IL-10 and FoxP3 expression was measured, and mean percent expression was calculated. *(D-F)* Mean percent expression of IL-17 and IFNγ were also examined. Representative flow cytometry plots and bar charts are shown. Data represent 2 independent experiments, totaling 4-8 mice per group. *P<0.05; **P<0.01 compared to untreated controls.

DISCUSSION AND CONCLUSIONS

In this work, we demonstrate the regulation of Treg function by non-conventional means. Here, we define non-conventional regulators of Treg function as factors that are not classically thought to influence T cell phenotype. This is in contrast to conventional regulation of $CD4^+$ T cell function by cytokines and immune evasion by pathogens. Here we have concentrated on the modulation of gut $CD4^+$ T cells, Tregs in particular, with a focus on inflammation in the context of IBD. Thus, we demonstrate new mechanisms for the regulation of Tregs. We show here that the intestinal chemokine CCR9 and glucose supplementation can modify the expansion and function of Tregs both *in vitro* and *in vivo*.

We found that CCR9 is differentially expressed on Tregs, Teffs, and naïve $CD4^+$ cells. Morever, differential expression on $CD4^+$ T cell subsets varies by organ and is regulated by RA, but not TGF-β. We also demonstrate a novel, functional role for CCR9 in the regulation of T cell function. Specifically, CCR9 inhibits Tregs by an mTOR-dependent mechanism but does not alter Teff differentiation or function. In animal models of IBD, we show that CCR9 deficiency does not affect Teff or Treg function in a chronic model of intestinal inflammation. However, we demonstrate that CCR9 KO confers protection from colitis in an acute intestinal inflammation, and that susceptibility to colitis is restored upon depletion of Tregs. Finally, we show that glucose supplementation can regulate immune responses. Addition of exogenous glucose to culture media differentially regulates Th17 and Treg expansion in a dose-dependent manner *in vitro.* Treatment with low doses of glucose enhances Th17 development, whereas high doses enhance Treg development. Additionally dietary glucose supplemented in drinking water alters Treg development in the MLN and SB *in vivo.*

These novel findings have broad implications for human health. The regulation of T lymphocytes is important in many aspects of medicine, including autoimmunity, infectious disease, and cancer. Sufficient Treg function is vital to the development of oral tolerance, preventing autoimmunity, and reduction of immunopathology during infection. Conversely, an overabundance of Tregs results in an immunosuppressed state, leading to increased susceptibility to infection, failure to clear pathogens, and tumor immunoevasion. New knowledge regarding the regulation of Tregs aids in our understanding of these finely-tuned processes. Additionally, these discoveries have the potential to drive development of new therapeutics that downregulate or enhance Tregs via unconventional means.

Immune regulation by CCR9 is a novel discovery, as most research regarding chemokine receptor ligand-pairs has focused on their role in chemotaxis. Modulation of $CD4^+$ T cells function by chemokines has only been reported by one other group. Nathan Karin's lab has demonstrated immunomodulatory properties of several chemokine receptors-ligand pairs in an EAE model[40–42]. Currently, CCR5, CXCL10, CXCL11, and CXCL12 have demonstrated the capability to affect $CD4^+$ T cell differentiation[40–42]. Of these, CCR5, CXCL10, and CXCL12 are immunosuppressive, while CXCL11 enhances inflammation[40–42]. Interestingly, CXCL10 and CXCL11 induce opposite results in $CD4^+$ T cells despite sharing the same chemokine receptor[40]. Treatment with chimeric Ig constructs for CXCL10, CXCL11, and CCR5 has been shown to reduce inflammation and disease severity in EAE[40–42]. However, our work is the first to demonstrate immune regulation by CCR9. Additionally, we provide the first evidence that chemokine-receptor pairs can impact disease outcome by influencing $CD4^+$ T cell differentiation and function independently of its chemoattractant properties in a model other than EAE.

Therapeutics targeting CCR9 are already being investigated as treatments for IBD due to its role in migration to the intestine. Our research brings up several important concerns regarding the design and function of CCR9 inhibitors. First, the enhanced

expression of CCR9 on Tregs means CCR9 inhibitors must be carefully examined to ensure that they do not result in depletion of $CCR9^+$ cells, as can be the case with mAb. Furthermore, all CCR9 inhibitors designed for clinical use must be thoroughly tested to confirm that they are true CCR9 antagonists, as opposed to CCR9 agonists. Failure of CCR9 inhibitors to fulfill these requirements would result in fewer Tregs and thus enhanced inflammation.

In addition, further investigation into the effect of CCR9 on Treg suppressive function is required. Inhibition of Treg function by CCR9 signaling is a potential explanation for why Tregs fail to control inflammation in IBD patients, despite their high numbers in inflamed intestinal tissue and normal suppressive function *ex vivo*[33]. If this is the case, therapeutic inhibition of CCR9 could result in the restoration of Treg function and resolution of intestinal inflammation. Thus, it is important to determine whether CCR9 signaling affects production of anti-inflammatory cytokines by Tregs or their ability to suppress Teff proliferation. Additionally, the signaling pathways downstream of CCR9 require further elucidation.

Our finding that dietary glucose can regulate the differentiation of T cells in a dose-dependent manner is both novel and relevant. Our data show influence of glucose on T cell differentiation is TGF-β independent. Therefore, it is suspected that regulation of Tregs and Th17 cells by glucose occurs on the HIF-1/AMPK axis. This is supported by the fact that Th17 generation is enhanced by low doses of glucose, but not high doses of glucose. HIF-1, which enhances Th17 differentiation, was previously shown to be downregulated by noncancerous cells in the presence of high concentrations of glucose[86–88,94]. Interestingly, despite their vastly different downstream effects, both HIF-1 and AMPK can be induced under hypoxic conditions[83,85,88]. The tumor microenvironment is one such hypoxic setting and boasts high numbers of Treg cells[88]. Given our findings, it is possible that high glucose levels within the tumor microenvironment suppresses Th17

cells by inhibiting HIF-1, leading to enrichment of Tregs and an immunosuppressive tumor environment. If so, this is an exciting discovery in tumor immunology.

Additionally, there has been great public concern regarding the use of high-fructose sweeteners in commercial food. We demonstrate the first evidence that dietary intake of simple carbohydrates can modify immune function. However, it is currently unknown if consumption of other mono- and disaccharides are capable of regulating immune responses. This must be investigated further. If other dietary sugars do indeed demonstrate immunomodulatory properties, these discoveries would be a boon to public health and preventative medicine.

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