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Inducible T Cell Costimulator Stabilizes Regulatory T Cell Phenotype And Controls Host-Microbiota Interactions To Reduce Susceptibility To Intestinal Inflammation

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ABSTRACT

Genome wide association studies (GWAS) have identified a loss-of-function mutation in the gene encoding inducible T cell costimulator ligand (ICOSL) as correlating with inflammatory bowel disease (IBD). The reason for this association was largely unknown. ICOS signals have been implicated in controlling the dynamics of regulatory T (Treg) cells, which are essential to intestinal homeostasis. Compared to WT mice, ICOS-deficient mice possessed fewer Treg cells in the colonic lamina propria (cLP). The deficit in ICOS-deficient Treg cell numbers was attributable to preferential loss of Foxp3 expression. Bisulfite sequencing revealed that ICOS-deficient Treg cells did not demethylate conserved noncoding sequence 2 (CNS2) of Foxp3, an epigenetic change essential to maintaining the Treg cell function. Thus, while $Foxp3^{+}$ cells from WT mice could prevent and reverse colitis in the $CD45RB^{hi}$ CD4 transfer model, ICOS-deficient Treg cells were incapable of reversing ongoing intestinal inflammation. Our work also investigated the potential of ICOS-ICOSL signals to contribute to antibody-mediated control of intestinal commensals. ICOSL-deficient mice possessed reduced IgA and IgG binding to colonic commensals, including mucus-associated microbial antigens and flagellins. In compensation, ICOSL-deficient mice surprisingly possessed more IL-10 producing CD4 T cells in the cLP than WT. Mice doubly deficient in ICOSL and CD4 T cell derived IL-10 developed spontaneous colitis in early life that could be mitigated through provision of

milk from WT dams, which rescued the antibody levels of ICOSL-deficient pups. ICOSL helps protect against colitis throughout life, as adult WT mice remained unaffected while ICOSL-deficient mice exhibited rapid-onset colitis upon temporary depletion of IL-10 producing cells. Our studies have determined ICOS signaling helps stabilize Treg cells to allow suppressive function in the inflamed intestine. We have also found that ICOSL promotes antibody mediated control of commensals and cooperates with IL-10 to quell inflammatory reactions to gastrointestinal microbes. Collectively, our work identifies multiple roles for ICOS-ICOSL signaling in the suppression of intestinal inflammation.

Keywords: Inflammatory Bowel Disease (IBD), Inducible T cell costimulator (ICOS), inducible T cell costimulator ligand (ICOSL), Foxp3, IL-10, anti-commensal antibodies

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INTRODUCTION

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) encompasses a variety of chronic relapsing-remitting conditions that cause autoinflammatory damage to the gastrointestinal tract. In the United States, an estimated 1 in 83 adults have received a diagnosis of IBD[1]. This number has increased from the estimated 1 in 111 adults in 1999[2]. In fact, both pediatric and adult-onset cases of IBD have gradually increased worldwide, particularly in North America and Europe[3]. While indeterminate forms of IBD exist, the two classical forms that compose most cases are Crohn's disease (CD) and ulcerative colitis (UC). Despite being grouped together by the term IBD, the presentations of CD and UC are quite different. Ulcerative colitis manifests as superficial ulcerating inflammation beginning at the rectum and ascending contiguously into the colon. Crohn's disease, however, can occur in "skip lesions" anywhere along the gastrointestinal tract and involves more penetrating inflammation. This can lead to intestinal modification, such as tunnel-like fistulae that connect the bowel to nearby epithelial tissue. An estimated 17-50% of CD patients develop fistulae, most of which connect externally to the skin[4, 5]. Remodeling also comes in the form of fibrotic strictures, which cause narrowing of the bowel in one third of CD patients[6]. As such, it is unsurprising that, despite postoperative recurrence odds of 40-76% within the decade[7], 3 out of 4 CD patients undergo related surgery in their lifetime[8]. In contrast, 32-45% of UC patients undergo such intervention[9, 10], often due to fibrotic stricturing or dysplasia. Technically, unless inflammation arises in a surgically created ileal pouch, the removal of the colon and rectum (proctocolectomy) is

curative of UC intestinal symptoms [11]. However, both CD and UC are systemic diseases. Extraintestinal manifestations (EIM), such as ankylosing spondylitis, pyoderma gangrenosum, or ocular complications, impact 31-43% of IBD patients[12]. These potential symptoms, in conjunction with chronic intestinal pain, render IBD capable of massive influence on patient quality of life. In addition to quantifiable impact on work, intimacy, and sleep [13], adult-onset IBD patients have a 2.3 year lower life expectancy on average [14]. In addition, childhood-onset IBD patients followed up through adulthood are 3 times more likely to die before 30 years old, on average follow-up, than the general population despite the influence of modern treatment [15]. There is no cure for IBD. Unfortunately, despite substantial innovations in current disease management, caveats persist in present therapeutics. Patients that respond to steroids may become steroid-dependent for disease control and risk complications of long-term steroid use. Broad immunosuppressants and blockade of α4-integrin, expression of which is not exclusive to the GI tract, are accompanied by vulnerability to opportunistic infections [16]. Additionally, despite the impressive efficacy of various anti-TNF biologics in disease suppression, up to 30% of IBD patients may not respond to an anti-TNF agent and 26-43% of patients lose responsiveness over time [17]. Treatment of chronic disease with such biologics can also be up to 36 times more expensive than standard immunomodulators [18]. With rising prevalence of IBD, research into IBD treatment and etiology addresses an increasingly pressing issue with substantial impact on healthcare costs and quality of life.

Cytokine Environment of the IBD Intestine

One of the barriers to IBD treatment is the complexity of the inflammatory environment associated with the disease. Although other cell subsets undoubtedly share blame, IBD is commonly thought of as a CD4 T cell driven disease. CD4 T cells are enriched in inflamed tissue in both CD and UC, and CD4 T cell depletion has reduced disease activity in limited studies and cases [19, 20]. Past paradigms would place CD and UC as Th1/Th2 -driven respectively, but the current understanding of IBD appreciates that Th17 responses contribute and that Th1/Th2 responses are not always a simple dichotomy.

Interferon gamma

Some factors do associate more with one form than the other, however, such as the prevalence of elevated accumulation of IL-12 producing macrophages in the lamina propria in CD rather than UC [21, 22]. This contributes to the expanded Th1 populations found in inflamed CD tissue that produce large amounts of IFNγ [23]. Genome wide association studies (GWAS) have identified SNPs in *IFNG* (encoding IFNγ) that increase secretion of the cytokine and SNPs in loci for IFNγ receptors as correlative with IBD [24, 25]. The critical contributions of IFNγ to intestinal inflammation can also be observed in many mouse models, including the DSS-induced and CD45RB^{hi} CD4 T cell transfer models of colitis [26, 27]. However, while blockade of IFNγ is effective in these models, IFNγ blockade (via fontolizumab) in CD patients has only demonstrated slight decreases

in disease activity index [28]. Thus, clearly, IFNγ does not drive the entire pathology of Crohn's disease, much less all IBD.

Interleukin -17

Although more commonly associated with CD, both CD and UC exhibit elevated levels of Th17-associated inflammatory mediators. IL-17 producing cells are enriched in the inflamed mucosa of CD and UC patients and IL-17 levels are higher in sera of those with active disease [29]. IL-21, an autocrine factor that induces Th17 differentiation, is also elevated in both CD and UC patients—although more so in CD [30, 31]. IL-23, which is involved in stabilizing the phenotype of established Th17 cells, is also a major cytokine player in IBD [32, 33]. *IL23R*, encoding the IL-23 specific component of the IL-23 receptor complex, constitutes a significant susceptibility locus in both CD and UC [25]. Protective SNPs in *IL23R* have been characterized as loss-of-function mutations [34, 35] and risk variants as gain-of-function through loss of regulation [36]. Additionally, elevated levels of IL-23 mRNA have been found in inflamed CD and UC mucosa as well as increased production of IL-23 by antigen presenting cells (APCs) in the CD lamina propria [37, 38, 39]. Although correlations of IBD with the Th17 pathway are strong, the effects of these factors are often nuanced *in vivo*. Despite the strong IL-17 production often observed in IBD, blockade of IL-17 worsens DSS colitis and IL-17A deficiency worsens both the $CD45RB^{hi}$ T cell transfer and IL-10 deficiency models of colitis [40, 41, 42]. Likewise, deficiencies in IL-21R exacerbate DSS-induced and $CD45RB^{hi}$ T cell transfer colitis [43, 44]. This is strong evidence that these cytokines, despite often being caught at the site of intestinal inflammation, can play a protective role in the situation. In fact, even though

IL-21 blockade has yet to be rigorously tested in human IBD, trials for the IL-17A blocking antibody secukinumab were halted early for adverse events including worsened inflammation [45]. Among the Th17-associated cytokines, however, IL-23 definitively worsens colitis in a variety of chemically-induced and adaptive IBD mouse models [46, 47, 48].

Interleukin-1β

One cytokine that promotes the differentiation of Th17 cells and is heavily associated with intestinal inflammation is IL-1β. IL-1β is generated as the inert form, pro-IL-1β, and activated by the inflammasome component caspase-1. [49] IL-1β is elevated in inflamed CD and UC tissue and levels of IL-1β correlate closely with disease severity. [50] Despite this, however, results vary from model-to-model on whether IL-1β or NLRP3, the inflammasome complex that activates IL-1β and IL-18, is detrimental or protective in animal models of colitis. [51] In addition, human IL-1 signaling inhibition through biologics like anakinra has thus far not proven effective as therapy for IBD, although trials are still ongoing. This leaves the relation of IL-1β, which is commonly considered a proinflammatory cytokine, to intestinal inflammation somewhat unclear.

Tumor Necrosis Factor

TNF is another broadly proinflammatory cytokine significantly associated with IBD and is currently one of the most successful targets for IBD therapy. Stool and inflamed tissue of patients with IBD frequently exhibit elevated TNF levels, although as

TNF is commonly present near inflammation this is fairly expected. [52, 53] However, mice with increased TNF production resulting from enhanced stability of TNF transcripts ($TNF^{\Delta ARE}$) develop spontaneous colitis and continual blockade of TNF attenuates disease in the $CD45RB^{hi}$ CD4 T cell transfer model of colitis [27, 54]. Thus, although it is worth noting that TNF is also involved in mucosal wound healing, TNF significantly contributes to intestinal inflammation. [55] Blockade of human TNF signaling has proven an effective therapy for IBD, although clinical response and remission rates vary from patient-to-patient and type of anti-TNF biologic used. Unfortunately, patients can generate antibodies against anti-TNF therapies which neutralize their effectiveness to cause loss of response. [17] Overall, the cytokine environment of the IBD intestine is an intricate web that invites more investigation into individual cytokine contributions to disease and how they can be manipulated to diminish inflammation.

IBD Etiology and the Microbiota

The etiology of IBD is even more complex than its presentations are varied. Current common consensus is that IBD susceptibility lies at the intersection of genetic predisposition, gastrointestinal flora, and environmental influence. Genome-wide association studies (GWAS) have implicated over 230 susceptibility loci as contributive to the risk of IBD [56, 57]. Single nucleotide polymorphisms (SNPs) at these loci can also act additively with other SNPs [58, 59], engage in epistatic interactions with other SNPs [60], or be influenced epigenetically [61]. Although trends exist that link the presence of individual SNPs to locations (e.g. ileal, ilealcolonic, colonic, etc. [62]) and phenotypes (e.g. fibrotic [63, 64], fistulizing [65], etc.) of IBD, the specific mechanisms by which most of these

SNPs contribute to disease are often unclear. Of the select polymorphisms that have been well-characterized, multiple involve insufficient control of gastrointestinal commensals. For example, loss-of-function mutations in *NOD2* are some of the greatest individual genetic risk factors for Crohn's disease [66]. NOD2 is a cytosolic sensor for muramyl dipeptide (MDP), a component of peptidoglycan, and is highly expressed by myeloid cells and Paneth cells. NOD2 recognition of MDP facilitates secretion of anti-microbial peptides (AMPs) by Paneth cells in the ileum. Indeed, NOD2-deficient mice have reduced bactericidal activity from crypt secretions, increased bacterial burden, and a shift in microbial species occupying the ileum [67, 68]. The shift in ileal microbiota also occurs in Crohn's patients with *NOD2* mutations [69] and implicates mismanagement of commensals as a consequence of such SNPs. In fact, genetic contribution alone to IBD is significant but not always sufficient for development of disease; only approximately half of affected monozygotic twins share a CD diagnosis [70, 71] and merely 5-16% of IBD patients have family history of IBD [72]. The involvement of many susceptibility loci, such as *NOD2*, with microbial control underlines that gastrointestinal flora also play a role in IBD etiology.

IBD is associated with gastrointestinal "dysbiosis", an abnormal microbiota as compared to healthy controls. IBD-related dysbiosis manifests as reduced intestinal microbial diversity [73] and significantly more fluctuations in community composition than in healthy individuals [74], even during remission [75]. Overall trends, however, include reduction in phylum Firmicutes with or without a corresponding increase in Proteobacteria [73, 76]. One Firmicutes member of particular interest is *Faecalibacterium prausnitzii,* the prevalence of which is significantly reduced in Crohn's disease. Lower

levels of *F. prausnitzii* in ileal biopsies correlates with endoscopy-detected recurrence in CD patients and treatment with *F. prausnitzii* lowers the severity of TNBS-induced colitis in mice [77]. As such, the species exemplifies how individual members of the microbiota can suppress intestinal inflammation or leave a host vulnerable through their absence. Not all bacteria are protective, however. Bacterial commensals can also exacerbate IBD through their presence. This is perhaps demonstrated most directly by the fact that administration of antibiotics, such as metronidazole, have shown some success in reducing disease activity [78]. Still, despite copious correlative evidence, overwhelming variability has rendered direct causal links between defined commensals and IBD difficult to discern in human studies.

Causal links have been much easier to define in the controlled environments provided by mouse models. Multiple mouse models of IBD require the presence of a microbiota to manifest colitis. For example, IL-10 deficient mice fail to develop spontaneous colitis under germ-free conditions [79]. Monocolonization with certain microbes (e.g. *Akkermansia muciniphila*) can restore disease development whereas monocolonization with others (e.g. *Bacteroides acidifaciens*) does not [80]. Likewise, transfer of $CD45RB^{hi}$ CD4 T cells into Rag-deficient mice does not induce colitis if recipient mice are germ-free. Again, in this model, certain microbes (e.g. *Helicobacter muridarium*) allow for colitis induction while other microbes (e.g. SFB) do not [81]. Such models show the contribution of gastrointestinal microbes to colitis and that differences in microbial colonization can directly contribute to differences in disease outcomes. The *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ Ulcerative Colitis (TRUC) mouse model also demonstrates this principle in a manner surpassing monocolonization into the context of an intact microbial

community. TRUC mice have elevated production of TNF by colonic dendritic cells (DC) that results in increased apoptosis within the colonic epithelium, resulting in a UC-like colitis. This can be suppressed by broad spectrum antibiotics, thus is microbiota dependent. Remarkably, fostering of WT pups with TRUC dams or even cohousing adult WT mice with TRUC mice can induce histologically detectable colitis in WT mice [82], essentially signifying transmission of a colitis-inducing microbiota.

While commensals as colitogenic factors can be studied in isolation in mice, in humans they can be confounded by the presence of other environmental factors that have also been determined to correlate with IBD incidence. For example, smoking is one of the most well-known risk factors for CD, for yet undetermined reasons that may include modified mucus production [83], increased oxidative stress [84], or even disruption of the gut microvasculature [85]. Additionally, dietary factors can modify IBD risk. Prime examples of this include high fiber intake correlating with a 40% reduced risk of CD [86] and high trans-unsaturated fat intake associating with increased UC risk [87]. In mice, physiological concentrations of frequently used food emulsifiers have been demonstrated to thin the colonic mucus layer and accelerate colitis in genetically predisposed animals [88]. However, all of these factors have also been implicated in altering gastrointestinal microbiota [83, 88, 89, 90, 91]. Essentially, predisposition to IBD remains an exceedingly convoluted matter in which genetic predisposition, environment, and gastrointestinal microbes all indistinctly intertwine.

Although gastrointestinal microbes greatly influence IBD, their host possesses wide variety of tools that can curate the composition of commensals. MicroRNA (miRNA) produced by intestinal epithelial cells (IEC) and goblet cells is one such

regulatory mechanism. Host miRNA is capable of physically entering bacterial cells and of modifying the prevalence of certain species in the gut. Mice with IEC deficient in Dicer1, the miRNA processing enzyme, possess an altered microbiota and get more acute dextran sodium sulfate (DSS)-induced colitis. Administration of fecal RNA from WT mice partly rescues representation of some microbial species and reduces DSS-induced colitis severity [92]. This demonstrates one way a host can modify their own microbiota, and impact colonic inflammation, through secreted products. Antibodies are another well-known secreted component by which hosts control commensals. Humans with common variable immunodeficiency (CVID), in which reduced levels of antibody are produced, demonstrate diminished diversity of fecal microbes through an alpha diversity (Chao1) not significantly different from that of IBD patients. This decreased diversity was particularly evident in patients with lower levels of IgA, the predominant antibody of mucosal surfaces [93]. Indeed, humans selectively deficient in IgA also have significant differences in gastrointestinal microflora, although microbial diversity is largely unimpacted. Rather, minor differences have been observed such as decreased presence of Firmicutes, including the earlier-mentioned regulatory *F. prausnitzii*, and increased presence of *Prevotella* [94], which has been previously implicated as colitogenic [95]. Shaping of the microbiota by the host can help manage microbes that would otherwise contribute to inflammatory conditions.

However, while hosts help define their commensal landscape, this regulation goes both ways. There are many, if not more, instances defined in which intestinal microbes or their products shape host immunity. One prime illustration of this is the ability of segmented filamentous bacteria (SFB) to elicit a profound Th17 response in

mice. This adherent commensal drives production of serum amyloid A (SAA) from IECs in the terminal ileum which provokes production of IL-1β from DCs in the lamina propria and favors subsequent Th17 development [96]. This ability seems to be predicated on adherence, a trait that commensal SFB also shares with certain pathogens [97], which SFB helps protect against through such colonization [96]. Another key example of microbiota influences on the host comes in the form of short-chain fatty acids (SCFA) such as butyrate. Bacterial fermentation of dietary fiber can form butyrate that can be absorbed through monocarboxylate transporter 1 (MCT1) or sodium-coupled MCT1 (SMCT1) for use as fuel by colonic IECs [98]. In fact, colonization with *Butyrivibrio fibrisolvens*, a butyrate-producing bacteria, is enough to return elevated autophagy in the colonic epithelium in germ-free mice to normal through supplying butyrate as an energy source [99]. Additionally, SCFAs stabilize hypoxia-inducible factor 1α (HIF-1α) that preserves integrity of the colonic IEC barrier in its low oxygen environment and protects from trinitrobenzene sulfonic acid (TNBS)-induced colitis [100, 101].

The effects of microbial SCFAs surpass helping IECs, however. They can act as stimulants of G-coupled protein receptors (GPCRs) and stimulate colonic macrophages and DCs through GPR109a to drive regulatory T cell (Treg) differentiation [102]. Additionally, SCFAs act as histone deacetylase (HDAC) inhibitors through signaling GPR43 to allow expansion of colonic Treg cells [103] and can also be absorbed into the bloodstream for systemic effects [104]. Notably, patients with IBD have lower levels of SCFA and amounts of prevalent SCFA-producing bacteria, such as *F. praunitsii* and *Roseburia intestinalis* [105, 106]. Carbohydrates from common commensals can also considerably impact host intestinal immunity. *Bacteroides fragilis* polysaccharide A

(PSA) is a capsular polysaccharide that both stimulates TLR2 and, uncommonly for carbohydrates, also drives a T cell-dependent response [107, 108]. Through promotion of CD4 T cell derived IL-10, PSA is capable of suppressing pathology in both the TNBS and CD45RB^{hi} CD4 T cell transfer models of colitis [109]. Although some evidence correlates an enterotoxigenic form of *B. fragilis* with IBD, recent data suggests that lower frequencies of *B. fragilis* in IBD patients have a promoter in the correct orientation to allow PSA production and, as such, PSA expression may be lower in disease [110, 111, 112]. The host and microbiota have an intensely reciprocal relationship that helps define susceptibility to intestinal inflammation.

Anti-Commensal Antibodies in IBD

Since antibodies as a major element in host-microbe interactions, it is unsurprising that patients with IBD often generate elevated levels of specific antibodies toward certain self-antigens and/or epitopes found in the microbiota. These antibodies are of the IgA or IgG isotypes and are detectable in the serum in conjunction with disease often enough to enable usage as a biomarker [113]. While specificities of these antibodies are not truly exclusive between CD and UC, the target antigens trend differently depending on the form of IBD. For example, perinuclear antineutrophil cytoplasmic antibodies (pANCA) are present in approximately 60-80% of UC patients but only 10-15% of CD patients, making pANCA correlate much more strongly with UC than CD [114, 115]. Likewise, antibodies toward the mannan of *Saccharomyces cerevisae* cell walls (ASCA) are more prevalent in CD, with 60-70% of CD patients having IgG ASCA as opposed to 10-15% of UC patients [113]. The relation of pANCA to UC and ASCA to CD

respectively is strong enough for routine use as a diagnostic tool [116]. Use of antibodies as biomarkers in IBD does not end with pANCA and ASCA, however. Many antibodies toward components of gastrointestinal commensals correlate with Crohn's disease especially. This includes antibodies toward *Escherichia coli* outer-membrane porin C (OmpC), *Pseudomonas fluorescens* component I2 (I2), and flagellin (CBir1), each of which individually can be detected in more than 50% of CD patients [117, 118, 119, 120, 121]. The same holds true for antibodies toward other flagellins than CBir1, such as A4Fla2 and FlaX, demonstrating the prevalence of anti-flagellin antibodies in CD patients [122]. Although the targets of many IBD-related antibodies are known, however, the role that said antibodies play in relation to intestinal inflammation is still undergoing investigation. In mouse models, elevated anti-flagellin IgG is detectable during recovery from DSS-induced colitis. However, if mice are hyperimmunized with flagellin before receiving DSS they develop worsened disease. [123]. In humans, GWAS have associated a loss-of-function mutation in an activating IgG Fc receptor as protective from UC [25]. This may be, in part, because mononuclear phagocytes (MNPs) from the intestine of those with the SNP produce less IL-1β, a prominent cytokine in IBD, upon exposure to IgG immune complexes [123]. This evidence would imply that anti-commensal antibodies may contribute to pathogenesis in IBD. However, B cell depletion via rituximab has proven ineffective in UC and anti-commensal IgG is also detectable in healthy individuals [124, 125]. The way that microbiota-binding antibodies play into IBD remains unclear and is in need of further investigation.

Treg cells: Function and Role in IBD

Treg cells are an essential subset of CD4 T cells that subdue inflammatory responses toward self-antigen or the microbiota. Treg cell development and suppressive phenotype relies on expression of the Treg cell "master transcription factor", Foxp3. Mice and humans with Foxp3 deficiency suffer from overwhelming fatal autoimmunity [126, 127]. Differentiated Foxp3$^+$ Treg cells emerge from two sources: directly from the thymus as Foxp3$^+$ thymic Treg cells (tTreg) or as peripherally-derived Treg cells (pTreg) from antigen stimulation of fully-developed naïve CD4 T cells. [128] Foxp3 expression can be temporary, however, and the demethylation of multiple conserved non-coding sequences (CNS) within the *Foxp3* locus are required to maintain stable expression. CNS1 is particularly involved in the induction of pTreg cells; mice with deletions of CNS1 have similar Treg frequencies in the thymus but greatly reduced Treg populations in peripheral lymphoid tissue and intestinal lamina propria. CNS2 is also known as the Treg-specific demethylated region (TSDR) and, while not required for Foxp3 induction, is essential for the maintenance of Foxp3 expression to the point that CNS-2 deficient mice develop systemic inflammation. [129, 130] As such, demethylation of the CNS2 region can be used as a measure of stability of Foxp3 expression. [131] CNS3 allows Foxp3 expression in response to TCR stimulation and is thus needed for Treg cell induction. [132] In addition to these three elements, CNS0 is a Foxp3 super-enhancer that binds transcription factor Satb1, a pioneer factor enabling efficient Foxp3 transcription, and is particularly important for tTreg development. [133] Demethylation of these various CNS to stabilize Foxp3 expression is essential to the Treg cell identity and enable their suppressive mechanisms reliant on Foxp3 expression. A main mechanism Treg cells

utilize is the secretion of IL-10, an immunosuppressive cytokine essential for colonic health. In fact, not only do comprehensively IL-10 deficient mice develop spontaneous colitis, but a Treg cell-restricted deficiency in IL-10 is enough to drive colitis in mice [134]. Treg cells can also produce cytokines such as TGF-β and IL-35 [135, 136]. Amongst its many functions, TGF-β both drives the differentiation of Treg cells and directly acts on naïve and effector T cells to suppress proliferation and inflammatory cytokine production [137, 138, 139]. In addition to generating TGF-β, Tregs can render pre-produced inactive TGF-β biologically active through sequestration by Glycoprotein A repetitions predominant (GARP) and subsequent integrin activity that frees the cytokine from latency associated peptide (LAP) [140]. IL-35 is a largely immunosuppressive cytokine that can suppress Th17 responses and is protective in various mouse models of colitis [141, 142]. Cytokine production is certainly not the only utility that Treg cells have available to subdue inflammation, however. Treg cells also express cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which has high affinity for the CD80 and CD86 expressed by APCs [143]. Its high affinity allows CTLA-4 to outcompete T cell-expressed CD28 for binding to these molecules to prevent it from facilitating T cell activation [144]. It also allows the physical removal of the CD80 and CD86 from APCs by transendocytosis [145]. Thus, Treg cells can use CTLA-4 suppressively by effectively removing activating stimuli from the environment of other T cells. Similarly, Treg cells express large amounts of a component of the high-affinity IL-2 receptor, CD25. IL-2 has a vast impact on both CD4 and CD8 T cell proliferation and differentiation [146]; CD25 allows Treg cells to compete with other cells for the cytokine, thus both denying naïve and effector T cells access to IL-2 and using it to fuel their own maintenance [147, 148]. Treg cells can also deplete extracellular

ATP (eATP), which would normally perpetuate inflammation via purinergic type 2 (P2) receptors that subsequent increase in inflammasome activity and proinflammatory cytokine production [149, 150]. Treg cells use ectoenzymes CD39, which converts eATP to adAMP, and CD73, which converts AMP to adenosine, to shift the surrounding extracellular purinergic environment toward the non-inflammatory [151]. Treg cells can also simply remove inflammation-promoting cells themselves by direct cytolysis of dendritic cells and effector T cells with perforin and granzyme B [152, 153]. Using these many mechanisms and more, Treg cells are critical to containing inflammatory reactions toward self and commensals. Despite the importance of these cells, however, no SNPs in the locus for Foxp3 have been associated with IBD [154]. However, SNPs associated with the expression of many suppressive tools employed by Treg cells, such as CTLA-4,w CD39, and IL-10, have been associated with the condition [155, 156, 157, 158]. In addition, Treg cells prevent or mitigate ongoing disease in multiple murine colitis models [27, 159, 160]. Treg presence is elevated in IBD patient lesions [161, 162]. The reasons that these Treg cells do not resolve the inflammation are still subject to ongoing investigation.

ICOSL and Treg cells

Inducible T cell costimulator ligand (ICOSL) is a costimulatory molecule found on APCs that interfaces with inducible T cell costimulator (ICOS) on T cells, including on Treg cells. *ICOSLG*, which encodes ICOSL, has been identified by GWAS as a susceptibility locus for IBD [25]. One of the IBD-predisposing SNPs in *ICOSLG* has been identified as a loss-of-function mutation that reduces the expression levels of ICOSL, even in heterozygous individuals, as demonstrated in monocyte-derived dendritic cells

(MDDC) [163]. The fact that it is a loss-of-function mutation in *ICOSLG* in the risk conferring allele implies that ICOSL plays a protective role in intestinal inflammation. Additionally, ICOSL is found at the effector sites of IBD. B cells, macrophages, and IECs from the inflamed mucosa of CD and UC patients have significantly more ICOSL, and CD4 T cells within affected tissue also express more ICOS than in healthy controls [164]. Although ICOSL and ICOS are increased at the inflamed area, proximity to inflammation does not always equate pro-inflammatory function. Many regulatory elements, such as Treg cells themselves, are also increased at the site of IBD lesions [161, 162]. In fact, Treg cells often express high amounts of ICOS, with most of that expression being on a subset of effector Treg cells (eTreg), which migrate to inflamed sites [165, 166]. The signaling between ICOS and ICOSL is essential to this particular population, with blockade of ICOSL significantly diminishing eTreg cell numbers in mice in a BCL-2-interacting mediator of cell death (BIM)- dependent manner [165]. As BIM is pro-apoptotic, this difference in eTreg cells may be partially attributable to survival; $ICOS^+$ Treg cells are more responsive to IL-2 which, in turn, increases expression of pro-survival factors such as BCL-2 and MCL-1 [167, 168]. As eTreg cells are already lower in BCL-2 and MCL-1 than their lymphoid-tissue patrolling central Treg (cTreg) cell counterparts, $ICOS^+$ eTreg cell populations may have a survival advantage [165]. Regardless, ICOS-deficient mice have fewer Treg cells overall but no defect in Treg cell count in the thymus, suggesting that the deficit may be in peripherally-derived Treg cells, which tend to skew toward an eTreg cell phenotype [169, 170]. Resilience of eTreg cell survival and phenotype are particularly important in inflammatory environments to allow eTreg cells to perform their principal task, local supply of the immunosuppressive cytokine IL-10 [166]. Historically,

ICOS highly-expressing cells in secondary lymphoid tissue have also been strongly associated with IL-10 production [171]. On the APC side of things, human peripheral blood mononuclear cells also generate more IL-10 and less IL-2 when stimulated with ICOS in lieu of CD28 [172]. In human holders of an *ICOSLG* loss-of-function SNP resulting in reduced expression MDDC generate reduced amounts of IL-10 upon stimulation of NOD2 or various TLRs, indicating a role for ICOSL in APC IL-10 production as well [163]. As such, ICOS-ICOSL signaling has been inextricably linked to Treg population dynamics and production of regulatory cytokines.

ICOSL and Antibody Production

Despite its relations to regulatory T cells, ICOS is perhaps best known for is its role in follicular helper T cell (Tfh) differentiation and antibody production. ICOS-ICOSL interaction is responsible for multiple functions in Tfh development. Much like stimulation of CD28, stimulation of ICOS activates the phosphoinositide 3-kinase (PI3K) pathway. However, while both CD28 and ICOS use this pathway, ICOS stimulation is biased toward recruitment of different class IA PI3-kinases than CD28, such as p50α. This difference in isoforms results in an overall stronger PI3K signal and Akt phosphorylation than CD28 [173]. This signaling allows ICOS to cause the temporary exclusion of transcription factor FOXO1 from the nucleus of differentiating CD4 T cells [174]. In this way, the absence of FOXO1 both derepresses the transcription of the Tfh "master transcription factor" Bcl-6 and reduces transcription of Klf2 [175]. This stops repression of CXCR5, one of the essential homing chemokine receptors that allows T cells entry into the lymphoid follicle to begin the germinal center (GC) reaction, the

cornerstone of T-dependent antibody responses. Concomitantly, ICOS PI3K signaling also goes through p85α, which brings osteopontin (OPN) into the nucleus to protect Bcl-6 from ubiquitin-dependent degradation [176]. Thusly, signals from ICOS are essential for maintaining Tfh cell trafficking to the germinal center and helps preserve expression of Bcl-6 and the Tfh signature transcriptional profile. Requisite ICOSL signals are provided by dendritic cells (DC) initially, with B cells providing ICOSL that retains Tfh cells during later stages of the GC reaction [177]. GC B cells also require T cell ICOS signaling to secrete lymphotoxin alpha/beta and structure a germinal center [178]. It is no surprise then that mice without ICOS develop stunted and poorly formed primary GCs and almost no secondary GCs upon repeat encounter with an antigen [179]. Results of this manifest in almost three-fold fewer CD4 T cells entering the active GC and lowered production of all IgG isotypes observed compared to WT controls after immunization of ICOS-deficient mice with T-dependent antigen [180]. The IgG1 isotype is particularly diminished which may be explained by the fact that ICOS-deficient T cells do not produce much IL-4, which promotes IgG1 [179, 181]. Even basal levels of serum IgG are lower in ICOS-deficient mice and mice with specifically impaired ICOS-dependent PI3K activity (ICOS-YF mice). The quality of IgG generated was also affected, as ICOS-YF mice had reduced affinity IgG upon secondary challenge with a T-dependent antigen [182]. T-independent responses appear largely unaffected [183]. While memory B cells (MBC) can form without ICOS-ICOSL signals, as they do not necessarily require germinal centers, ones that do form have fewer somatic mutations and provide low affinity recall responses [184, 185]. From this we can infer that ICOS is essential to affinity maturation, which is supported by the fact that higher-affinity B cell receptors (BCRs) are concentrated in GC B cells that

highly-express ICOSL [186]. Thus, while lack of ICOS may impair primary GCs, its absence is most keenly felt as a reduction of class-switched high-affinity antibody toward recurrent antigens, such as those supplied by persistent commensals.

Antibody-Mediated Control of the Microbiota

IgA is by far the most well characterized antibody at mucosal surfaces and the mechanisms by which IgA physically controls residing commensals have been thoroughly investigated. IgA is capable of agglutinating microbes or chaining dividing bacteria together with daughter cells to group them in the lumen for passage through the rest of the GI tract [187, 188]. IgA has also been demonstrated as capable of binding to mucus via secretory component (SC), the remnant of the polymeric immunoglobulin receptor (pIgR) that transports IgA across the mucosal epithelium, to entrap encroaching bacteria. [189] The binding of SC to mucus is mediated primarily by carbohydrate-carbohydrate interactions. [190] Similar interactions also render the highly glycosylated secretory IgA and SC capable of noncanonical binding to glycans on bacterial membranes independent of the specificity provided by IgA Fab region. [191] Even the majority of canonical binding through the Fab portion is commonly thought to be largely polyreactive low affinity binding with regards to commensal components. [192] Given the continued presence of these antibodies in T cell-deficient mice, these responses are thought largely to be T-independent. [193] In mice, these T-independent IgA plasma cells develop from either peritoneal B1b cells or follicular B2 cells and are driven by TLR stimulation from the microbiota and resultant secretion of a proliferation-inducing ligand (APRIL) or B-cell-activating factor (BAFF) by innate cells, stromal cells, and IECs in GALT or the lamina propria itself. [194, 195, 196] In fact, despite being part of the adaptive immune system,

antibodies to control commensals can be immensely influenced by innate immune sensors. One of the chief examples of this is the TLR5-deficient mouse, in which impairment of innate pathogen associated molecular pattern (PAMP) sensing directly translates to reduced antibodies toward flagellin. This translates into upregulation of flagellin in the cecal commensals of TLR5-deficient mice and breaching of the protective colonic mucus layer, presumably through enhanced motility. [197] Despite the role of the microbiota in providing TLR signals, both germ-free and antigen-free mice also produce intestinal IgA that is capable of binding commensals from SPF mice. These polyreactive clones tend to be mostly unmutated, indicating that they, like those found in T cell-deficient mice, have undergone minimal to no SHM. [192] In fact, newer cell-tracking methods such as single LN photoactivation or Confetti mice have made it readily apparent that antibody class switching can occur before germinal center formation and well before SHM, making it easily possible for these largely unmutated T-independent IgA^+ plasma cells to exist. [198] The gastrointestinal tract is home to both T-independent and T-dependent plasma cells, however. [192] Certain atypical commensals, particularly those that live close to the mucus layer or epithelium such as *Mucispirillum*, *Prevotella,* and *Helicobacter* are coated solely by high-affinity T-dependent IgA and remain uncoated in T cell-deficient mice. [199, 200] In fact, multiple of these species that are preferentially coated with T-dependent IgA are commonly perceived as colitogenic or live close to the epithelium. [200] Although T-dependence often goes hand-in-hand with affinity maturation, T cells also provide help to mucosal antibody production beyond the constraints of the germinal center reaction. T cell-deficient ($Tcrb^{-/-}d^{-/-}$) mice have significantly fewer IgA^+ plasma cells in the intestine than BCL-6 deficient mice, which

have T cells but no germinal centers. [192, 199] Additionally, certain bacteria, such as the IEC-adherent SFB, require T cells but not germinal centers or SHM for strong antibody coating. [199] This implicates roles other than the traditional Tfh-B cell interface for T cells in providing help for antibody production, such as the provision of IL-10 or TGF-β to drive class-switching. [195, 201] Despite the existence of T cell help through endocrine or paracrine routes, however, the germinal center reaction is undoubtedly at the heart of T-dependent humoral immunity. In fact, the somatic hypermutation (SHM) and affinity maturation of IgA in the GC reaction impacts the curating of gastrointestinal microflora. Mice with a mutated activation-induced cytidine deaminase (AID) that allows most class switch recombination (CSR) but much less SHM develop an outgrowth of intestinal microbes despite having similar overall amounts of IgA in serum and GALT [202]. Additionally, mice with altered plasmablast selection because of PD-1 deficiency have similar amounts of IgA^+ plasma cells in the small intestinal lamina propria but greatly reduced affinity maturation within the population. As such, they exhibit reduced bacterial coating with IgA and expansion of Proteobacteria and *Prevotella*, of which increases have been associated with IBD. [95, 203] Said mice also exhibited signs of systemic immune activation that was ablated by treatment with broad-spectrum antibiotics, implying the impact of antibody affinity and repertoire changes on commensal control. [204] The results from both the mutant AID and PD-1 deficient mouse studies indicate that, beyond differences in antibody isotype, differences in affinity and quality within a given isotype can affect the interactions between antibodies and the microbiota. While the mechanisms and significance of anti-commensal IgA have been extensively studied, less is currently known about the activity of anti-commensal IgG. Although often thought of as T-

dependent, IgG can be formed with or without T cell help in a manner similar to IgA. Additionally, although not as prevalent as IgA producers, IgG-producing ASCs are detectable in human and murine intestines and GALT [205]. IgG reactive to intestinal commensal antigens can also be detected in healthy individuals [206, 207]. One way in which this homeostatic anti-microbiota IgG can function is to curtail the dissemination of microbes from the colon. Antibody-deficient mice ($J_H^{-/-}$) exhibit more bacteria in the blood than WT when treated with DSS, and transfer of WT IgG protects $J_H^{-/-}$ mice from death upon intraperitoneal challenge with those same bacteria. Additionally, IgG toward microbiota antigens such as murein lipopeptide (MLP) can protect against systemic infection by pathogens that share that antigen [206]. As such, one function of anti-commensal IgG appears to be to restrict commensals and similar pathogens. T-dependent responses to intestinal pathogens themselves can also play critical roles in clearance. [208] Levels of IgG toward certain microbiota antigens are significantly higher in IBD patients, however. [122] Additionally, IBD patients also tend to have more IgG^+ ASCs in the colonic mucosa as compared to healthy controls. [209] Whether this IgG is contributive to intestinal inflammation is still a matter of investigation. A loss-of-function mutation in an activating Fcγ receptor, *FCGR2A,* has been determined to negatively correlate with UC by GWAS and Fcγ receptors (both inhibitory and activating) are upregulated in inflamed UC biopsies. [210] Also, significantly more microbes in UC stool are coated with IgG than in controls which correlates with disease severity. [210, 211] The reduction or overexpression of the inhibitory Fcγ receptor, FcγR2b, in mice either enhances or reduces colonic pro-IL-1β expression respectively in response to DSS. Additionally, prior transfer of anti-flagellin enriched IgG into Rag-deficient mice provokes enhanced pro-IL-1β production

by colonic MNPs during DSS colitis. [210] These results led the authors of one study to suggest that anti-commensal IgG contributes to UC, with an emphasis on the importance of the ratio of activating to inhibitory Fcγ receptors available. Despite this, evidence also exists that IgG may play a large role in inhibiting bacterial adhesion to the epithelium and enhancing neutrophil-mediated clearance of effacing pathogens [212]. Reduced distance between commensals and the epithelium due to a compromised mucus layer is often found in IBD. [213] As such, it is very possible that the elevated anti-commensal IgG witnessed in IBD is similarly attempting to reduce microbe adhesion and increase bacterial clearance in the face of commensal encroachment on the mucosal barrier. Indeed, as IgA is capable of localizing within mucus via secretory component-mediated binding, it is worth investigating the potential of IgG to perform similarly; Fcγ binding protein, a major structural component of intestinal mucus, was originally named for its ability to bind to IgG. [214, 215] Undoubtedly, the mysteries of how anti-commensal IgG functions at homeostasis or during inflammation require further investigation. Still, between the well-characterized functions of affinity-matured anti-commensal IgA and the presence of anti-commensal IgG, T-dependent anti-microbiota antibodies indubitably contribute to the host-commensal relationship in the intestine.

IL-10 and the Intestinal Barrier

Interleukin-10 is a primarily immunosuppressive cytokine that is paramount for suppression of inflammation at mucosal interfaces. Deficiencies in IL-10 signaling have long been linked to intestinal inflammation in animal studies, with both IL-10 deficient (*Il10*$^{-/-}$) and IL-10Rβ deficient (*Il10rb*$^{-/-}$) mice developing spontaneous colitis. [216, 217]

Simple antibody blockade of IL-10 signaling also suffices to induce colitis in mice. [218] Spontaneous colitis arises in mice with a CD4 T cell restricted deficiency in IL-10, as CD4 T cells are a main source of IL-10 in the gut. [219] In fact, Treg cells especially are an important provider of the cytokine and Treg cell -restricted deficiency in IL-10 production also results in spontaneous colitis in mice. [134] Many other cell populations make IL-10 to a lesser degree, including macrophages, dendritic cells, and B cells, but loss of IL-10 secretion by these populations does not generally provoke spontaneous intestinal inflammation. [220, 221, 222] IL-10 deficient mice do not develop disease in gnotobiotic conditions. [79] Thus, the role of IL-10 as restraining host responses toward the microbiota and consequential importance to intestinal health is readily evident. In humans, loss-of-function mutations in the genes encoding IL-10 receptor components have each been monogenically associated with infantile or very early onset (VEO) IBD, prior to 6 years of age [223]. GWAS have also identified SNPs in the gene encoding IL-10 as correlative with IBD [25, 157]. As such, IL-10 is inextricably linked to the maintenance of intestinal homeostasis, a task it helps accomplish by several means that affect many different cell types. At the epithelium, IL-10 suppresses claudin-2 expression by IECs to preserve the integrity of tight junctions and promotes IEC proliferation to heal mucosal injuries [224, 225]. IL-10 also reduces ER stress in goblet cells and allows for efficient production of MUC2, the primary component of intestinal mucus, by upregulating genes allowing for correct protein folding [226]. In conjunction, this signifies that IL-10 plays a large role in maintaining integrity of the intestinal barrier itself, additionally evidenced by the fact that IL-10 deficient mice exhibit increased barrier permeability even before the onset of overt inflammation [227]. One of the most imperative tasks for IL-10 in the gut is

restraining the activation of myeloid cells in the face of the overwhelming stimuli provided by the microbiota; IL-10 inhibits the MyD88 signaling that would otherwise convey activating signals from TLRs. [228] Accordingly, colitis onset in IL-10 deficient mice is prevented by deletion of MyD88 in MNPs. [229] Suppression of a proinflammatory myeloid profile by IL-10 is critical, as even macrophage-restricted (*Cx3cr1-Cre*) deficiencies of IL-10Rα result in spontaneous colitis in mice [221]. Through inhibiting the activation of myeloid cells, IL-10 also has a profound downstream effect on the adaptive immune system. For example, IL-10 encourages the production of March-I that can ubiquitinate MHC Class II and CD86 for degradation, reducing the capability of APCs to activate T cells. [230, 231, 232] IL-10 also lowers stimulated macrophage production of IL-1β, a cytokine that drives CD4 Th17 responses and correlates with IBD severity. [233, 234, 235] In addition to IL-1β, IL-10 inhibits myeloid production of TNF, IL-6, and IL-12, effectively cutting proinflammatory cytokine help that would otherwise drive Th1 and Th17 differentiation and function. [236, 237, 238] Through modulation of DCs, IL-10 can also inhibit T cell expression of CXCR3 to impede effector T cell traffic to inflamed regions. [239] While it has been well-demonstrated that IL-10 can control CD4 T cell expansion and Th1 or Th17 responses indirectly through modulating APCs, the direct effects of IL-10 on T cells themselves are less obvious. Unlike mice deficient in IL-10R signaling to macrophages, mice with a dominant negative mutation of IL-10R restricted to CD4 T cells do not exhibit spontaneous disease. [240] However, transfer of Th17 cells from these mice or WT mice into lymphopenic hosts induces colitis that cannot be controlled by Treg or T regulatory type 1 (Tr1) cell transfer unless the Th17s have functional IL-10R. [241] This suggests that there may be a role for direct action of IL-10 upon Th17

populations to suppress activity. Other evidence, such as the spontaneous colitis that emerges in mice with a Foxp3-restricted deletion of IL-10Rα (*Foxp3-Cre.Il10ra*$^{fl/fl}$) and the inability of Tr1 cells deficient in IL-10R signaling to control CD45RB^{hi} CD4 T cell transfer colitis, suggest that IL-10 may also directly act on some regulatory populations to reinforce their function [242, 243]. Antibody-mediated control of commensals is also a large part of maintaining host-microbe mutualism. IL-10 has long been known to induce B cell proliferation and plasma cell production of IgA and IgG. [244, 245] IL-10 from follicular T regulatory cells may also enhance the affinity maturation taking place in the germinal center. [246] It can also contribute to T-independent antibody formation through augmenting transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) -mediated class switching of B cells to IgA or IgG. [247] Collectively, IL-10 makes myriad contributions to intestinal homeostasis and helps avert overactive immune responses toward the microbiota.

My work has been focused on how ICOS-ICOSL signaling regulates host-commensal interactions and influences susceptibility to intestinal inflammation. Generation of these studies was heavily shaped by existing GWAS findings that a loss-of-function mutation in ICOSL correlates with human IBD. [25, 163] The first portion of my dissertation focuses on determining how loss of ICOS signaling negatively impacts Treg cell populations, with particular focus on colonic Treg cells. We determined that the Treg deficit previously described in ICOS-deficient mice is localized in the colon relative to lymphoid tissues. Our research identifies the probable cause of this reduction as insufficiency of TSDR demethylation and subsequent reduced stability of Foxp3 expression. We also

show the functional consequences of ICOS signaling on Treg function, demonstrating that ICOS-deficient Treg cells are incapable of suppressing ongoing intestinal inflammation in the context of the CD45RB^{hi} CD4 T cell transfer model of colitis. In this study, we also contest the established paradigm that ICOS is paramount for the expression of immunosuppressive cytokine IL-10 by CD4 T cells. Our research shows that IL-10 expression in the colon is unaffected, if not increased, by lack of ICOS-ICOSL signaling and Foxp3^{-} CD4 T cells have increased IL-10 production that offsets the reduced availability of Foxp3^{+} populations as IL-10 providers. The second section of this dissertation examines the impact of ICOSL-deficiency on antibody-mediated control of the colonic microbiota and concomitant increases in IL-10 production by colonic CD4 T cells. ICOSL-deficient mice exhibit impaired antibody binding to colonic commensals and components of mucus-associated bacteria. They also show increased reliance on IL-10 to stave off intestinal inflammation during both youth and adulthood. Mice with CD4-restricted IL-10 deficiency develop early-onset spontaneous colitis when also deficient in ICOSL, indicating ICOSL has suppressive functions independent of IL-10 secretion. This accelerated disease can be delayed by provision of WT milk which, in conjunction with host-provided antibodies, contributes to T-dependent antibody content in the recipient colon. Temporary depletion of IL-10 producing cells from ICOSL-deficient mice causes colitis whereas WT mice treated thusly do not exhibit disease. As such, our research identifies cooperation between IL-10 and ICOSL in the inhibition of intestinal inflammation. Collectively, the research presented in this dissertation outlines two different methods by which ICOS-ICOSL signaling reduces inclination toward colonic inflammation.

CUTTING EDGE: ICOS-DEFICIENT REGULATORY T CELLS DISPLAY NORMAL INDUCTION OF IL10 BUT READILY DOWNREGULATE EXPRESSION OF FOXP3

by

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ABSTRACT

The ICOS pathway has been implicated in the development and functions of regulatory T cells (Treg cells) including those producing IL-10. Treg cell-derived IL-10 is indispensable for the establishment and maintenance of intestinal immune homeostasis. We examined the possible involvement of the ICOS pathway in the accumulation of murine colonic Foxp3- and/or IL-10-expressing cells. We show that, ICOS deficiency does not impair induction of IL-10 by intestinal CD4 T cells, but instead triggers substantial reductions in gut-resident and peripherally-derived Foxp3+ Treg cells. ICOS deficiency is associated with reduced demethylation of Foxp3 CNS2 and enhanced loss of Foxp3. This instability significantly limits the ability of ICOS-deficient Treg cells to reverse ongoing inflammation. Collectively, our results identify a novel role for ICOS co-stimulation in imprinting the functional stability of Foxp3 that is required for the retention of full Treg cell function in the periphery.

INTRODUCTION

The B7 family of ligands expressed by antigen-presenting cells (APC) interact with the CD28 family of co-receptors expressed on T cells, delivering unique signals that were historically classified as either co-stimulatory or co-inhibitory. More recently, it has become accepted that these interactions can have mechanistic effects beyond just modulation of T cell activation. For example, signaling via T cell co-receptors including CD28, CTLA-4, herpes virus entry mediator (HVEM), programmed cell death-1 (PD-1) and the inducible-co-stimulator (ICOS) figure prominently in the development and functions of Treg cells - essential mediators of immune homeostasis (1–5).

Compared to wild type mice, Icos−/− mice harbor reduced Foxp3+ Treg cells in secondary lymphoid tissues (1, 6). Though dispensable for induction of Foxp3+, ICOS labels Treg cells with superior suppressive capacity (5) and promotes resistance of Treg cells to cell death (7). The ICOS pathway has also been implicated in the maintenance and/or functions of CD4+ effector cells at homeostasis, following antigenic challenge, and during chronic inflammation (6, 8–11). Thus, despite often being used as Treg cell marker, several of the functions of ICOS in T cell lineage maintenance also extend to effector and memory cells. Accordingly, a range of studies in animal models have positioned ICOS as a context-dependent negative or positive regulator, or even a non-factor in T cell-mediated diseases (12–14).

Loss-of-function polymorphisms in the gene encoding the ligand of ICOS (ICOSL) (15) confer susceptibility to inflammatory bowel disease but neither Icos−/− or Icosl−/− mice develop overt intestinal inflammation. This is despite many reported links between ICOSL-ICOS and CD4 T cell production of IL-10 (7, 16–19), which is critical for intestinal immune homeostasis (20, 21). Here, we show that ICOS deficiency actually results in increased induction of Il10 in colonic CD4 T cells but reduced accumulation of murine large intestinal Foxp3+ cells, including microbiota-dependent, peripheral Treg (pTreg) cells. Icos−/− Treg cells displayed reduced demethylation of Foxp3 CNS2 and preferentially down-regulated Foxp3 relative to Icos+/+ Treg cells. The extinction of Foxp3 rendered Icos−/− Treg cells incapable of reversing gut inflammation. Our study identifies ICOS as an important mediator of Foxp3 stability that is dispensable for T cell production of IL-10 in the intestine.

MATERIALS AND METHODS

Mice (C57BL/6)

CD45.1, Rag1−/−, and Icos−/−, and Icosl−/−, mice were purchased from Jackson Laboratories. Foxp3-IRES-GFP, CBir1 TCR transgenic, and Myd88−/−.Trif−/− mice were gifts from Dr. V. Kuchroo, Dr. C. Elson, and Dr. S. Michalek, respectively. 10BiT mice have been previously described (22). All mice were bred and maintained at the University of Alabama at Birmingham in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Antibodies and Flow Cytometry

The following mouse antibodies were purchased from eBioscience: PE-anti-IL-17A, FITC-anti-Foxp3, APC-anti-Helios, PE-Cy7-anti-CD4 and anti-CD11c, Biotin-anti-ICOSL. The following were purchased from BD Biosciences: PE-anti-CD103, PerCP-anti-CD90.1, and PerCP-Cy5.5-anti-CD45.1. Samples were acquired on an LSRII instrument and data was analyzed using FlowJo software.

Lamina Propria Cell Isolation

The intestines were removed, stripped of mesenteric fat and luminal contents flushed using sterile HBSS. The epithelial layer was removed by incubating in HBSS with 154 μg/L L-dithioerythreitol (DTT) and 2 μM EDTA. Remaining tissue was digested with 100 U/ml collagenase IV and 20 μg/ml DNase (Sigma) for 30 min at 37°C with gentle stirring. Total lamina propria cells were purified on a 40%/75% Percoll gradient by room temperature centrifugation at 2000 rpm with no brake for 20 min.

Analysis of DNA Methylation

Bisulfite conversion, pyrosequencing and analysis were performed by EpigenDx (Hopkinton, MA). Assays ADS568-FS1 and ADS568-FS2 were used to analyze 9 CpGs of the mouse Foxp3 CNS2 (−2369 to −2207 from the Foxp3 TSS).

T Cell Transfer Colitis

CD45RBhi T cells were FACS-purified from B6.CD45.1 splenocytes and 4×105 cells were injected into each Rag1−/− recipient. CD4+GFP+ cells were FACS sorted from Icos+/+.Foxp3gfp or Icos−/−.Foxp3gfp mice, both on the CD45.2 background. Each recipient received either PBS, 2×105 Icos+/+ GFP+, or 2×105 Icos−/− GFP+ cells on either day 0 (prevention) or day 28 (reversal). At necropsy, representative sections of proximal, middle, and distal colon were fixed in formalin, embedded in paraffin, and 5

μm sections were cut and stained with hematoxylin & eosin (H&E). Histological scoring was performed in a blinded fashion. Remaining tissue was processed to isolate lamina propria cells.

Statistical Analysis

Statistical significance was calculated by unpaired Student's t test, Mann-Whitney U or ANOVA as appropriate, using Prism software (GraphPad; San Diego, CA). All p values ≤ 0.05 are considered significant and are referred to as such in the text.

RESULTS and DISCUSSION

Reduced Accumulation of Foxp3+ Cells in the Large Intestine of Icos−/− Mice

Our preliminary analysis of splenic and intestinal CD4 T cells suggested that co-expression of ICOS is not an essential feature of IL-10-competent cells, particularly in the large intestine (Supplemental Fig. 1). To further examine this, we compared the impact of ICOS deficiency on gut Treg cells with that of an extra-intestinal tissue (spleen) and an inductive site (thymus). Consistent with previous findings, there was no difference in Foxp3+ cell frequencies in the thymus of Icos+/+ and Icos−/− mice (Figure 1, A–B) but significant reductions in splenic and large intestine (LI) Foxp3+ Treg cells in absence of ICOS. Importantly, in the LI, where we observed the greatest difference in frequency, we detected similar numbers of total CD4 T cells but significantly reduced numbers of Foxp3+ cells (Figure 1C).

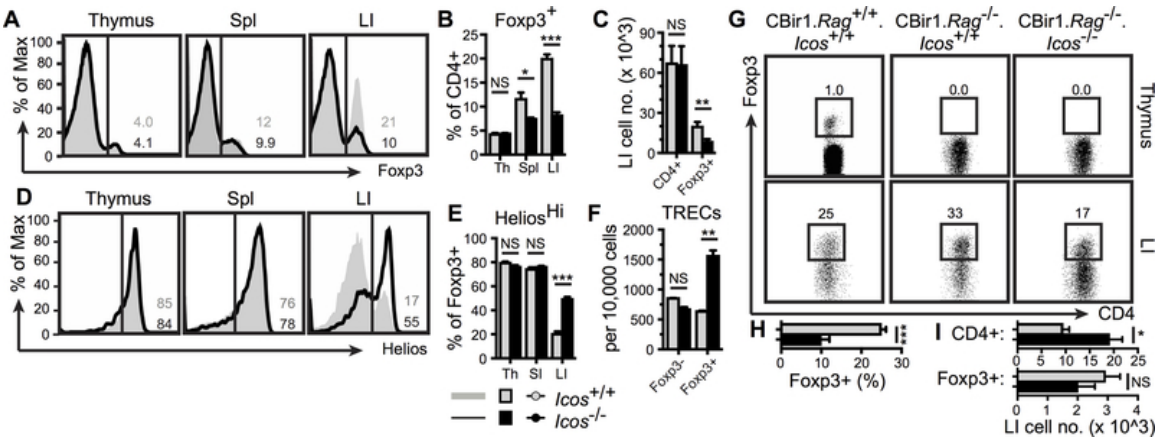

Figure 1: Altered Distribution of Colonic Foxp3+ Subsets in Absence of ICOS.
(**A**) Total CD4 single-positive T cells from the thymus, spleen (Spl), and large intestine (LI) of co-housed wild type (grey fill) and Icos−/− (black line) mice were examined for expression of Foxp3. (**B**) Graph summarizing the frequencies of Foxp3+ cells in wild type and Icos−/− mice analyzed as in A. (**C**) Graph displaying actual numbers of LI CD4+ and Foxp3+ cells. (**D**) Helios expression by Foxp3-gated cells. (**E**) Graph summarizing the frequencies of Helios+ cells among Foxp3+ cells. (**F**) TREC counts among purified splenic CD4 T cells from Icos+/+ and Icos−/− mice. (**G**) Analysis of thymic and lamina propria CD4 T cells from 6-week-old CBir1 transgenic mice. Graphs summarize frequencies of LI Foxp3+ cells (**H**) and numbers of colonic CD4+ and Foxp3+ cells (**I**) from Icos+/+ and Icos−/− CBir1.Rag−/− mice analyzed as in G. Graphs represent data from 2 (C, E) or 3 (G) similar experiments each with 3–5 mice per group. Graphs show mean +/− SEM. *p<0.05, ** p<0.01, ***p<0.001, NS=not significant.

The transcription factor Helios is expressed by the majority of thymic Treg (tTreg) cells but only a minor fraction of colonic lamina propria Treg cells, although the utility of Helios as a marker of tTreg cells remains controversial. As expected (23), in Icos+/+ mice the majority of Foxp3+ cells in the thymus and spleen co-expressed high levels of Helios, and importantly, the same was true of Icos−/− Foxp3+ cells in these 2 compartments (Figure 1, D–E). However, in the colonic lamina propria of Icos−/− mice, we detected a significantly increased representation of HeliosHi and a concomitant decrease in HeliosLo/− Foxp3+ cells (Figure 1, D–E). Analysis of T cell receptor excision circles (TREC) (24) indicated that there is a statistically-significant increase in thymic output of Treg cells in Icos−/− mice relative to wild type mice (Figure 1F), which may help explain the increase in HeliosHi cells in the lamina propria.

To confirm the impact of ICOS deficiency on definitively colonic pTreg cells and avoid the controversy surrounding the use of Helios as a marker, we employed the CBir1 T cell receptor (TCR) transgenic system (25). This strain expresses a TCR specific for CBir1 flagellin, a microbiota-derived antigen detectable in healthy mice and humans. As with most TCR transgenics, these mice can rearrange a non-transgenic (endogenous) TCR alpha chain meaning they can generate self-antigen reactive Treg cells in the thymus (Figure 1G, left panel). However, by rendering these mice deficient for the recombination-activating gene-1 (Rag1), we eliminated thymic Treg cell development (Figure 1G, center and right panels). Therefore, any Treg cell detected in the periphery of these mice is a bona fide pTreg cell. In healthy, 6–8-week-old CBir1.Rag1−/− mice, ICOS deficiency resulted in reduced numbers and frequencies of Foxp3+ LI Treg cells, despite an elevated number of total CD4+ T cells (Figure 1, H–I).

Importantly, developing pTreg cells likely receive an ICOSL signal since lamina propria CD11c+CD103+ dendritic cells express ICOSL. This expression occurs independent of the microbiota or TLR signaling, suggesting that it is developmentally regulated (Supplemental Fig. 2, A-E). The same signal is likely available to developing tTreg cells since ICOSL is also expressed by MHCII-expressing thymocytes (Supplemental Fig. 2, F-G).

Expression of Il10 by Intestinal CD4 T Cells Independent of ICOS

To determine whether the impact of ICOS on colonic Foxp3+ cells extends to IL-10-producing cells, we utilized IL-10 BAC-In transgenic (10BiT) mice in which induction of Il10 results in surface expression of Thy-1.1 (CD90.1) (22). In Icos+/+ mice, the vast majority of colonic IL-10-competent cells co-express Foxp3, with limited numbers of Foxp3-Thy1.1+ cells detected. In contrast, in Icos−/− mice, we found that the majority of IL-10-competent cells were actually Foxp3− cells (Figure 2A). Overall, ICOS deficiency led to a significant reduction in the proportion of Foxp3+Thy1.1+ cells among CD4 T cells as a direct result of reduced Foxp3. (Figure 2, A–B). However, this was counterbalanced by significant increases in Foxp3−IL-10+ cells. The net effect was that the total expression of IL-10 by CD4 T cells, as determined by cell frequencies or fluorescence intensity of Thy1.1 expression, was not diminished (Figure 2, A–C).

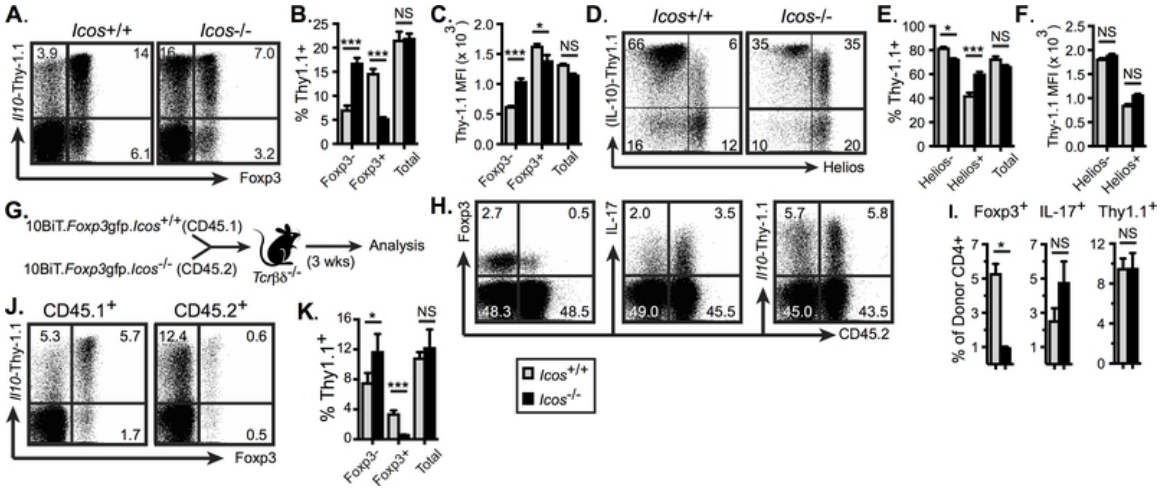

Figure 2: Induction of Il10 in Intestinal CD4 T cells Independent of ICOS. (**A**) LI lamina propria CD4 T cells from 10BiT and 10BiT.Icos−/− mice were examined for co-expression of Thy1.1 and Foxp3. (**B** and **C**) Graphs summarizing frequencies and MFI respectively of Thy1.1+ cells among Foxp3−, Foxp3+, and total CD4 T cells as shown in A. (**D**) LI lamina propria Foxp3-gated CD4 T cells from 10BiT and 10BiT.Icos−/− mice were examined for co-expression of Helios and Thy1.1. (**E** and **F**) Graphs summarizing frequencies and MFI of Thy1.1+ cells among Helios−, Helios+, and total Foxp3+ T cells. (**G**) Schematic overview of thymocyte transfer experiment. CD4 single-positive Foxp3− thymocytes were FACS-sorted from congenically-marked WT (CD45.1) and Icos−/− (CD45.2) 10BiT.Foxp3 mice and transferred to Tcrβδ−/− recipients. After 3 weeks, donor CD4+TCRβ+ cells from the LI lamina propria were analyzed by FACS. (**H**) Analysis of Foxp3, IL-17 and Thy1.1 expression by donor T cells 3 weeks after transfer. (**I**) Graphs summarizing frequencies of the various cell populations from all mice analyzed as in H. (**J**) Analysis of Foxp3 and Thy1.1 expression by wild type and Icos−/− T cells recovered from the LI lamina propria. (**K**) Graphs summarizing frequencies of the various cell populations from mice analyzed as in J. Graphs represent data pooled from 1 of 2 similar experiments with 3–5 mice per group and display mean + SEM. *p<0.05, ***p<0.001, NS=not significant.

Consistent with previous results (26), the majority of colonic Foxp3+IL-10+ cells in wild type mice were HeliosLo/−. However, Icos−/− mice displayed a substantial population of Helios-expressing Foxp3+IL-10+ cells (Figure 2D). Thus, despite the reduced frequency of Foxp3+ cells in Icos−/− mice, independent of Helios expression, the proportion that expressed IL-10 and the levels of IL-10 expression remained largely unchanged relative to Icos+/+ mice (Figure 2, D–F). To eliminate any possible host-intrinsic effects on the foregoing results, we performed adoptive transfer of CD4 single-positive, Foxp3- thymocytes from congenically-marked 10BiT.Foxp3gfp.Icos+/+ (CD45.1) and 10BiT.Foxp3gfp.Icos−/− (CD45.2) mice into the same T cell-deficient (Tcrβδ−/−) recipients (Figure 2G). After 3 weeks, despite similar frequencies of colonic CD4 T cells derived from each source, the frequency of Icos+/+ Foxp3+ cells was approximately 5 times that of Icos−/− and conversely, IL-17 frequencies were elevated in the latter. The frequency of IL-10-competent CD4 T cells was similar in both Icos+/+ and Icos−/− cells (Figure 2, H–I) and there was a significant increase of Foxp3−IL-10+ cells among Icos−/− colonic CD4 T cells, collectively mimicking intact mice (Figure 2, J–K).

Interestingly, among lamina propria CD4 T cells, the frequencies of IL-10-competent cells actually increased with aging (Supplemental Fig. 3, A-B), suggesting a potentially compensatory role for IL-10 in the face of the colonic Treg cell deficits in Icos−/− mice. Collectively, these results argue that ICOS is dispensable for induction of Il10 in intestinal Treg cells.

ICOS-Deficient Treg Cells Display Robust Methylation of Foxp3 CNS2 and Preferentially Downregulate Foxp3 Ex Vivo and in Vivo

The intensity of Thy1.1 expression by Foxp3− cells in the LI lamina propria of Icos−/− mice strongly resembled that of LI Foxp3+Thy1.1+ (Figure 2A), raising the possibility that some of the Icos−/− Foxp3-Thy1.1+ cells were 'ex-Foxp3' cells. To determine whether ICOS impacts the stability of Foxp3, we conducted pyrosequencing analysis of conserved non-coding sequence 2 (CNS2) of the Foxp3 locus, which is demethylated in Treg cells that stably express Foxp3 (27). In Icos−/− Treg cells, there was significant methylation of Foxp3 CNS2 relative to Icos+/+ cells. In fact, the methylation levels closely resembled that of naïve T cells (Figure 3A). To confirm the instability of Icos−/− Foxp3+ cells, we FACS-sorted CD4+GFP+ cells from wild type (CD45.1) and Icos−/− (CD45.2) Foxp3gfp reporter mice (Figure 3B, left panel) and co-cultured equal numbers in the presence of IL-2, anti-CD3 and anti-CD28. On day 3, the majority of CD45.2+ cells were Foxp3−, in contrast to cells of wild type origin that were still mostly Foxp3+ (Figure 3B, right panel). Neither a cocktail of pro-inflammatory cytokines IL-1β, IL-6, IL-12, and IL-23 (stim + cytokines) nor blocking antibodies targeting IL-6R, IL-12/23p40, and IL-21R (stim + blockade) had any major impact on the Foxp3 loss by Icos−/− T cells (Figure 3B). Altogether, these data argue that ICOS helps to imprint stable expression of Foxp3 mainly by promoting demethylation of Foxp3 CNS2. The highly methylated CNS2 leading to the rapid downregulation of Foxp3 may help to explain why the elevated thymic output of Foxp3+ cells (Figure 1F) is incapable of restoring to wild type levels the numbers of Foxp3+ cells in the spleen and especially the large intestine.

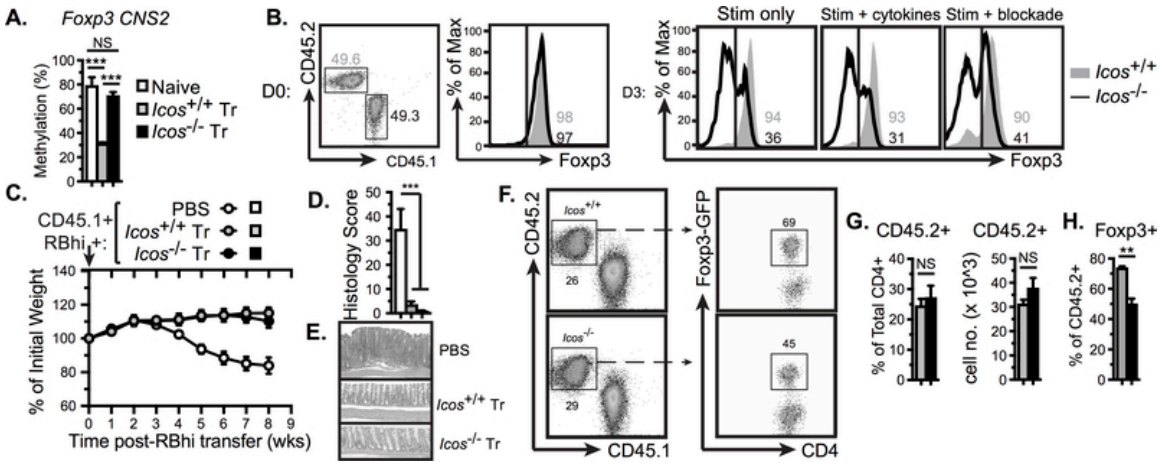

Figure 3: ICOS-Deficient Treg Cells Display Robust Methylation of Foxp3 CNS2 and Readily Downregulate Foxp3 Ex Vivo and In Vivo. (**A**). Graph shows average methylation of 9 CpG sites of Foxp3 CNS2 as determined by pyrosequencing of purified CD4+GFP+ cells from Icos+/+ and Icos−/− Foxp3gfp mice. (**B**) Purified CD4+GFP+ cells from the WT (CD45.1) and Icos−/− (CD45.2) Foxp3gfp mice were co-cultured with anti-CD3 and anti-CD28 plus IL-2 and IL-7. IL-1β, IL-6, IL-12, and IL-23 (stim + cytokines), or anti-IL-6R, anti-IL-12/23p40, and anti-IL-21R (stim + blockade) were added to select wells. Expression of Foxp3 was examined on Day 3. (**C**) Rag1−/− mice received naïve CD4+ CD45RBhi T cells purified from B6.CD45.1 mice. Mice in each cage were then randomly assigned to 1 of 3 groups and received either vehicle (PBS), or identical doses of wild type or Icos−/− Treg cells, each on the CD45.2 background. Mice were weighed weekly until they were euthanized at week 8. (**D** and **E**) Histology scores and representative photomicrographs of H&E-stained colonic tissues from mice in the 3 recipient groups, 10X magnification. (**F**) FACS analysis of LI CD4+ cells (left panel) and of Foxp3+ cells among CD45.2+ cells (right panel). Graphs summarize relative frequencies and numbers of LI CD45.2+ cells (**G**) and frequencies of Foxp3+ among CD45.2+ analyzed as in F (**H**). Bar graphs display mean + SEM and represent data from 1 of 2 (A) or 1 of 3 (D, G, H) similar experiments. **p<0.01, ***p<0.001, NS, not significant.

We then decided to definitively examine the functional consequence of this instability in vivo, first under homeostatic conditions. We employed the T cell co-transfer model of colitis and injected equal numbers of CD45.2+ wild type or Icos−/− Foxp3+ cells into Rag1−/− recipients that simultaneously received CD45.1+ naïve CD45RBhi T cells. As expected, naïve cells alone induced severe weight loss and colonic inflammation (Figure 3, C–E). However, as previously shown (28), both Icos+/+ and Icos−/− Treg cells prevented the development of colitis. Despite similarly-sized donor CD45.2 fractions of Icos+/+ and Icos−/− origin, we detected significantly reduced frequencies of the latter that still expressed Foxp3 (Figure 3, F–H). Thus, Icos−/− Treg cells were able to inhibit the development of colitis, despite the enhanced loss of Foxp3. In longer term analyses under similarly homeostatic conditions, we examined the ability of Icos−/− Treg cells to prevent the spontaneous autoimmunity and premature death experienced by Foxp3−/− mice. Our results showed that Icos−/− Treg cells could temporarily rescue Foxp3−/− mice but preferentially lost expression of Foxp3 and ultimately failed to promote long-term survival, in contrast to Icos+/+ Treg cells (Supplemental Fig. 4).

ICOS-Deficient Treg cells are Unable to Reverse Ongoing Colitis

To determine the potential consequences of Icos−/− Treg cell instability during inflammation, we first induced colitis in recipient mice then transferred equal numbers of wild type or Icos−/− Treg cells. In this setting, Icos−/− Treg cells failed to prevent the wasting disease characteristic of mice that did not receive a secondary Treg cell transfer (Figure 4A). In contrast, recipients of Icos+/+ Treg cells were rescued from disease, as

further confirmed by histological analysis (Figure 4, B–C). Furthermore, the failure of Icos−/− Treg cells to mitigate the ongoing inflammation in recipient mice correlated with their almost complete loss of Foxp3 expression, reflected in reduced frequencies and numbers of Icos−/− Foxp3+ cells (Figure 4, D–E), despite similar numbers of cells of CD45.2 origin (Figure 4F). These results provide compelling evidence that ICOS imprints Treg stability that is particularly important for Treg cell function during inflammation.

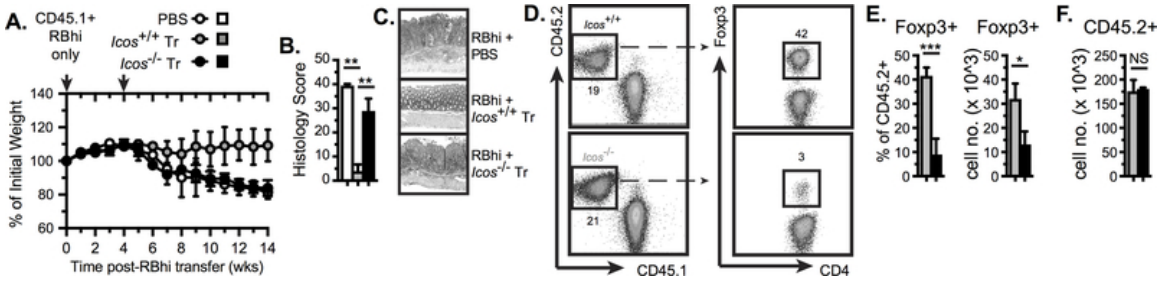

Figure 4: Downregulation of Foxp3 Correlates with the Inability of ICOS-Deficient Treg Cells to Reverse Ongoing Inflammation. (**A**) Rag1−/− mice were injected with CD45.1+ CD45RBhi T cells as in Figure 3 and monitored for 4 weeks. At week 4, mice were randomly assigned to 1 of 3 groups that received PBS, or identical numbers of CD45.2+ of wild type or Icos−/− Treg cells. Mice were weighed weekly until week 14. (**B** and **C**) Histology scores and representative photomicrographs of H&E-stained colonic tissues from mice in the 3 recipient groups, 10X magnification. (**D**) Representative plots depicting relative frequencies of CD4+ cells of Treg origin (CD45.2+) remaining at the end of the experiment (left panel) and the percentage of CD45.2+ cells that still expressed Foxp3 (right panel). (**E**) Bar graphs displaying relative Foxp3+ cell frequencies and numbers of Foxp3+ cells from all mice analyzed as in D. (**F**) Graph shows numbers of cells of CD45.2 origin recovered from the LI or recipient mice. All data are from 1 of 2 replicate experiments each with 5 recipients per group. Graphs show mean +/− SEM. ** p<0.01, ***p<0.001, NS=not significant.

In this study we identified a novel role for ICOS signaling in imprinting the epigenetic stability of Foxp3+ Treg cells, with no impairment in induction of Il10 in gut CD4 T cells. Despite this novel role, Icos−/− mice retain a sizeable pool of Treg cells and under specific pathogen-free housing conditions, do not succumb to the spontaneous autoimmunity characteristic Foxp3-deficient mice. This may be explained by (1) the thymic output of Foxp3+ cells even in aged mice, and (2) the increase in IL-10-producing cells throughout life. However, during inflammation, this instability of Foxp3 produces detrimental consequences for the host. Our data, together with recent findings of a role for PD-1 signaling in stabilizing induced Treg cells (4) identify yet another contribution of T cell co-receptors in imprinting the long-term fate of Treg cells. Ultimately, these discoveries will present unique opportunities to target these pathways in cell-based treatment of chronic inflammatory diseases.

ACKNOWLEDGEMENTS

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REFERENCES

1. Guo F, Iclozan C, Suh WK, Anasetti C, and Yu XZ. 2008. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. J Immunol 181: 2285–2291.

2. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, and Sakaguchi S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. Science 322: 271–275.

3. Jones A, Bourque J, Kuehm L, Opejin A, Teague RM, Gross C, and Hawiger D. 2016. Immunomodulatory Functions of BTLA and HVEM Govern Induction of Extrathymic Regulatory T Cells and Tolerance by Dendritic Cells. Immunity 45: 1066–1077.

4. Stathopoulou C, Gangaplara A, Mallett G, Flomerfelt FA, Liniany LP, Knight D, Samsel LA, Berlinguer-Palmini R, Yim JJ, Felizardo TC, Eckhaus MA, Edgington-Mitchell L, Martinez-Fabregas J, Zhu J, Fowler DH, van Kasteren SI, Laurence A, Bogyo M, Watts C, Shevach EM, and Amarnath S. 2018. PD-1 Inhibitory Receptor Downregulates Asparaginyl Endopeptidase and Maintains Foxp3 Transcription Factor Stability in Induced Regulatory T Cells. Immunity 49: 247–263 e247.

5. Kornete M, Sgouroudis E, and Piccirillo CA. 2012. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. J Immunol 188: 1064–1074.

6. Burmeister Y, Lischke T, Dahler AC, Mages HW, Lam KP, Coyle AJ, Kroczek RA, and Hutloff A. 2008. ICOS controls the pool size of effector-memory and regulatory T cells. J Immunol 180: 774–782.

7. Redpath SA, van der Werf N, Cervera AM, MacDonald AS, Gray D, Maizels RM, and Taylor MD. 2013. ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production during helminth infection. Eur J Immunol 43: 705–715.

8. Lischke T, Hegemann A, Gurka S, Vu Van D, Burmeister Y, Lam KP, Kershaw O, Mollenkopf HJ, Mages HW, Hutloff A, and Kroczek RA. 2012. Comprehensive analysis of CD4+ T cells in the decision between tolerance and immunity in vivo reveals a pivotal role for ICOS. J Immunol 189: 234–244.

9. Smigiel KS, Richards E, Srivastava S, Thomas KR, Dudda JC, Klonowski KD, and Campbell DJ. 2014. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. J Exp Med 211: 121–136.

10. Moore TV, Clay BS, Ferreira CM, Williams JW, Rogozinska M, Cannon JL, Shilling RA, Marzo AL, and Sperling AI. 2011. Protective effector memory CD4 T cells depend on ICOS for survival. PloS one 6: e16529.

11. Zhang M, Wu Y, Bastian D, Iamsawat S, Chang J, Daenthanasanmak A, Nguyen HD, Schutt S, Dai M, Chen F, Suh WK, and Yu XZ. 2018. Inducible T-Cell Co-Stimulator Impacts Chronic Graft-Versus-Host Disease by Regulating Both Pathogenic and Regulatory T Cells. Front Immunol 9: 1461.

12. Dong H, Zhu G, Tamada K, and Chen L. 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 5: 1365–1369.

13. Iwai H, Kozono Y, Hirose S, Akiba H, Yagita H, Okumura K, Kohsaka H, Miyasaka N, and Azuma M. 2002. Amelioration of collagen-induced arthritis by blockade of inducible costimulator-B7 homologous protein costimulation. J Immunol 169: 4332–4339.

14. Hawiger D, Tran E, Du W, Booth CJ, Wen L, Dong C, and Flavell RA. 2008. ICOS mediates the development of insulin-dependent diabetes mellitus in nonobese diabetic mice. J Immunol 180: 3140–3147.

15. Hedl M, Lahiri A, Ning K, Cho JH, and Abraham C. 2014. Pattern recognition receptor signaling in human dendritic cells is enhanced by ICOS ligand and modulated by the Crohn's disease ICOSLG risk allele. Immunity 40: 734–746.

16. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, Berry G, DeKruyff RH, and Umetsu DT. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. Nat Med 8: 1024–1032.

17. Kohyama M, Sugahara D, Sugiyama S, Yagita H, Okumura K, and Hozumi N. 2004. Inducible costimulator-dependent IL-10 production by regulatory T cells specific for self-antigen. Proc Natl Acad Sci U S A 101: 4192–4197.

18. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, Qin FX, Gilliet M, and Liu YJ. 2008. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity 28: 870–880.

19. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, Sharpe AH, Karp CL, Miaw SC, Ho IC, and Kuchroo VK. 2009. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol 183: 797–801.

20. Roers A, Siewe L, Strittmatter E, Deckert M, Schluter D, Stenzel W, Gruber AD, Krieg T, Rajewsky K, and Muller W. 2004. T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. J Exp Med 200: 1289–1297.

21. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR Jr., Muller W, and Rudensky AY. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28: 546–558.

22. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, and Weaver CT. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. Nature immunology 8: 931–941.

23. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, and Shevach EM. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 184: 3433–3441.

24. Sempowski GD, Gooding ME, Liao HX, Le PT, and Haynes BF. 2002. T cell receptor excision circle assessment of thymopoiesis in aging mice. Mol Immunol 38: 841–848.

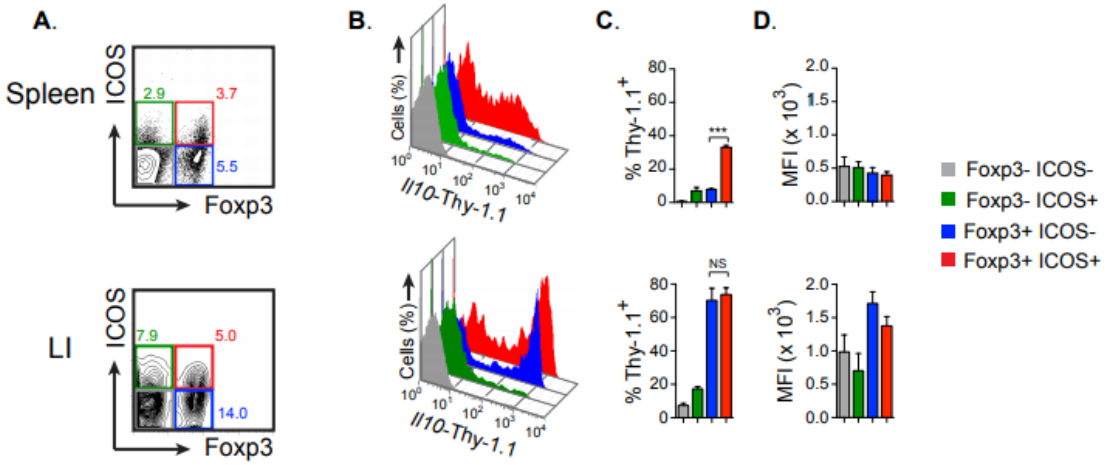
25. Cong Y, Feng T, Fujihashi K, Schoeb TR, and Elson CO. 2009. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc Natl Acad Sci U S A 106: 19256–19261.

26. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, and Honda K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. Science 331: 337–341.

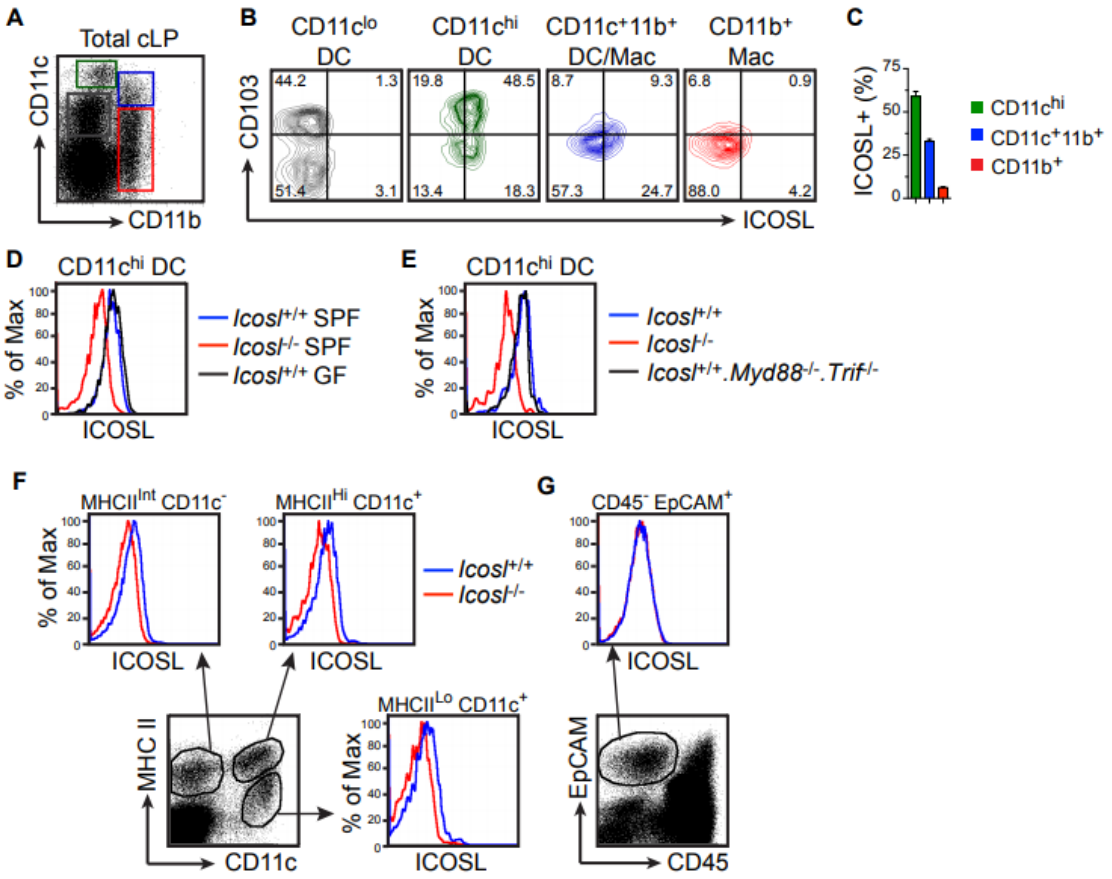
27. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, and Rudensky AY. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 463: 808–812.

28. de Jong YP, Rietdijk ST, Faubion WA, Abadia-Molina AC, Clarke K, Mizoguchi E, Tian J, Delaney T, Manning S, Gutierrez-Ramos JC, Bhan AK, Coyle AJ, and Terhorst C. 2004. Blocking inducible co-stimulator in the absence of CD28 impairs Th1 and CD25+ regulatory T cells in murine colitis. Int Immunol 16: 205–213.

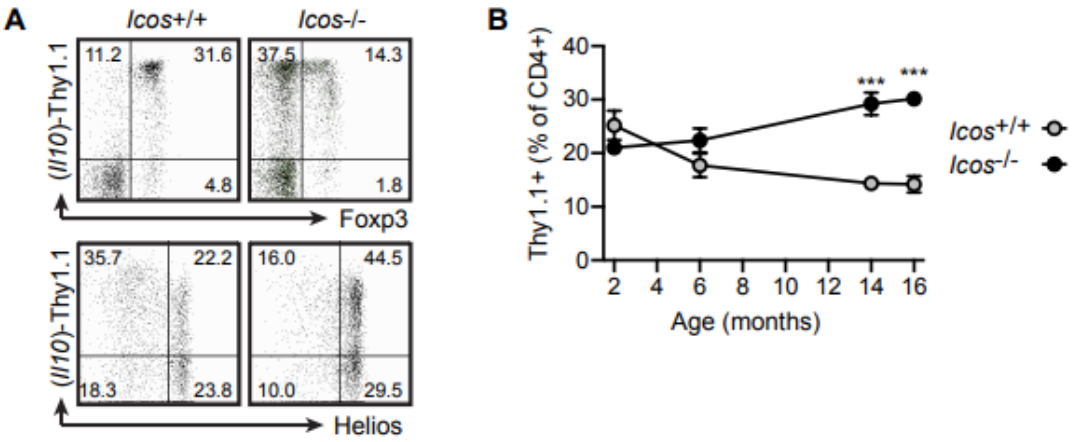
SUPPLEMENTARY FIGURES



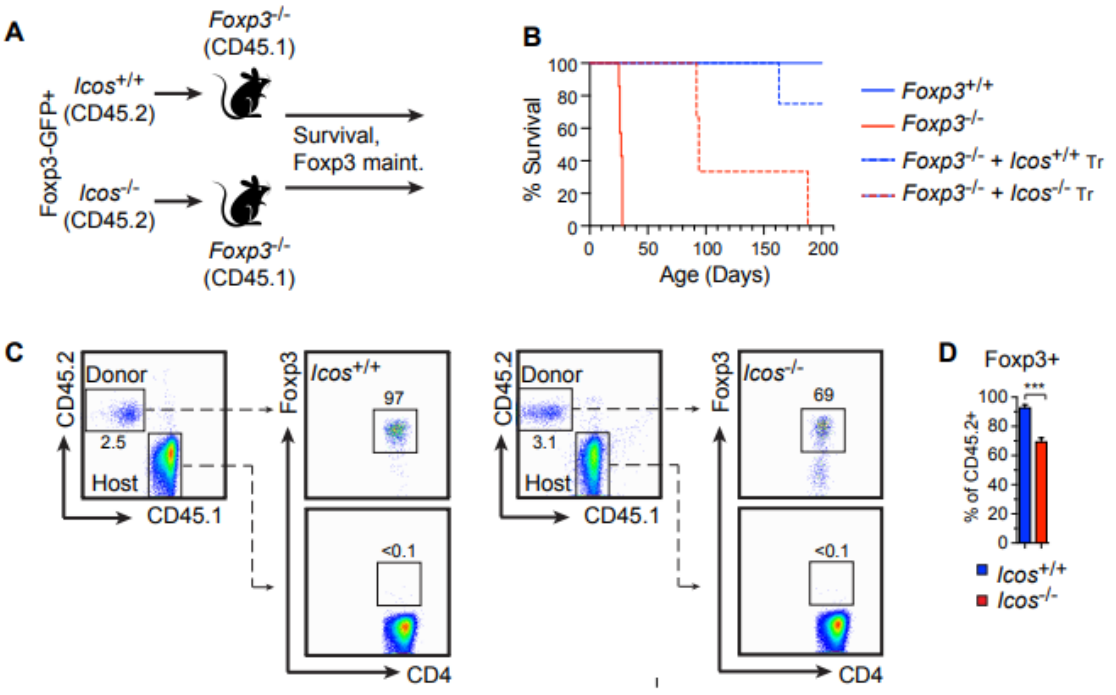
Supplemental Fig. 1: Co-expression of ICOS is not a Feature of All of IL-10-Competent Intestinal CD4 T cells. (**A**) Total CD4 T cells from the spleen and colonic lamina propria of IL-10 reporter transgenic (10BiT) mice were stained for ICOS and Foxp3 and gated into 4 populations based on expression of these markers. Numbers indicate the frequency of cells in the given quadrant. (**B**) each subset in (A) was further examined for expression of Thy1.1. (**C** and **D**). Bar graphs summarizing frequencies of Thy1.1+ cells, and mean fluorescence intensity of Thy1.1 among the indicated subsets of cells. Graphs show mean+/-SEM, n=5 mice per group. Results are from 1 of 3 experiments with similar results.



Supplemental Fig. 2: ICOSL is Constitutively Expressed by Mucosal Antigen Presenting Cells, Independent of the Microbiota and TLR Signaling, and by Thymic MHC-II-Expressing Cells. (**A**) Surface expression of CD11c and CD11b was used to distinguish 4 populations of colonic lamina propria myeloid cells – CD11clo DC (grey), CD11chi DC (green), CD11c+CD11b+ (blue) and CD11b+ macrophages (red). (**B**) Expression of ICOSL and CD103 by the 4 subsets demarcated in A. (**C**) Graph summarizing frequencies of ICOSL+ cells in the indicated APC subset. (**D**) FACS comparison of ICOSL expression by colonic CD11chi DC from ICOSL-sufficient, specific pathogen-free (SPF, blue) and germ-free (GF, black) mice, with ICOSL-deficient SPF mice (red) serving as negative controls. (**E**) Comparison of ICOSL expression by colonic CD11chi DC from ICOSL sufficient mice that are either wild type (blue) or deficient (black) in both MyD88 and TRIF. (**F**) Total viable thymocytes were enriched from wild type (blue) of ICOSL-deficient (red) mice and examined for expression of MHCII, CD11c, and ICOSL. (**G**) Examination of ICOSL expression by thymic EpCAM+CD45- epithelial cells. All data are from one of 2-3 experiments with at least 4 mice per group.



Supplemental Fig. 3: Increasing Expression of IL-10 Among ICOS-Deficient CD4 T Cells with Aging. (**A**) Flow cytometric analysis of Foxp3 and IL-10 reporter expression by total CD4 T cells (upper) and of Helios and IL-10 reporter expression by Foxp3-gated T cells (lower) in the LI lamina propria of 16-month-old Icos+/+ and Icos-/- 10BiT mice. Data are from one of 5 mice per group and were collected in one of 2 similar experiments. (**B**) Graph summarizing frequencies of Thy1.1+ cells among lamina propria CD4 T cells in mice between 2 and 16 months of age. Each data point represents 6-11 mice per group. ***$p<0.001$.



Supplemental Fig. 4: ICOS-Deficient Treg Cells Eventually Fail to Prevent Death of Foxp3-Deficient Mice. (**A**) Identical numbers of purified Foxp3-GFP+ Treg cells were transferred into congenically-marked (CD45.1) Foxp3-/-mice on postnatal day 2 and recipient survival was monitored. (**B**) Survival curve for Foxp3+/+ (solid blue line), Foxp3-/- (solid red line) and Foxp3-/- mice transplanted with wild type (dashed blue line) or ICOSdeficient (dashed red line)Treg cells on postnatal day 2. As expected, untreated Foxp3-/- mice succumbed to lethal autoimmunity by 4 weeks of age while Foxp3+/+ mice survived for the duration of the experiments. In all experiments, all Foxp3-/- recipients of Icos-/- Treg cells eventually succumbed. (**C**) At postnatal day 105 (15 weeks of age) we examined peripheral blood CD4+ cells from surviving mice for expression of Foxp3. Importantly, ICOS-deficiency did not result in reduced frequencies of donor CD45.2+ T cells relative to ICOS-sufficient cells. (**D**) Graphical summary of Foxp3+ cell frequencies among donor CD45.2+ cells in all mice examined. Data in graphs are pooled from 3 experiments for a total of 8-13 mice per group.

ICOS LIGAND AND IL-10 SYNERGIZE TO PROMOTE HOST-MICROBIOTA MUTUALISM

by

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Proceedings of the National Academy of Sciences

Format adapted and errata corrected for dissertation

SIGNIFICANCE

Both *IL10* and ICOSL are risk alleles for inflammatory bowel disease (IBD). The role of IL-10 in preventing gut inflammation is well established experimentally and clinically. However, it is unclear whether and how ICOSL functions in the intestines, and how it might impact susceptibility to gut inflammation. We found that in mice, the absence of ICOSL is associated with increased accumulation of IL-10–producing CD4 T cells but dramatic reductions in anti-commensal antibodies, resulting in limited recognition of antigens implicated in the progression of IBD. Simultaneous disruption of both pathways, either genetically or transiently, resulted in colitis. Therefore, we have identified an IBD-relevant axis in intestinal immune regulation predicated on the cooperative functions of ICOSL and CD4 T cell–derived IL-10.

ABSTRACT

Genome-wide association studies have identified *ICOSLG*, which encodes the inducible costimulator ligand (ICOSLG or ICOSL) as a susceptibility locus for inflammatory bowel disease. ICOSL has been implicated in the enhancement of pattern recognition receptor signaling in dendritic cells, induction of IL-10 production by CD4 T cells, and the generation of high-affinity antibodies to specific antigens—all of which can potentially explain its involvement in gastrointestinal inflammation. Here, we show that murine ICOSL deficiency results in significant enrichment of IL-10–producing CD4 T cells particularly in the proximal large intestine. Transient depletion of IL-10–producing cells from adult ICOSL-deficient mice induced severe colonic inflammation that was prevented when mice were first treated with metronidazole. ICOSL-deficient mice displayed reduced IgA and IgG antibodies in the colon mucus and impaired serum antibody recognition of microbial antigens, including flagellins derived from mucus-associated bacteria of the *Lachnospiraceae* family. Confirming the synergy between ICOSL and IL-10, ICOSL deficiency coupled with CD4-specific deletion of the *Il10* gene resulted in juvenile onset colitis that was impeded when pups were fostered by ICOSL-sufficient dams. In this setting, we found that both maternally acquired and host-derived antibodies contribute to the life anti-commensal antibody repertoire that mediates this protection in early life. Collectively, our findings reveal a partnership between ICOSL-dependent anti-commensal antibodies and IL-10 in adaptive immune regulation of the

microbiota in the large intestine. Furthermore, we identify ICOSL deficiency as an effective platform for exploring the functions of anti-commensal antibodies in host–microbiota mutualism.

INTRODUCTION

The establishment and maintenance of immune homeostasis with the diverse community of microbes that inhabit the intestine is a dynamic process involving multiple cellular and molecular mediators. A major player in this process is the immunoregulatory cytokine interleukin-10 (IL-10), a dominant functional product of intestinal CD4+ T regulatory (Treg) cells at steady state. Germline deletion of *Il10*, targeted ablation in CD4 T cells (1), or just Foxp3+ cells (2), but not myeloid cells (3–6), can result in spontaneous adult-onset inflammation. *IL10* has been identified by genome-wide association studies as a risk allele for both Crohn's disease and ulcerative colitis, the two major types of inflammatory bowel disease (IBD). Moreover, humans with impaired expression of the receptor for IL-10 develop severe pediatric onset IBD (7). Thus, because of its critical role in gut immune regulation, elucidation of the molecular mechanisms that intersect the IL-10 pathway, and the circumstances surrounding those intersections, can identify avenues whereby host–microbiota mutualism is achieved and/or can potentially be reinforced to prevent gut inflammation.

The inducible T cell costimulator ligand (ICOSL) is a member of the B7 family of molecules that is constitutively expressed on antigen-presenting cells including B cells and dendritic cells (8–10). Via its interaction with the T cell–expressed ICOS receptor, ICOSL is able to modulate T cell activation and differentiation (11, 12). In humans,

partial loss-of-function polymorphisms at chromosome 21q22, which encompasses *ICOSLG* (the gene encoding ICOSL), have also been associated with IBD as well as celiac disease—immune-mediated chronic inflammatory disorders of the gastrointestinal tract (13–15). One such IBD-associated polymorphism contributes to impaired dendritic cell production of proinflammatory cytokines in response to pathogen recognition receptor stimulation (16), demonstrating that impaired ICOSL function can adversely affect microbiota-induced innate immune responses.

ICOSL is also critical for the successful interaction of B and T cells in response to antigenic challenge. Specifically, during the germinal center reaction, B cell–expressed ICOSL interacts with ICOS on T follicular helper (Tfh) cells, promoting the retention of Tfh cells in the late germinal center reaction. In the absence of this interaction, developing Tfh cells lose their lineage-specific phenotype and leave the germinal center, eliminating T cell help in antibody production (17, 18). As evidence of the importance of this signal, complete deficiency of ICOSL or ICOS in humans results in combined immunodeficiency involving recurrent bacterial and viral infection and abnormal B cell maturation (19, 20).

Unlike in humans, ICOSL deficiency is largely inconsequential in laboratory mice maintained under specific pathogen-free conditions and thus “naïve” to the types of antigens encountered by humans. However, even in this state of restricted antigen exposure, antibody responses are still an essential component of the immune apparatus that enables peaceful coexistence with the microorganisms that inhabit barrier sites such as the intestines. These antibodies can be either broadly reactive, thymus (T)-independent

immunoglobulins recognizing conserved bacterial moieties or high-affinity, antigen-specific antibodies, the generation of which requires T cell help (21–28). In experimental settings, the importance of T cells for antibody responses is usually based on whether such responses exist, or can be induced, in T cell-deficient mice (22, 26, 27, 29). Given the role of ICOSL in Tfh retention, the T-dependent anti-commensal antibody repertoire of ICOSL-deficient mice at steady state should resemble that of T cell–deficient mice. Moreover, ICOSL-deficient mice also retain much of the CD4 T cell compartment, including the various subsets of mucosal effector and regulatory T cells known to participate in intestinal immune tolerance (30–32). Arguably, this makes murine ICOSL deficiency a superior model with which to study the functional positioning of T-dependent anti-commensal antibodies within the larger immunoregulatory network of the gut.

Here we show that at homeostasis, and similar to ICOS-deficient mice (33), ICOSL-deficient mice harbor increased frequencies and numbers of IL-10–producing CD4 T cells particularly in the proximal colon lamina propria. Strikingly, transient depletion of IL-10–producing cells resulted in rapid onset of severe colonic inflammation that was also largely restricted to the proximal colon. Conversely, we observed significantly diminished colon-associated Tfh cells and IgA+ and IgG+ plasma cells as well as serum- and mucus-associated IgA and IgG in ICOSL-deficient mice, which suggest that the elevated IL-10 is induced to counterbalance these antibody deficits. In support of this, ICOSL-deficient mice displayed reduced serum antibody recognition of multiple bacterial antigens including flagellin antigens derived from several members of the family *Lachnospiraceae*, which are known to enrich within the colon mucus layer

(34–36). Furthermore, CD4-specific ablation of *Il10* coupled with germline deletion of *Icosl* predisposed to early onset intestinal inflammation that was delayed when the mutant pups was fostered by ICOSL-sufficient dams. Importantly, both maternally transmitted and host-derived ICOSL-dependent antibodies appear to be involved in limiting this early-life reactivity to the microbiota. Collectively, our data identify a synergy between two IBD-related pathways—T cell–derived IL-10 and ICOSL-dependent anti-commensal antibodies—that promotes mutualism with the gut microbiota.

RESULTS

Transient Depletion of IL-10–Producing Cells from Adult ICOSL-Deficient Mice Results in Colonic Inflammation.

We previously reported that deficiency of ICOS, the T cell–expressed receptor for ICOSL, impairs the functional stability of Foxp3+ Treg cells (33). Surprisingly, we have found that ICOSL-deficient mice harbor wild-type (WT) levels of colonic Foxp3-expressing cells (SI Appendix, Fig. S1 A–C). Importantly, using our IL-10 reporter transgenic (10BiT) mice (37), we noticed that similar to ICOS-deficient mice, ICOSL-deficient mice displayed significant increases in colonic IL-10–producing cells and overall IL-10 protein output of total lamina propria CD4 T cells (SI Appendix, Fig. S1 A and D–F). Therefore, independent of any effect on Treg cells, ICOSL–ICOS pathway deficiency is associated with increased IL-10 in the colon. Importantly, the differences in IL-10–producing cells did not appear to be due to microbiota differences as analysis of the fecal microbiota of WT and *Icosl*$^{-/-}$ cohorts revealed no major compositional differences (SI Appendix, Fig. S2).

To begin to explore the significance of the increased IL-10 that emerges in the absence of ICOSL, we took advantage of a key feature of the 10BiT mice—surface expression of the Thy1.1 reporter molecule (37). This allows for transient depletion of IL-10–expressing cells from healthy adult mice, enabling strict temporal control of the IL-10 disruption. 10BiT.*Icosl*$^{+/+}$ or 10BiT.*Icosl*$^{-/-}$ mice received intraperitoneal injections

of anti-Thy1.1 or vehicle (phosphate-buffered saline [PBS]) every 5 d for 15 d. Body weight and fecal Lcn-2 were measured every 5 d, and mice were euthanized for analysis of colitis indices on day 25 (Fig. 1A). Anti-Thy1.1 treatment resulted in efficient depletion of IL-10–competent cells from the colonic lamina propria (Fig. 1 B and C). Interestingly, in 10BiT.*Icosl*$^{-/-}$ mice, the initial re-emergence of IL-10–competent cells could be seen by 5 d post antibody treatment, whereas 10BiT.*Icosl*$^{+/+}$mice remained fully depleted (Fig. 1 B and C), supporting the notion of a constant and increased demand for IL-10 production in the absence of ICOSL. Accordingly, anti-Thy1.1–treated 10BiT.*Icosl*$^{-/-}$ mice rapidly developed signs of inflammation including diminished weight gain and a rapid spike in fecal Lcn-2 even after a single injection of anti-Thy1.1 (Fig. 1 D and E). In contrast, nondepleted 10BiT.*Icosl*$^{-/-}$ and 10BiT.*Icosl*$^{+/+}$mice as well as depleted 10BiT.*Icosl*$^{+/+}$mice continued to gain weight and their fecal Lcn-2 levels remained low (Fig. 1 D and E). The development of colitis in depleted 10BiT.*Icosl*$^{-/-}$ mice was confirmed by histological evaluation on day 25 and revealed that colitis was predominantly confined to the proximal colon (Fig. 1 F–H).

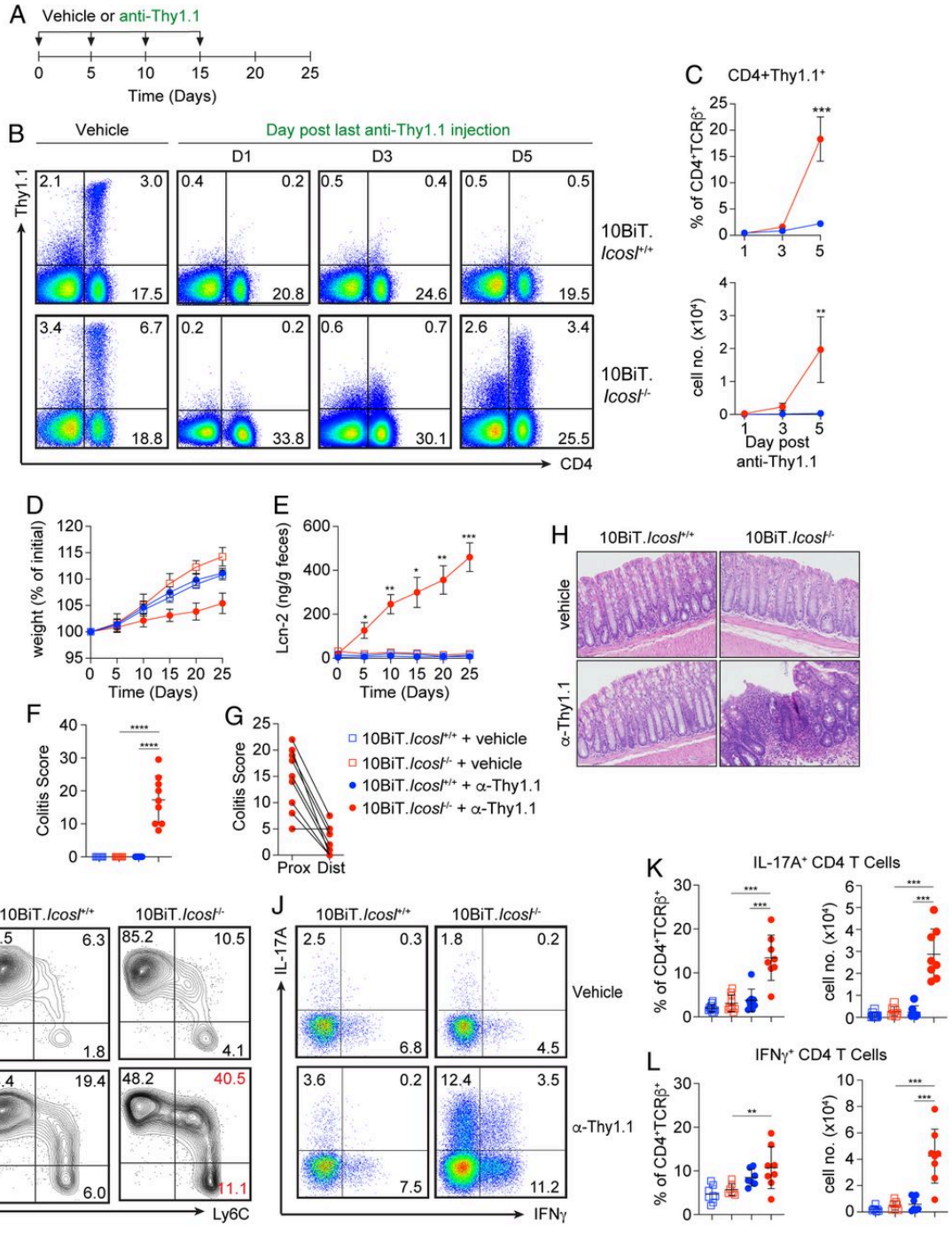
A
Vehicle or anti-Thy1.1
0 5 10 15 20 25
Time (Days)
B
Vehicle
Day post last anti-Thy1.1 injection
D1
D3
D5
Thy1.1
CD4
10BiT. Icosl+/+
10BiT. Icosl-/-
2.1 3.0 17.5
0.4 0.2 20.8
0.5 0.4 24.6
0.5 0.5 19.5
3.4 6.7 18.8
0.2 0.2 33.8
0.6 0.7 30.1
2.6 3.4 25.5
C
CD4+Thy1.1+
% of CD4+TCRβ+
cell no. (x10⁴)
Day post anti-Thy1.1
D
weight (% of initial)
Time (Days)
E
Lcn-2 (ng/g feces)
Time (Days)
F
Colitis Score
G
Colitis Score
Prox Dist
10BiT.Icosl+/+ + vehicle
10BiT.Icosl-/- + vehicle
10BiT.Icosl+/+ + α-Thy1.1
10BiT.Icosl-/- + α-Thy1.1
H
10BiT.Icosl+/+
10BiT.Icosl-/-
vehicle
α-Thy1.1
I
10BiT.Icosl+/+
10BiT.Icosl-/-
MHC II
Ly6C
91.5 6.3 1.8
85.2 10.5 4.1
74.4 19.4 6.0
48.2 40.5 11.1
J
10BiT.Icosl+/+
10BiT.Icosl-/-
IL-17A
IFNγ
Vehicle
α-Thy1.1
2.5 0.3 6.8
1.8 0.2 4.5
3.6 0.2 7.5
12.4 3.5 11.2
K
IL-17A+ CD4 T Cells
% of CD4+TCRβ+
cell no. (x10⁴)
L
IFNγ+ CD4 T Cells
% of CD4+TCRβ+
cell no. (x10⁴)

Figure 1: Transient depletion of IL-10–producing cells from ICOSL-deficient mice results in colonic inflammation. (**A**) Experimental design for Thy1.1+ cell depletion experiments. (**B**) Representative flow cytometry plots demonstrating the impact of anti-Thy1.1 treatment on Thy1.1 expression on colon lamina propria cells of 10BiT.*Icosl*$^{+/+}$or 10BiT.*Icosl*$^{-/-}$ mice 1, 3, or 5 d after anti-Thy1.1 injection. (**C**) Graphs displaying frequencies (*Upper*) and numbers (*Lower*) of CD4+TCR-β+Thy1.1+ cells from mice analyzed as in B; n = 9 mice/group. (**D**) Percent change in weight and (**E**) fecal Lcn-2 levels measured every 5 d from start of anti-Thy1.1 injection. (**F**) Total histopathological scores of colonic tissue sections from all mice at day 25. (**G**) Histopathological scores of proximal (Prox) and distal (Dist) colon sections. (**H**) Representative hematoxylin and eosin–stained photomicrographs depicting the colonic inflammation observed in mice examined as in F and G. n = 8 to 10 mice/group. (**I**) Representative flow cytometry plots demonstrating colonic "monocyte waterfall" cell subsets from WT and *Icosl*$^{-/-}$ mice at 5 d after vehicle or anti-Thy1.1 injection. (**J**) Representative flow cytometry plots showing IFNγ and IL-17A expression by CD4 T cells in the colon lamina propria of mice analyzed in D–H. Scatter plots show frequencies (*Left*) and numbers (*Right*) of IL-17A+ (**K**) and IFNγ+ (L) CD4 T cells from mice analyzed as in A. n = 7 to 9 mice/group. Error bars represent mean ± SD; P values were calculated by ANOVA for repeated measures with Bonferroni correction (B–E) or by ANOVA followed by Tukey's multiple comparisons test (F, G, K, L). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are compiled from two (B, C, I, K, L) or three (E–G) independent experiments.

Previous studies have shown that ICOSL deficiency can impair the activation and proinflammatory potential of myeloid lineage cells (16, 38, 39), yet in our studies, mice devoid of ICOSL deficiency predisposed to a worse inflammatory outcome than did ICOSL sufficiency. Nevertheless, we sought to determine whether there was a myeloid cell defect that contributed to disease susceptibility in our model. Importantly, at steady state, and prior to the depletion of IL-10–producing cells, we did not detect any differences in the frequencies and numbers of lamina propria monocytes or macrophages in ICOSL-deficient mice relative to ICOSL-sufficient mice (SI Appendix, Fig. S3 A and B). Furthermore, using CD86 upregulation as an indicator of myeloid cell activation, we found similar levels of expression by monocytes, newly differentiated as well as mature macrophages, and dendritic cells in the lamina propria of the proximal and distal colon, suggesting that these cells were not hyper activated (SI Appendix, Fig. S3 C and D). However, within 5 d of anti-Thy1.1 treatment in vivo, and consistent with disruption of IL-10 signaling (5, 40), we observed an increase in monocyte recruitment and differentiation into inflammatory macrophages in the colonic lamina propria of ICOSL-deficient mice (Fig. 1H). By day 25, the colitis appeared to be sustained in a T cell–driven manner by robust frequencies of Th1 and Th17 lymphocytes in the colon lamina propria (Fig. 1 I–K). Collectively, these findings demonstrate that sustained expression of both IL-10 and ICOSL is essential for the maintenance of intestinal immune homeostasis, especially in the ascending colon.

Divergent Colon-Associated IL-10 and Tfh Cell Responses in the Absence of ICOSL.

Based on the localization of inflammation to the proximal colon in Fig. 1, we next determined the impact of ICOSL deficiency on the expression of IL-10 by CD4 T cells along the length of the colon. We observed elevated frequencies of IL-10–producing CD4 T cells in the proximal relative to the distal colon in *Icosl*$^{+/+}$mice. Relative to the distal colon, the proximal colon of ICOSL-sufficient mice is enriched with IL-10–producing CD4 T cells (Fig. 2A, Top). However, the frequencies of these cells in the proximal colon are significantly increased in the absence of ICOSL (Fig. 2 A–C). In fact, on average, IL-10–competent cells accounted for ∼50% of all CD4+TCRβ+ cells in the proximal colon of *Icosl*$^{-/-}$ mice (Fig. 2 A–C).

The interaction of ICOSL on B cells with ICOS on Tfh cells is essential for the retention of Tfh cells within the germinal center. In the absence of this interaction, developing Tfh cells lose their lineage specific phenotype and leave the germinal center, and thus antibody production is impaired (17, 18). In general, Tfh cells are rare in naïve specific pathogen-free mice but detectable in gut-associated lymphoid tissues in which they develop in a commensal-dependent manner (41). In WT mice, Tfh cells were most prevalent in the lymph nodes draining the proximal colon with reduced frequencies and numbers in the middle and distal regions (Fig. 2 D–F). In ICOSL-deficient mice, Tfh cells were reduced across all colon-draining nodes, with the most significant difference relative to WT mice detected in the proximal region, the same region where we observed the largest and increase in IL-10–producing CD4 T cells

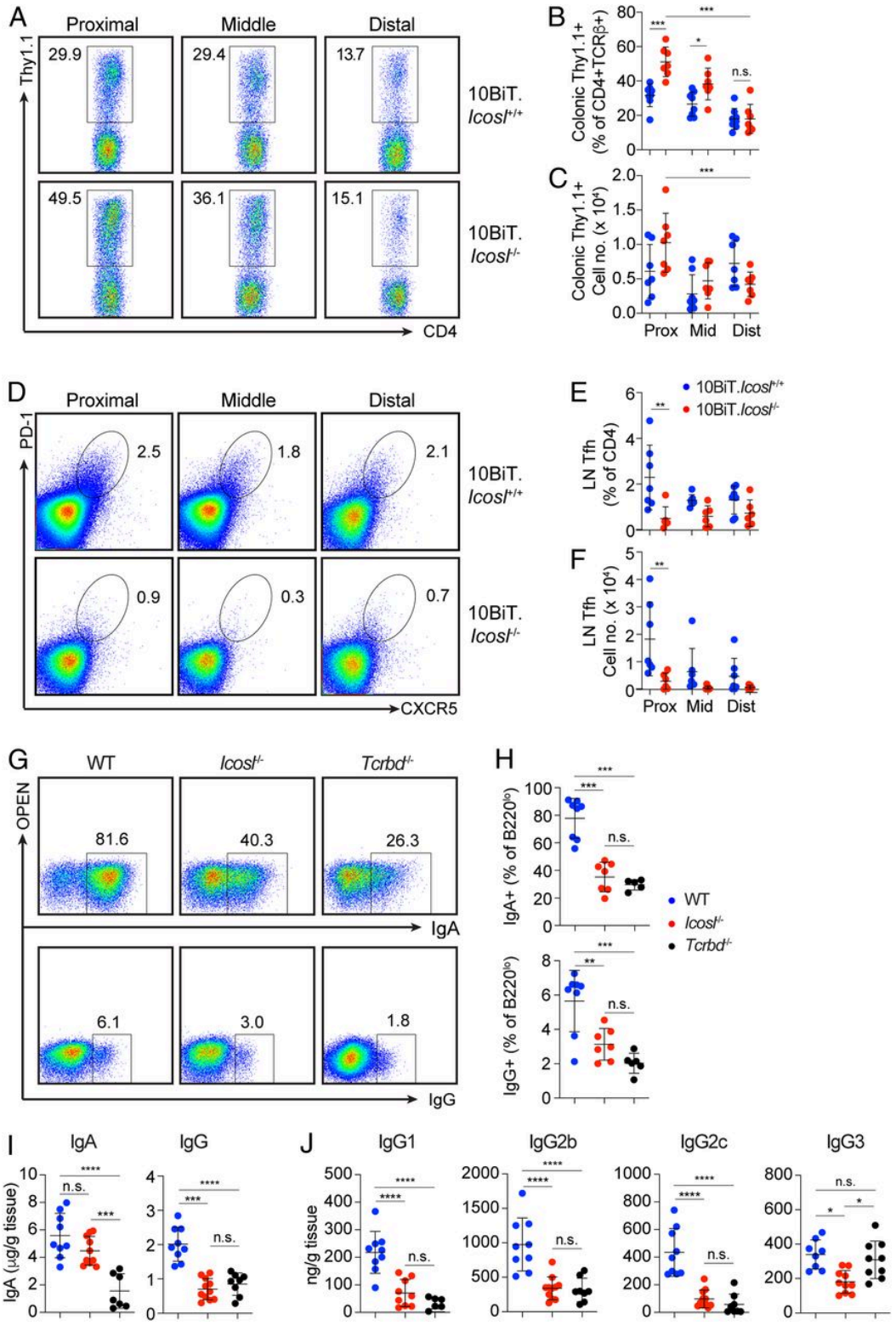
A
Proximal
Middle
Distal
Thy1.1
CD4
29.9
29.4
13.7
49.5
36.1
15.1
10BiT. Icosl+/+
10BiT. Icosl-/-
B
Colonic Thy1.1+ (% of CD4+TCRβ+)
C
Colonic Thy1.1+ Cell no. (x 10⁴)
Prox
Mid
Dist
D
PD-1
CXCR5
2.5
1.8
2.1
0.9
0.3
0.7
E
LN Tfh (% of CD4)
10BiT.Icosl+/+
10BiT.Icosl-/-
F
LN Tfh Cell no. (x 10⁴)
G
WT
Icosl-/-
Tcrbd-/-
OPEN
IgA
IgG
81.6
40.3
26.3
6.1
3.0
1.8
H
IgA+ (% of B220lo)
IgG+ (% of B220lo)
WT
Icosl-/-
Tcrbd-/-
I
IgA
IgG
IgA (μg/g tissue)
J
IgG1
IgG2b
IgG2c
IgG3
ng/g tissue
n.s.

Figure 2: T cell–derived IL-10 is elevated, whereas Tfh and anti-commensal antibodies are reduced in the colons of ICOSL-deficient mice. (**A**) Representative flow cytometry plots showing CD4+Thy1.1+ cells from the lamina propria of the proximal, middle, and distal colon of 10BiT.*Icosl*$^{+/+}$and 10BiT.*Icosl*$^{-/-}$ mice. Graphs show (**B**) frequencies and (**C**) numbers of cells per mouse analyzed as in A. (**D**) Representative flow cytometry plots showing Tfh cells in the lymph nodes draining the proximal, middle, and distal colon of 10BiT.*Icosl*$^{+/+}$and 10BiT.*Icosl*$^{-/-}$ mice. Graphs show (**E**) frequencies and (**F**) numbers of cells per mouse analyzed as in D. Plots in A and D are gated on CD4+TCRβ+ cells, and numbers represent the percentage of cells in the gate shown. 10BiT.*Icosl*$^{+/+}$n = 7, 10BiT.*Icosl*$^{-/-}$ n = 6. Data from one of two independent experiments are shown. (**G**) Representative flow cytometric analysis of IgA and IgG expression by B220lo cells from the colonic lamina propria of WT, *Icosl*$^{-/-}$, or *Tcrbd*$^{-/-}$ mice. (**H**) Frequency of B220lo cells expressing IgA or IgG as depicted in A. WT, n = 8; *Icosl*$^{-/-}$, n = 7; *Tcrbd*$^{-/-}$, n = 6. Data are compiled from two independent experiments. (**I**) ELISA of total IgA and IgG in colonic mucus from WT, *Icosl*$^{-/-}$, or *Tcrbd*$^{-/-}$ mice. WT, n = 9; *Icosl*$^{-/-}$, n = 11; *Tcrbd*$^{-/-}$, n = 8. (**J**) ELISA of IgG1, IgG2b, IgG2c, and IgG3 in colonic mucus from WT, *Icosl*$^{-/-}$, or *Tcrbd*$^{-/-}$ mice. WT, n = 9; *Icosl*$^{-/-}$, n = 10; *Tcrbd*$^{-/-}$, n = 9. Data are compiled from three independent experiments. In all graphs, each symbol represents an individual mouse; error bars represent mean ± SD. P values were calculated by ANOVA followed by Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Reduced Colon-Associated Antibody Responses in ICOSL-Deficient Mice.

We next examined the impact of ICOSL on homeostatic levels of anti-commensal antibodies, by measuring the levels of colon-associated IgA and IgG, the two most common classes of switched antibodies induced by commensals both without and with the help of T cells. For comparison, we included TCRβ x TCRδ double-deficient (*Tcrbd*$^{-/-}$) mice which are incapable of generating T-dependent antibodies. Like *Tcrbd*$^{-/-}$ mice, *Icosl*$^{-/-}$ mice displayed significantly reduced frequencies of IgA- and IgG-secreting cells in the colonic lamina propria (Fig. 2 G and H). At steady state, IgG is not readily detectable in murine feces (42); therefore, we focused our comparisons on the colon mucus aspirate. In the colon mucus, unlike the significant drop in IgA observed in *Tcrbd*$^{-/-}$ mice, the IgA levels detected in *Icosl*$^{-/-}$ mice were not substantially changed (Fig. 2I) and may represent a compensatory increase in T-independent IgA production amid reduced ICOSL-dependent IgA in *Icosl*$^{-/-}$ mice. However, both mutant strains displayed similar reductions in total IgG levels in the colon mucus (Fig. 2I). We also examined the relative levels of the four murine IgG subtypes in the serum and colon mucus of ICOSL-deficient mice and compared to T cell–deficient mice. We found statistically significant reductions in serum IgG1 and IgG2c but not IgG2b or IgG3 in both *Icosl*$^{-/-}$ and *Tcrbd*$^{-/-}$ mice (Fig. 2J). In the colon mucus, there were significant reductions in all IgG subtypes in both *Icosl*$^{-/-}$ and *Tcrbd*$^{-/-}$ with the exception of IgG3, which was unchanged in *Tcrbd*$^{-/-}$ mice (Fig. 2J). These results demonstrate the existence of similar colon-associated anti-microbial antibody alterations in ICOSL- and TCR-deficient mice and suggest similar deficits in antibody-mediated interactions with commensals.

ICOSL Deficiency Leads to Reduced Serum Antibody Recognition of Antigens Derived from Diverse Bacteria including Flagellated and Mucus-Associated Commensals.

Anti-commensal IgA and IgG antibodies are known to be present in the circulation (27), and both *Tcrbd*$^{-/-}$ and *Icosl*$^{-/-}$ mice displayed reduced serum IgA and IgG relative to WT mice (SI Appendix, Fig. S4). To understand whether and how host–microbe interactions are disrupted in the absence of ICOSL, we examined the impact of ICOSL deficiency on the antibody-mediated recognition of whole commensal organisms as well as commensal antigens. Since a large fraction of fecal microbes is actively bound by predominantly T-independent IgA (25, 26), we first compared the relative frequencies of IgA-bound bacteria in the colon lumen of *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ mice. ICOSL deficiency did not lead to reduced IgA coating of luminal bacteria (Fig. 3 B and C). This eliminated IgA-based sorting as a means to identify microbes that are specifically targeted by ICOSL-dependent antibodies. Moreover, we did not reliably detect IgG-bound microbes or binding of serum IgG to luminal bacteria, as has been previously described (21). Thus, the differences in antibody levels between WT and ICOSL-deficient mice did not appear to impact antibody coating of luminal microbes. We then harvested luminal and mucus-associated bacteria from *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ mice and generated crude lysates that we then used in an enzyme-linked immunosorbent (ELISA)–based assay to "capture" antibodies present in paired serum. Using this system, we found significant reductions in the levels of anti-luminal lysate IgA and IgG, in *Icosl*$^{-/-}$ relative to *Icosl*$^{+/+}$mice (Fig. 3D), and an even more dramatic diminution in serum IgA and IgG binding to mucus lysates (Fig. 3E). Considering that luminal and mucus communities continually admix via bidirectional transfer (36), these data support the notion that mucus-associated communities in particular elicit robust ICOSL-dependent antibody responses.

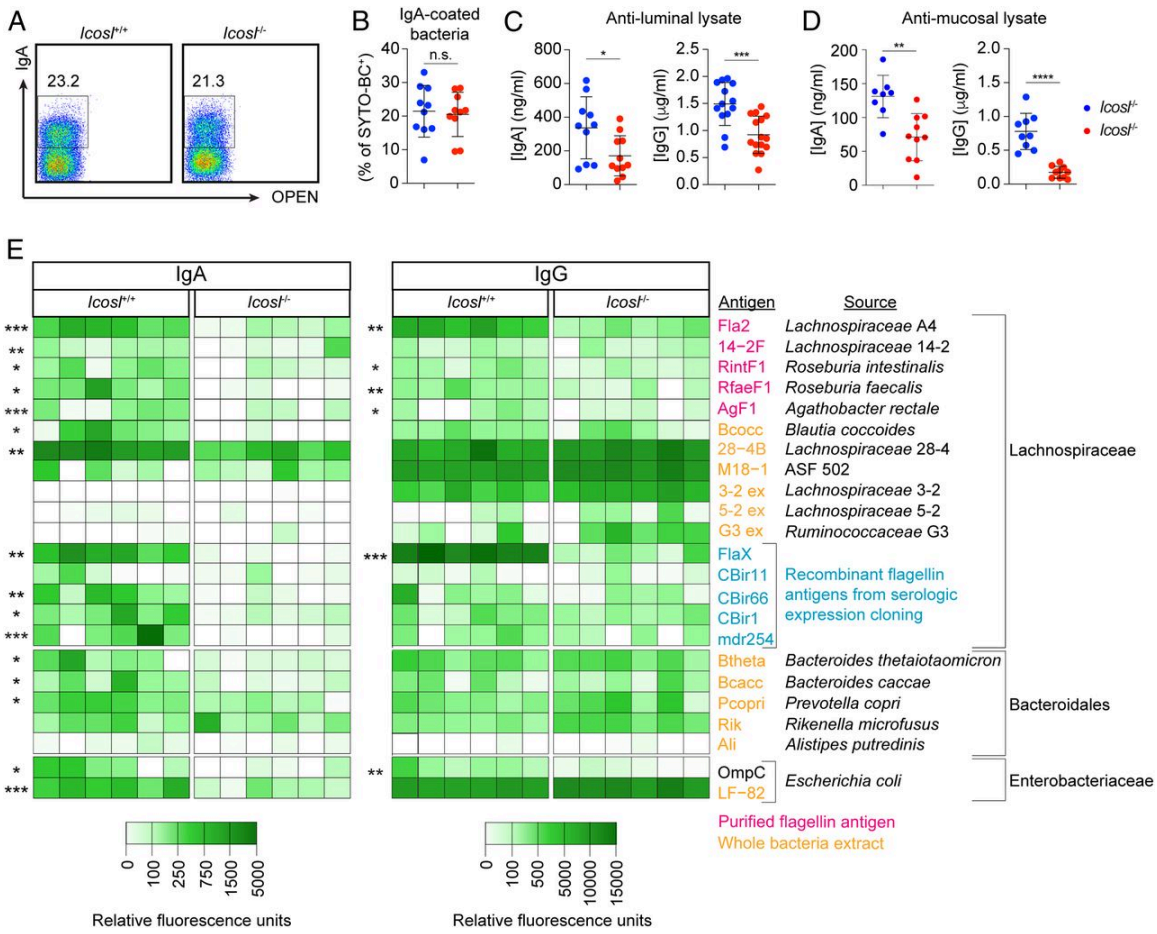
A
Icosl+/+
Icosl-/-
IgA
23.2
21.3
OPEN
B
IgA-coated bacteria
n.s.
(% of SYTO-BC+)
C
Anti-luminal lysate
[IgA] (ng/ml)
[IgG] (μg/ml)
D
Anti-mucosal lysate
[IgA] (ng/ml)
[IgG] (μg/ml)
Icosl-/-
Icosl-/-
E
IgA
IgG
Icosl+/+
Icosl-/-
Antigen
Source
Fla2 Lachnospiraceae A4
14−2F Lachnospiraceae 14-2
RintF1 Roseburia intestinalis
RfaeF1 Roseburia faecalis
AgF1 Agathobacter rectale
Bcocc Blautia coccoides
28−4B Lachnospiraceae 28-4
M18−1 ASF 502
3-2 ex Lachnospiraceae 3-2
5-2 ex Lachnospiraceae 5-2
G3 ex Ruminococcaceae G3
FlaX
CBir11
CBir66
CBir1
mdr254
Recombinant flagellin antigens from serologic expression cloning
Lachnospiraceae
Btheta Bacteroides thetaiotaomicron
Bcacc Bacteroides caccae
Pcopri Prevotella copri
Rik Rikenella microfusus
Ali Alistipes putredinis
Bacteroidales
OmpC
LF−82
Escherichia coli
Enterobacteriaceae
Purified flagellin antigen
Whole bacteria extract
0 100 250 750 1500 5000
Relative fluorescence units
0 100 500 5000 10000 15000
Relative fluorescence units

Figure 3: Reduced antibody binding to antigens derived from predominantly mucus-associated bacteria in ICOSL-deficient mice. (**A**) Representative flow cytometric analysis of IgA-coating of bacteria (SYTO-BC+) from fecal pellets of *Icosl*$^{+/+}$or *Icosl*$^{-/-}$ mice. (**B**) Frequency of bacteria coated with IgA as analyzed in A. Data are compiled from three independent experiments. n = 10 mice/group. ELISA of matched serum IgA (*Left*) and IgG (*Right*) binding to crude bacterial lysates prepared from (**C**) luminal contents or (**D**) mucus from WT or *Icosl*$^{-/-}$ mice. Icosl$^{+/+}$, n = 13; *Icosl*$^{-/-}$, n = 15. In B–D, data are compiled from three independent experiments. Error bars represent mean ± SD, and P values were calculated by ANOVA test followed by Tukey's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001. (**E**) Heatmap showing relative fluorescence intensity of IgA (*Left*) and IgG (*Right*) binding to microbial antigens or lysates. Values were normalized to fluorescence, if any, detected when each antigen was probed with *Rag1*$^{-/-}$ mouse serum. Asterisks denote antigens or lysates for which there was a statistically significant difference in fluorescence intensity between *Icosl*$^{-/-}$ and *Icosl*$^{-/-}$ serum. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are from one of two individual experiments.

Bacteria belonging to the *Lachnospiraceae* family are enriched among mucus-associated communities (34–36). Moreover, Crohn's disease patients display seroreactivity to *Lachnospiraceae* flagellin antigens A4-Fla2 and FlaX, which, along with reactivity to CBir1 flagellin and *Escherichia coli* outer membrane protein C (OmpC), correlate with early postoperative recurrence of Crohn's disease (43–45). Therefore, to determine the origin and types of antigens that drive ICOSL-dependent antibody responses, we performed a microbial antigen array (46) in which purified antigens or whole extracts of bacteria from an "in-house" library were screened for reactivity to serum IgA and IgG from *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ mice. We tested a collection of purified flagellins or recombinant flagellins generated by serologic expression cloning as well as whole-cell extracts of several species of *Lachnospiraceae*, five members of the order *Bacteroidales*, and *E. coli*. There was reduced serum IgA from *Icosl*$^{-/-}$ mice reactive to almost all *Lachnospiraceae* flagellin antigens tested as well as to extracts of *Bacteroides thetaiotaomicron*, *Bacteroides caccae*, and *Prevotella copri* (Fig. 3E, Left). In addition, IgG reactivity to several *Lachnospiraceae* flagellin antigens, including most notably A4Fla2 as well as FlaX, was also significantly reduced in ICOSL-deficient mice (Fig. 3E, Right). Collectively, our results demonstrate that in the absence of ICOSL-dependent anti-commensal antibodies, there is reduced IgA and IgG targeting antigens derived from multiple species of anaerobic bacteria, including antigens previously associated with active IBD. Consistent with this, treatment with metronidazole, which depletes anaerobes, completely inhibited development of colitis when ICOSL-deficient mice were depleted of IL-10–producing cells (SI Appendix, Fig. S5). Disease was also partially suppressed by vancomycin but unimpacted by colistin, which broadly target Gram+ and

Gram− bacteria, respectively. Thus, the colitis that develops when IL-10–producing cells are transiently removed from mice with a deficiency of ICOSL-dependent anti-commensal antibodies appears to be driven by predominantly Gram+ anaerobic bacteria.

Coablation of *Icosl* and CD4 T Cell–Derived *Il10* Predisposes to Early-Onset Colitis.

All of the foregoing results suggest a cooperative relationship between IL-10 and ICOSL-dependent anti-commensal antibodies in the regulation of colonic immune homeostasis at least in adulthood. To determine whether CD4 T cells are the primary source of this synergistic IL-10 and to examine the possible necessity of this axis for gut immune homeostasis in early life, we eliminated CD4 T cell expression of IL-10 in ICOSL-deficient mice. We crossed mice in which IL-10 deficiency is restricted to CD4–expressing cells (*Il10* conditional knockout, *Il10*cKO) (1) with *Icosl*$^{-/-}$ mice to generate mice deficient for only ICOSL (*Icosl*$^{-/-}$), only T cell–derived IL-10 (*Il10*cKO), both (*Icosl*$^{-/-}$*.Il10*cKO), or neither (Icosl$^{+/+}$). Kinetic analysis of fecal lipocalin-2 (Lcn-2) showed elevated expression as early as the fourth week of life in *Icosl*$^{-/-}$*.Il10*cKO mice but not in Icosl$^{+/+}$, *Icosl*$^{-/-}$, or *Il10*cKO animals (Fig. 4A). The fecal Lcn-2 levels increased rapidly over the next few weeks, reaching a plateau by the sixth week of life. Histological analysis at 7 wk of age confirmed the development of severe colitis in *Icosl*$^{-/-}$*.Il10*cKO mice while the other groups remained disease-free during the period of analysis (Fig. 4 B and C). CD4-specific IL-10 deficiency in mice has long been known to predispose to adult-onset colitis (1), and as expected, *Il10*cKO mice eventually experienced dramatic disease-related weight loss starting at around 18 wk of age, and all were severely colitic when euthanized at 27 to 28 wk of age (SI Appendix, Fig. S6). This

argues that the absence of ICOSL can measurably hasten colitis onset in the absence of T cell–derived IL-10 and demonstrates that CD4 T cells are the primary source of the IL-10 that counters ICOSL deficiency.

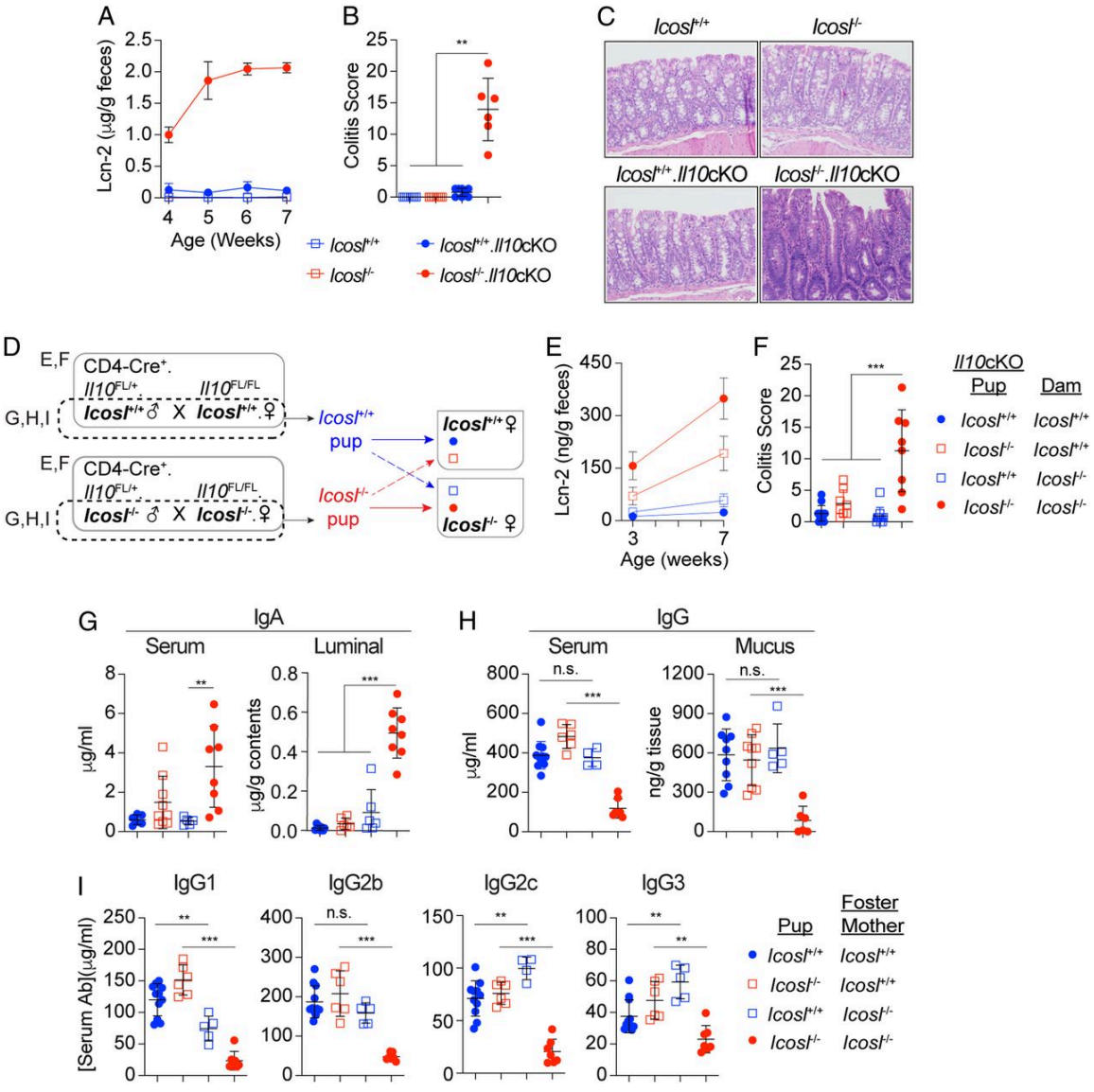

Figure 4: Maternal and host-derived ICOSL-dependent antibodies in combination with IL-10 help to promote intestinal immune homeostasis in early life. (**A**) Longitudinal Lcn-2 levels detected in feces of Icosl$^{+/+}$, *Icosl*$^{-/-}$, Icosl$^{+/+}$.*Il10*cKO, and *Icosl*$^{-/-}$.*Il10*cKO mice from 4 to 7 wk of age. (**B**) Histopathological scoring of hematoxylin and eosin–stained sections and (**C**) representative photomicrographs from mice of all four genotypes analyzed at 8 wk of age; n = 8 mice/group (20× magnification). (**D**) Experimental design for cross-fostering experiments featuring *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ mice with (solid line) or without (broken line) mutations associated with condition deletion of *Il10*. Graphs summarizing (**E**) Fecal Lcn-2 at 3 and 7 wk and (**F**) histopathological scores at week 7; n = 8 to 13 mice/group. Scatter plots show levels of serum and luminal IgA (**G**), serum and mucus IgG (**H**), and serum IgG1, IgG2b, IgG2c, and IgG3 (**I**) for 3-wk-old *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ pups housed as described in F. n = 5 to 11 mice/group. For all graphs, error bars represent mean ± SD. P values were calculated by ANOVA followed by Tukey's multiple comparisons test; *P < 0.05, **P < 0.01, ***P < 0.001. Data are representative of two (A–C) or four (D–I) experiments.

Early-Onset Colitis that Develops in *Icosl*$^{-/-}$.*Il10*cKO Mice Is Delayed in Mice Fostered by ICOSL-Sufficient Dams.

Anti-commensal antibodies transferred from mother to offspring via the placenta and/or the breast milk can help to tolerize the newborn to the encroaching microbiota. In theory, maternally derived antibodies can be either T dependent or T independent, but it has been reported that the major players in early-life homeostasis are T-independent IgA, IgG2b, and IgG3 (22). We thus sought to determine whether an ICOSL-sufficient nursing mom could help rescue the early-onset disease that develops in *Icosl*$^{-/-}$.*Il10*cKO mice, which would provide further proof of the importance of ICOSL-dependent antibodies in mitigating this inflammatory reaction. We conducted a series of timed matings utilizing *Icosl*$^{+/+}$or *Icosl*$^{-/-}$ mice that importantly were negative for Cre recombinase (Fig. 4D). In the first instance, the dams were also homozygous for LoxP-flanked *Il10* alleles (*Il10*$^{FL/FL}$.*Icosl*$^{+/+}$or *Il10*$^{FL/FL}$.*Icosl*$^{-/-}$) (1). These mice were mated with *Il10*$^{FL/+}$ males that were positive for Cre recombinase under the control of the Cd4 promoter. This allowed for the introduction of the mutations enabling conditional deletion of *Il10* (*Il10*cKO) in the progeny born to both WT and ICOSL-deficient dams. Within 12 h after birth, half of the male and female progeny in each litter was removed and fostered by the dam of the opposing *Icosl* genotype. Consistent with our earlier observation, Icosl$^{+/+}$.*Il10*cKO mice that remained with their *Icosl*$^{+/+}$birth mother remained disease free throughout the observation period (Fig. 4 E and F). *Icosl*$^{-/-}$.*Il10*cKO pups fostered by *Icosl*$^{+/+}$dams displayed low but detectable levels of fecal Lcn-2 at weaning, but the levels increased gradually thereafter. At 7 wk of age, these mice were found to have limited disease which suggests that maternally acquired ICOSL-dependent factors help to limit early-life immune reactivity to the microbiota in the large intestine.

Early-Life Intestinal Immune Homeostasis Involves ICOSL-Dependent Antibodies that Are both Maternally Acquired and Host Derived.

The discordance between fostered mice was more dramatic in mice housed with *Icosl*$^{-/-}$ dams. Here, as expected, *Icosl*$^{-/-}$.*Il10*cKO mice showed elevated Lcn-2 as early as 3 wk which increased further after weaning, and the majority of mice were severely colitic by 7 wk of age (Fig. 4 E and F). In contrast, their Icosl$^{+/+}$.*Il10*cKO cage mates were mostly free of early signs of inflammation, suggesting a possible contribution of host-derived antibodies as well in limiting microbiota reactivity. Therefore, to determine the relative contributions of maternal and host-derived ICOSL-dependent IgA and IgG antibodies to early-life homeostasis, we performed additional cross-fostering experiments but without the additional variable of disease susceptibility as a result of conditional deletion of *Il10*. *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ littermate females were weaned together and cohoused until they reach sexual maturity. These females were then separated and subjected to timed mating with proven males of the same genotype. Pups were swapped within the first 12 h of life, and antibody levels were determined at 21 d of age. There was no significant difference in the levels of serum or colon luminal IgA among *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ mice raised by *Icosl*$^{+/+}$dams (Fig. 4G). In contrast, we observed significant increases in both serum and luminal IgA in *Icosl*$^{-/-}$ pups, relative to their *Icosl*$^{+/+}$foster siblings all raised by *Icosl*$^{-/-}$ dams. This suggests that in the absence of maternal ICOSL-dependent anti-commensal IgA, host production of ICOSL-independent IgA in early life is increased. These results are consistent with our earlier observation of near-WT levels of colon mucus IgA in ICOSL-deficient adult mice (Fig. 2I).

Interestingly, *Icosl*$^{-/-}$ pups raised by their *Icosl*$^{-/-}$ mothers had significantly reduced levels of total IgG in both the serum and colon mucus (Fig. 4H). This demonstrates that, in contrast to the elevated IgA, the IgG antibodies present at this stage of life are predominantly ICOSL dependent. As with IgA, the amount of total IgG in the serum and colon mucus of pups raised by *Icosl*$^{+/+}$dams was similar in *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ pups, suggesting maternal origin. However, these IgG levels were matched by those of *Icosl*$^{+/+}$pups placed with *Icosl*$^{-/-}$ dams arguing that these antibodies can also originate in the pups and are likely increased to compensate for the absence of the maternal source. Analysis of the serum levels of individual subtypes of IgG showed a significant reduction in IgG1 in cross-fostered *Icosl*$^{+/+}$pups relative to their siblings that were left with their birth mothers (Fig. 4I, Left), indicating an important contribution from the mother to the early-life IgG1 pool. In contrast, the cross-fostered *Icosl*$^{+/+}$pups displayed significant increases in IgG2c and IgG3 relative to those left with their birth mothers (Fig. 4I) suggesting these subtypes are largely pup derived and likely amplified in the absence of maternal ICOSL-dependent antibodies. Altogether, these results demonstrate that that both maternal and host-derived IgG antibodies contribute to the early-life anti-commensal antibody pool and that the sustained levels of most subtypes are dynamically impacted by the relative contributions from each source. Moreover, they provide evidence that ICOSL-dependent anti-commensal antibodies are part of a mechanism capable of at least temporarily maintaining intestinal immune homeostasis in mice even in the absence of T cell–derived IL-10. Coupled with results presented in Fig. 1, these data further imply that the synergy between ICOSL-dependent antibodies and IL-10 is essential in early life and continues to be necessary for the long-term maintenance of host–microbiota mutualism.

DISCUSSION

In this study, we have presented murine ICOSL deficiency as a T cell–replete model of anti-commensal antibody deficiency, displaying diminished recognition by IgA and IgG antibodies of antigens derived from predominantly mucus-associated bacteria. By studying the CD4 T cell compartment in these mice, we identified a heretofore unknown synergy between ICOSL-dependent anti-commensal antibodies and IL-10 in regulating proinflammatory responses to the colonic microbiota starting in early life and extending into adulthood. Our study demonstrates how ICOSL deficiency can facilitate comprehensive mechanistic exploration of the relationship between anti-commensal antibodies and other immune pathways in promoting and maintaining host–microbiota mutualism. We posit that transient disruption of IL-10 function coupled with ICOSL deficiency represents an inducible and highly reproducible model of microbiota-dependent colonic inflammation in otherwise immune-sufficient mice.

We and others have observed defective Treg cell survival and/or epigenetic stability in the absence of ICOS (33, 47–50), the T cell–expressed receptor for which ICOSL is presumed to be the sole ligand. Surprisingly, we found no Foxp3+ Treg cell deficits in ICOSL-deficient mice. Further work will be necessary to determine whether the ICOS receptor is indeed triggered by an alternative mechanism in the absence of ICOSL. Nevertheless, this unexpected result forced us to explore an alternative basis for the enhanced accumulation of IL-10–producing CD4 T cells observed in the colon of both ICOS-deficient (33) and ICOSL-deficient mice. Fortunately, however, it also

enabled utilization of *Icosl*$^{-/-}$ mice without any confounding deficiencies in the Treg cell compartment. We also confirmed the absence of any effect of ICOSL deficiency on the differentiation and activation status of gut myeloid cells at steady state. These results strengthen our argument that the observed antibody deficiencies and elevated IL-10–producing CD4 T cells we detected at steady state are likely not due to defects in Treg or macrophage function. Instead, these phenomena appear to be inextricably linked, such that their combined disruption at any point in time predisposes to rapid-onset colonic inflammation.

Owing to the importance of ICOSL–ICOS in Tfh responses, ICOSL-deficient mice are a bona fide model of T-dependent anti-commensal antibody deficiency, featuring obvious advantages over the commonly utilized T cell–deficient mice. Most notably, the major compensatory change seemingly triggered by the Tfh deficiency in ICOSL-deficient mice is increased IL-10–producing CD4 T cells in the proximal colon. Obviously, this axis would have gone undetected if T cell–deficient mice were used instead, and thus our study highlights the value of lymphocyte-replete model systems for exploring the functions of anti-commensal antibodies. In general, ICOSL-deficient mice harbored antibody levels comparable to those of *Tcrbd*$^{-/-}$ mice, although there are noteworthy differences. For example, despite significant reductions in colonic antibody-secreting cells relative to WT mice, *Icosl*$^{-/-}$ mice harbored increased plasma cells relative to *Tcrbd*$^{-/-}$ mice. This led to significant differences in serum and mucus IgA between *Icosl*$^{-/-}$ and *Tcrbd*$^{-/-}$ mice. In fact, in the colon mucus, there was actually no statistically significant difference in IgA between WT and *Icosl*$^{-/-}$ mice. Furthermore, in 3-wk-old *Icosl*$^{-/-}$ pups raised by their *Icosl*$^{-/-}$ birth mothers, there was a significant increase in

luminal IgA relative to the littermates that were fostered by *Icosl*$^{+/+}$dams. This is likely due to a compensatory increase and/or accumulation of mostly T-independent IgA when all sources of ICOSL-dependent antibodies are disrupted. Importantly, despite the near-normal levels of colon mucus IgA, *Icosl*$^{-/-}$ mice were still prone to colitis development when the IL-10–producing cells were depleted, and *Icosl*$^{-/-}$*.Il10*cKO mice still developed early-onset inflammation. Therefore, regardless of the nature and level of the extra IgA generated in the absence of ICOSL, it is inadequate to control the organisms that drive the disease observed in both scenarios.

Both humans and mice express four different subclasses of IgG and it is generally accepted that the majority of T-dependent IgG antibodies, including those elicited by murine pathogens, are of the IgG1 subclass (42). In contrast, microbiota-reactive IgG2b and IgG3 antibodies are generated by predominantly TI mechanisms (22). Overall, we observed similar trends in the amounts of serum IgG antibodies in both *Icosl*$^{-/-}$ and *Tcrbd*$^{-/-}$ mice relative to WT mice. This included consistent reductions in IgG1 and IgG2c, but not IgG2b or IgG3, in the serum of both mutant strains. In the colon mucus, there were notable deviations from these trends in that the levels of IgG2b were reduced in both *Tcrbd*$^{-/-}$ and *Icosl*$^{-/-}$ mice. Furthermore, the levels of IgG3 were also reduced in *Icosl*$^{-/-}$ colon mucus as well as in the serum of 3-wk-old *Icosl*$^{-/-}$ pups raised by their isogenic mothers. The reasons for these discrepancies remain unclear.

The microbial density of the mucus layer is greater in the proximal relative to the distal colon (51). Our findings that Tfh cell responses are more robust in the lymph nodes draining this region, and that IL-10–producing CD4 T cells enrich in this region

especially when Tfh are impaired, highlight the necessity of robust immune regulation in this locale to actively limiting deleterious responses to mucus-associated bacteria. Owing to their proximity to the epithelium, mucus-associated commensals would arguably be more likely to require T-dependent antibody-mediated control and more prone to trigger an inflammatory response if not adequately restrained. Acute antigenic challenge results in the expression of IL-10 by CD4+ T follicular regulatory (TFR) cells which in turn promotes the differentiation of plasma cells (52). In light of our findings, it seems plausible that the role of T cell–derived IL-10 in synergy with antibody responses to commensal antigens involves both promoting the generation of a subset of these antibodies and actively compensating for any shortfall in their production, all in an effort to limit immune reactivity to mucus-associated microorganisms.

A previous study demonstrated an essential role for IL-10 in limiting inflammatory responses in recently colonized mice (53). By examining ICOSL-deficient mice incapable of IL-10 production in CD4 T cells, we now show that this IL-10 is predominantly T cell derived. In addition, it was previously shown that peaceful intestinal colonization of neonatal mice requires maternally derived T-independent IgA and IgG antibodies that are acquired via the breast milk and efficiently bind to luminal microbes (22). Our study does not dispute these findings but identifies additional roles in this process for maternally acquired and host-derived ICOSL-dependent antibodies acting in concert with T cell–derived IL-10. Ablation of IL-10 expression either in the germline or solely in CD4 T cells has long been known to predispose to colitis (1, 54–56) contingent on the presence in the microbiota of specific pathobiont species (57–59). Importantly, we observed the expected delay in disease onset in mice deficient in only T cell–derived IL-

10. However, in the absence of ICOSL, signs of disease were evident as early as weaning, suggesting that the presence of a robust repertoire of ICOSL-dependent anti-commensal antibodies can temporarily suppress the colitogenicity of even a "permissive" microbiota. These findings further suggest that the failure to progress to colitis, as sometimes occurs in this model, may be evidence of sustained ICOSL-dependent antibody-mediated control of mucus-associated communities.

Various microbes or microbial consortia can directly enhance IL-10–producing CD4 T cell accumulation in the colon or require IL-10 for their mutualistic existence in the intestine. These include *Bacteroides fragilis*, *Bifidobacterium breve*, a mixture of 17 microbes isolated from human feces and containing several species of *Lachnospiraceae*, and the commonly used eight-member consortium referred to as the altered Schaedler's flora (53, 60–62). Our analysis identified microbial antigen extracts from *Lachnospiraceae*, *Bacteroidales*, and *Enterobacteriaceae* that were differentially recognized by antibodies from WT mice relative to ICOSL-deficient littermates. Elevated T cell production of IL-10 in the absence of these antibodies implies that impaired antibody control of such microbial antigens can give way to antigen-dependent expansion of IL-10–producing colonic CD4 T cells. It also suggests that induction of ICOSL-dependent antibodies and T cell–derived IL-10 may be simultaneous host adaptations to microbial occupation of a niche near the epithelium. Future exploration of the specific microbes that drive these responses could potentially identify novel antigen-specific approaches to bolster mucosal immune defenses.

Finally, our study demonstrates that ablation of ICOSL expression impacts the mucosal levels of both IgA and IgG. Moreover, we detected deficiencies in the binding of IgA and IgG antibodies to flagellin and other antigens when antibody production was impaired. Based on the quantities detected in WT mice and the dramatic reductions in *Icosl*$^{-/-}$ mice, we would predict that ICOSL-dependent antibodies of both isotypes are important for ensuring gut homeostasis, but additional studies will be needed to determine the potential synergy and/or division of labor between these two isotypes and even between the various subtypes of IgG. Our results showing reduced anti-flagellin IgA in *Icosl*$^{-/-}$ mice, coupled with a recent study demonstrating a protective role for anti-flagellin IgA in preventing the development of murine colitis in response to disrupted IL-10 signaling (63), supports a beneficial role for IgA in preventing the intestinal inflammation observed in this study. Importantly, our data also argue in favor of IgG recognition of microbial antigens, including robust responses to flagellin antigens associated with IBD patients (44, 45). Unlike IgA, anti-flagellin IgG was shown to enhance the severity of disease in mice subsequently administered dextran sulfate sodium (64), supporting the long-held notion that IgG, acting via Fcγ receptors, is involved in the pathogenesis and/or perpetuation of IBD (15, 44, 65–67). However, a protective role for anti-commensal IgG was suggested by our analysis of *Icosl*$^{+/+}$and *Icosl*$^{-/-}$ weanlings that were raised either by their birth mother or by a dam of the opposite genotype. In these experiments, ICOSL-dependent IgG antibodies, derived from both mother and pup, were revealed to be major contributors to the homeostatic IgG repertoire at weaning and likely figure prominently in limiting adverse events during the weaning reaction. Therefore, our study, in combination with recent studies demonstrating important functions of anti-

commensal IgG antibodies under homeostatic conditions (21, 22), stresses the need for future reconciliation of these seemingly opposing roles of IgG in intestinal health and disease.

MATERIALS AND METHODS

Mice.

All mice used in this study were on the C57BL/6 genetic background. Icos$^{-/-}$, *Icosl*$^{-/-}$, *Tcrb-/-Tcrd-/-* (*Tcrbd*$^{-/-}$), *Rag1*$^{-/-}$, and CD4Cre mice were originally purchased from Jackson Laboratory. *Il10*FLOX mice were generously provided by Dr. A. Roers. 10BiT mice have been previously reported (37). Unless otherwise stated, 10BiT.*Icosl*$^{+/+}$and 10BiT.*Icosl*$^{-/-}$ mice for individual experiments were either littermates or age- and sex-matched mice cohoused starting at weaning (DOL 21) until experiments were initiated at 8 wk of age. *Tcrbd*$^{+/+}$ (WT) and *Tcrbd*$^{-/-}$ mice were littermates generated by mating *Tcrbd*+/− males and females. In comparisons of wild-type, *Icosl*$^{-/-}$, and *Tcrbd*$^{-/-}$ mice, wild-type mice are littermates/cage mates pooled from both cohorts. Mice were bred and maintained under specific pathogen-free conditions at the University of Alabama at Birmingham in accordance with Institutional Animal Care and Use Committee guidelines.

Collection of Colon Luminal Contents and Mucus.

Fecal pellets were collected, and large intestines were removed and opened longitudinally. Solid (luminal) content removed by forceps. Tissue was rinsed with PBS before collection of mucus by gentle suction, as described. Importantly, the suction did not appear interfere with the plasma cells present in the underlying lamina propria (SI Appendix, Fig. S7). Mucus was resuspended in 200 μL PBS. Fecal pellets were suspended in 0.1 mg/mL PBS. All samples were homogenized and centrifuged briefly (2,000 × g) to remove debris. Supernatants were collected and centrifuged (11,000 × g) to pellet bacteria. This supernatant was collected for quantification of free antibody, whereas the bacterial pellet was resuspended in 200 μL B-PER II Bacterial Protein Extraction Reagent (Thermo Fisher Scientific) to prepare microbial lysate. All samples were frozen at −70 °C before use.

Mucosal Lymphocyte Isolation.

Large intestine was removed, stripped of mesenteric fat, flushed with sterile PBS, and opened longitudinally. Tissue was cut into 1 cm sections and incubated with rotation at 37 °C in 154 μg/L l-dithioerythritol and 2 μM EDTA in HBSS to remove epithelium. Remaining tissue was then incubated with rotation at 37 °C with 20 μg/mL DNase-I and 100 U/mL collagenase IV (Sigma-Aldrich), and tissue disruption was completed using a GentleMACS Dissociator (Miltenyi Biotec). Total lamina propria cells were purified on a 40%/75% percoll gradient by room temperature centrifugation for 20 min at 2000 rpm with no brake. Lymph nodes draining the proximal, middle, and distal colon, identified as

previously described (68), were mechanically dissociated in RPMI. All cell suspensions were then filtered through 70 μM mesh strainer prior to counting and further analysis.

Antibodies and Flow Cytometry.

Cells were washed with PBS, blocked with 2.4G2 (BioXCell, 10 μg/mL), and then stained with LIVE/DEAD Fixable Near-IR (Invitrogen) and anti-mouse antibodies purchased from BioLegend: CD4 (GK1.5), TCRβ chain (H57-597), IL-17A (TC11-18H10.1), CD90.1 (OX-7), GL7 Antigen (GL7), and IgG (goat polyclonal); eBioscience: Foxp3 (FJK-16S), IFNγ (XMG1.2), and CXCR5 (SPRCL5); Invitrogen: PD-1 (J43), CD45R/B220 (RA3-682), and IgD (11–26); BD Pharmingen: CD95 (Jo2); or Southern Biotech: IgA (goat polyclonal). Cells were permeabilized for intracellular stains using the Foxp3/Transcription Factor Fixation/Permeabilization Kit. Cells for effector cytokine staining were stimulated at 37° in RPMI with 10% fetal bovine serum (FBS) (R10) with ionomycin (750 ng/mL), phorbol 12-myristate 13-acetate (50 ng/mL), and GolgiPlug (BD Biosciences) prior to blocking. All cells were fixed in 2% paraformaldehyde and acquired with an LSR II cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar).

ELISA of Free or Microbiota-Binding Antibody.

Plates were coated overnight at 4 °C in 100 uL of 10 μg/mL goat anti-mouse Ig (Southern Biotech) or microbial lysate diluted to 10 ug protein/mL as determined by Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). Plates were blocked 2 h with 1% bovine serum albumin (BSA) in PBS before addition of free

antibody supernatants or, to lysate coated plates, mouse-matched serum. After 2 h incubation, Goat Anti-Mouse horseradish peroxidase-conjugated antibodies from Southern Biotech were used to detect IgA (1040-05), IgG (1031-05), IgG1 (1070-05), IgG2b (1090-05), IgG2c (1079-05), or IgG3 (1100-05). All assays were normalized to negative controls from *Rag1*$^{-/-}$ mice.

IL-10 ELISA.

CD4 T cells were magnetically sorted (Dynabeads Mouse CD4, Invitrogen) from colonic lamina propria cells and plated at 1×10^6/mL in R10 and anti-CD28 (37.51, BD Pharmingen) in flat-bottom 96-well plates coated with anti-CD3 (145-2C11) for 24 h. Supernatants were assayed with Mouse DuoSet IL-10 ELISA kit (R&D) according to the manufacturer's instructions.

Microbial Flow Cytometry.

Fecal pellets were collected into 500 µL of protease inhibitor mixture (Roche) before homogenizing and brief centrifugation (2,000 × g) to remove debris. Supernatant was centrifuged at 4,000 × g for 10 min, and the resulting bacterial pellet was washed in sterile bacterial staining buffer (1% BSA in PBS). Bacteria were stained on ice with anti-mouse IgA, washed, and resuspended in SYTO BC (Invitrogen) to stain for nucleic acids. SYTO BC gates for bacteria were determined by comparison to germ-free control pellets. Samples were acquired with an LSR II cytometer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar).

Microbial Antigen Array.

Bacterial proteins and extracts were arrayed in triplicate on FAST 16 pad nitrocellulose slides (Maine Mfg.) at a concentration of 0.2 mg/mL using a Spotbot Personal Microarrayer (Korteks). Proteins were diluted from recombinant stocks to 10 mM Tris (pH 7.4), 20% glycerol, and 0.1% sodium dodecyl sulfate (SDS). Extracts were obtained by freeze-thawing whole cells in 10 mM Tris (pH 7.4) and 0.1% SDS three times then removing insoluble cell debris by centrifugation. Extracts were then brought to 20% glycerol before arraying. To probe the microarray, pads were blocked for 1 h in SuperBlock (Thermo Fisher) and then probed with mouse sera diluted 1 to 10 in SuperBlock for 1 h. Serum obtained from *Rag1*$^{-/-}$ mice was used as a negative control. The pads were washed three times in PBS w/0.05% Tween 20 and a mixture of goat anti-mouse IgA-Alexa 555 (Southern Biotech, 1040-32) and goat anti-mouse IgG (H+L)-Dylight 650 (Thermo Fisher Scientific, 84545) were applied at 1 to 1,000 (~0.5 μg/mL) for 1 h in SuperBlock. The pads were then washed three times with PBS-Tween, air-dried, and scanned using a GenePix 4000B imager (Axion).

Statistical Analysis.

Statistical significance was calculated by unpaired Student's t test or ANOVA as appropriate, using Prism software (GraphPad). All $P \leq 0.05$ are considered significant and are referred to as such in the text.

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AUTHOR CONTRIBUTIONS

A.E.L., B.J.K., L.W.D., R.Q.M., S. B., H.M.T., and C.L.M. designed and conducted experiments. KMK performed bioinformatics analysis of 16S data. GL performed histological scoring of mouse intestinal tissue. C.D.M. conducted 16S sequencing and COE oversaw microbiota antigen array and provided reagents. C.L.M. designed and supervised the study and wrote the manuscript with input from A.E.L., L.W.D., and K.M.K.

REFERENCES

1. A. Roers *et al.*, T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J. Exp. Med.* **200**, 1289–1297 (2004).

2. Y. P. Rubtsov *et al.*, Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546–558 (2008).

3. L. Siewe *et al.*, Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. *Eur. J. Immunol.* **36**, 3248–3255 (2006).

4. M. C. Pils *et al.*, Monocytes/macrophages and/or neutrophils are the target of IL-10 in the LPS endotoxemia model. *Eur. J. Immunol.* **40**, 443–448 (2010).

5. E. Zigmond *et al.*, Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* **40**, 720–733 (2014).

6. P. Krause *et al.*, IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nat. Commun.* **6**, 7055 (2015).

7. E. O. Glocker *et al.*, Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N. Engl. J. Med.* **361**, 2033–2045 (2009).

8. V. Ling *et al.*, Cutting edge: Identification of GL50, a novel B7-like protein that functionally binds to ICOS receptor. *J. Immunol.* **164**, 1653–1657 (2000).

9. S. Wang *et al.*, Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* **96**, 2808–2813 (2000).

10. S. K. Yoshinaga *et al.*, Characterization of a new human B7-related protein: B7RP-1 is the ligand to the co-stimulatory protein ICOS. *Int. Immunol.* **12**, 1439–1447 (2000).

11. S. K. Yoshinaga *et al.*, T-cell co-stimulation through B7RP-1 and ICOS. *Nature* **402**, 827–832 (1999).

12. C. Dong *et al.*, ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* **409**, 97–101 (2001).

13. P. C. Dubois *et al.*, Multiple common variants for celiac disease influencing immune gene expression. *Nat. Genet.* **42**, 295–302 (2010).

14. A. Franke *et al.*, Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* **42**, 1118–1125 (2010).

15. L. Jostins *et al.*; International IBD Genetics Consortium (IIBDGC), Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119–124 (2012).

16. M. Hedl, A. Lahiri, K. Ning, J. H. Cho, C. Abraham, Pattern recognition receptor signaling in human dendritic cells is enhanced by ICOS ligand and modulated by the Crohn's disease *ICOSLG* risk allele. *Immunity* **40**, 734–746 (2014).

17. J. Y. Lee *et al.*, The transcription factor KLF2 restrains $CD4^+$ T follicular helper cell differentiation. *Immunity* **42**, 252–264 (2015).

18. J. P. Weber *et al.*, ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2. *J. Exp. Med.* **212**, 217–233 (2015).

19. K. Warnatz *et al.*, Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. *Blood* **107**, 3045–3052 (2006).

20. L. Roussel *et al.*, Loss of human ICOSL results in combined immunodeficiency. *J. Exp. Med. 215*, 3151–3164 (2018).

21. E. Ansaldo *et al.*, Akkermansia muciniphila induces intestinal adaptive immune responses during homeostasis. *Science* **364**, 1179–1184 (2019).

22. M. A. Koch *et al.*, Maternal IgG and IgA antibodies dampen mucosal T helper cell responses in early life. *Cell* **165**, 827–841 (2016).

23. J. J. Bunker *et al.*, Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science* **358**, eaan6619 (2017).

24. F. Fransen *et al.*, BALB/c and C57BL/6 mice differ in polyreactive IgA abundance, which impacts the generation of antigen-specific IgA and microbiota diversity. *Immunity* **43**, 527–540 (2015).

25. N. W. Palm *et al.*, Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* **158**, 1000–1010 (2014).

26. J. J. Bunker *et al.*, Innate and adaptive humoral responses coat distinct commensal bacteria with immunoglobulin A. *Immunity* **43**, 541–553 (2015).

27. M. Y. Zeng *et al.*, Gut microbiota-induced immunoglobulin G controls systemic infection by symbiotic bacteria and pathogens. *Immunity* **44**, 647–658 (2016).

28. O. L. Wijburg *et al.*, Innate secretory antibodies protect against natural Salmonella typhimurium infection. *J. Exp. Med.* **203**, 21–26 (2006).

29. A. J. Macpherson *et al.*, A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* **288**, 2222–2226 (2000).

30. Y. Cong, T. Feng, K. Fujihashi, T. R. Schoeb, C. O. Elson, A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 19256–19261 (2009).

31. K. Hirota *et al.*, Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat. Immunol.* **14**, 372–379 (2013).

32. S. Kawamoto *et al.*, Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* **41**, 152–165 (2014).

33. A. E. Landuyt, B. J. Klocke, T. B. Colvin, T. R. Schoeb, C. L. Maynard, Cutting edge: ICOS-deficient regulatory T cells display normal induction of *Il10* but readily downregulate expression of Foxp3. *J. Immunol.* **202**, 1039–1044 (2019).

34. G. M. Nava, H. J. Friedrichsen, T. S. Stappenbeck, Spatial organization of intestinal microbiota in the mouse ascending colon. *ISME J.* **5**, 627–638 (2011).

35. P. Van den Abbeele *et al.*, Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. *ISME J.* **7**, 949–961 (2013).

36. A. Riva *et al.*, A fiber-deprived diet disturbs the fine-scale spatial architecture of the murine colon microbiome. *Nat. Commun.* **10**, 4366 (2019).

37. C. L. Maynard *et al.*, Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat. Immunol.* **8**, 931–941 (2007).

38. K. Larimore, L. Liang, S. Bakkour, W. C. Sha, B7h-expressing dendritic cells and plasma B cells mediate distinct outcomes of ICOS costimulation in T cell-dependent antibody responses. *BMC Immunol.* **13**, 29 (2012).

39. C. L. Gigliotti *et al.*, ICOS-ligand triggering impairs osteoclast differentiation and function in vitro and in vivo. *J. Immunol.* **197**, 3905–3916 (2016).

40. D. S. Shouval *et al.*, Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **40**, 706–719 (2014).

41. J. L. Kubinak *et al.*, MyD88 signaling in T cells directs IgA-mediated control of the microbiota to promote health. *Cell Host Microbe* **17**, 153–163 (2015).

42. T. Castro-Dopico, M. R. Clatworthy, IgG and Fcγ receptors in intestinal immunity and inflammation. *Front. Immunol.* **10**, 805 (2019).

43. A. L. Hamilton *et al.*, Serologic antibodies in relation to outcome in postoperative Crohn's disease. *J. Gastroenterol. Hepatol.* **32**, 1195–1203 (2017).

44. M. J. Lodes *et al.*, Bacterial flagellin is a dominant antigen in Crohn disease. *J. Clin. Invest.* **113**, 1296–1306 (2004).

45. L. W. Duck *et al.*, Isolation of flagellated bacteria implicated in Crohn's disease. *Inflamm. Bowel Dis.* **13**, 1191–1201 (2007).

46. B. S. Christmann *et al.*, Human seroreactivity to gut microbiota antigens. *J. Allergy Clin. Immunol.* **136**, 1378–1386.e1-5 (2015).

47. Y. Burmeister *et al.*, ICOS controls the pool size of effector-memory and regulatory T cells. J. Immunol. **180**, 774–782 (2008).

48. F. Guo, C. Iclozan, W. K. Suh, C. Anasetti, X. Z. Yu, CD28 controls differentiation of regulatory T cells from naive CD4 T cells. *J. Immunol*. **181**, 2285–2291 (2008).

49. M. Kornete, E. Sgouroudis, C. A. Piccirillo, ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. *J. Immunol.* **188**, 1064–1074 (2012).

50. S. A. Redpath *et al.*, ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production during helminth infection. *Eur. J. Immunol.* **43**, 705–715 (2013).

51. A. Swidsinski *et al.*, Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. *Inflamm. Bowel Dis.* **13**, 963–970 (2007).

52. B. J. Laidlaw *et al.*, Interleukin-10 from $CD4^+$ follicular regulatory T cells promotes the germinal center response. *Sci. Immunol.* **2**, eaan4767 (2017).

53. M. B. Geuking *et al.*, Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity* **34**, 794–806 (2011).

54. R. Kühn, J. Löhler, D. Rennick, K. Rajewsky, W. Müller, Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263–274 (1993).

55. C. L. Maynard, C. T. Weaver, Intestinal effector T cells in health and disease. *Immunity* **31**, 389–400 (2009).

56. C. O. Elson *et al.*, Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol. Rev.* **206**, 260–276 (2005).

57. M. C. Kullberg *et al.*, Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. *Infect. Immun.* **66**, 5157–5166 (1998).

58. L. Zhang *et al.*, Natural colonization with Helicobacter species and the development of inflammatory bowel disease in interleukin-10-deficient mice. *Helicobacter* **10**, 223–230 (2005).

59. M. Xu *et al.*, c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* **554**, 373–377 (2018).

60. K. Atarashi *et al.*, Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* **500**, 232–236 (2013).

61. J. L. Round *et al.*, The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* **332**, 974–977 (2011).

62. S. G. Jeon *et al.*, Probiotic Bifidobacterium breve induces IL-10-producing Tr1 cells in the colon. *PLoS Pathog.* **8**, e1002714 (2012).

63. H. Q. Tran, R. E. Ley, A. T. Gewirtz, B. Chassaing, Flagellin-elicited adaptive immunity suppresses flagellated microbiota and vaccinates against chronic inflammatory diseases. *Nat. Commun.* **10**, 5650 (2019).

64. K. Kobayashi *et al.*, An FcRn-dependent role for anti-flagellin immunoglobulin G in pathogenesis of colitis in mice. *Gastroenterology* **137**, 1746–1756.e1741 (2009).

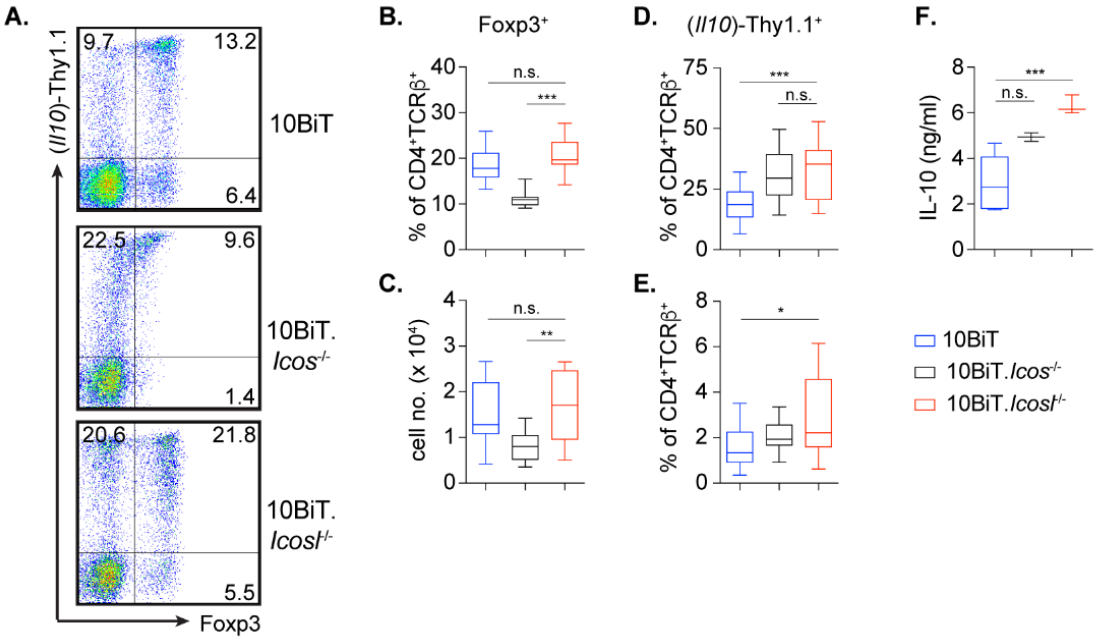
65. T. Castro-Dopico *et al.*, Anti-commensal IgG drives intestinal inflammation and type 17 immunity in ulcerative colitis. *Immunity* **50**, 1099–1114.e10 (2019).

66. J. Benckert *et al.*, The majority of intestinal IgA+ and IgG+ plasmablasts in the human gut are antigen-specific. *J. Clin. Invest.* **121**, 1946–1955 (2011).

67. M. Uo *et al.*, Mucosal CXCR4+ IgG plasma cells contribute to the pathogenesis of human ulcerative colitis through FcγR-mediated CD14 macrophage activation. *Gut* **62**, 1734–1744 (2013).

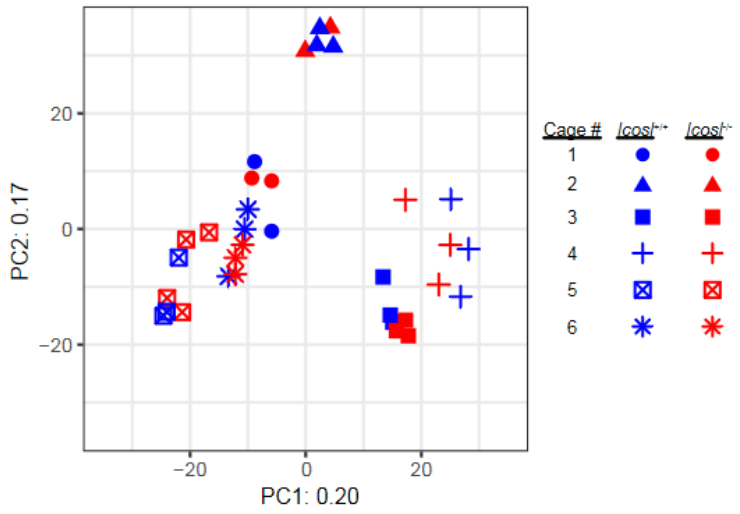
68. S. A. Houston *et al.*, The lymph nodes draining the small intestine and colon are anatomically separate and immunologically distinct. *Mucosal Immunol.* **9**, 468–478 (2016).

SUPPLEMENTARY FIGURES



Supplemental Fig. 1: Differential effects of ICOS-and ICOSL-deficiency on colonic Treg cells. (**A**) Representative FACS plots showing expression of Foxp3 and Thy1.1 by CD4+TCRβ+cells from the colonic lamina propria of 10BiT, 10BiT.Icos-/-and 10BiT.Icosl-/-mice. Box and whisker plots illustrate frequencies and numbers respectively of Foxp3+ cells (**B** and **C**) and Thy1.1+ cells (**D** and **E**), analyzed as in (A). 10BiT, n=15; 10BiT.Icos-/-, n=11, 10BiT.Icosl-/-, n=13. Data are compiled from four independent experiments. (**F**) IL-10 measured by ELISA of CD4 T cells isolated from colonic lamina propria of 10BiT, 10BiT.Icos-/-and 10BiT.Icosl-/-mice and stimulated for 24 hours with anti-CD3 and anti-CD28. 10BiT, n=5; 10BiT.Icos-/-, n=2, 10BiT.Icosl-/-, n=3. Data are from one of 2 independent experiments. Error bars represent mean ± SD; p values were calculated by ANOVA followed by Tukey's multiple-comparisons test. *p<0.05, **p<0.01, ***p<0.001.

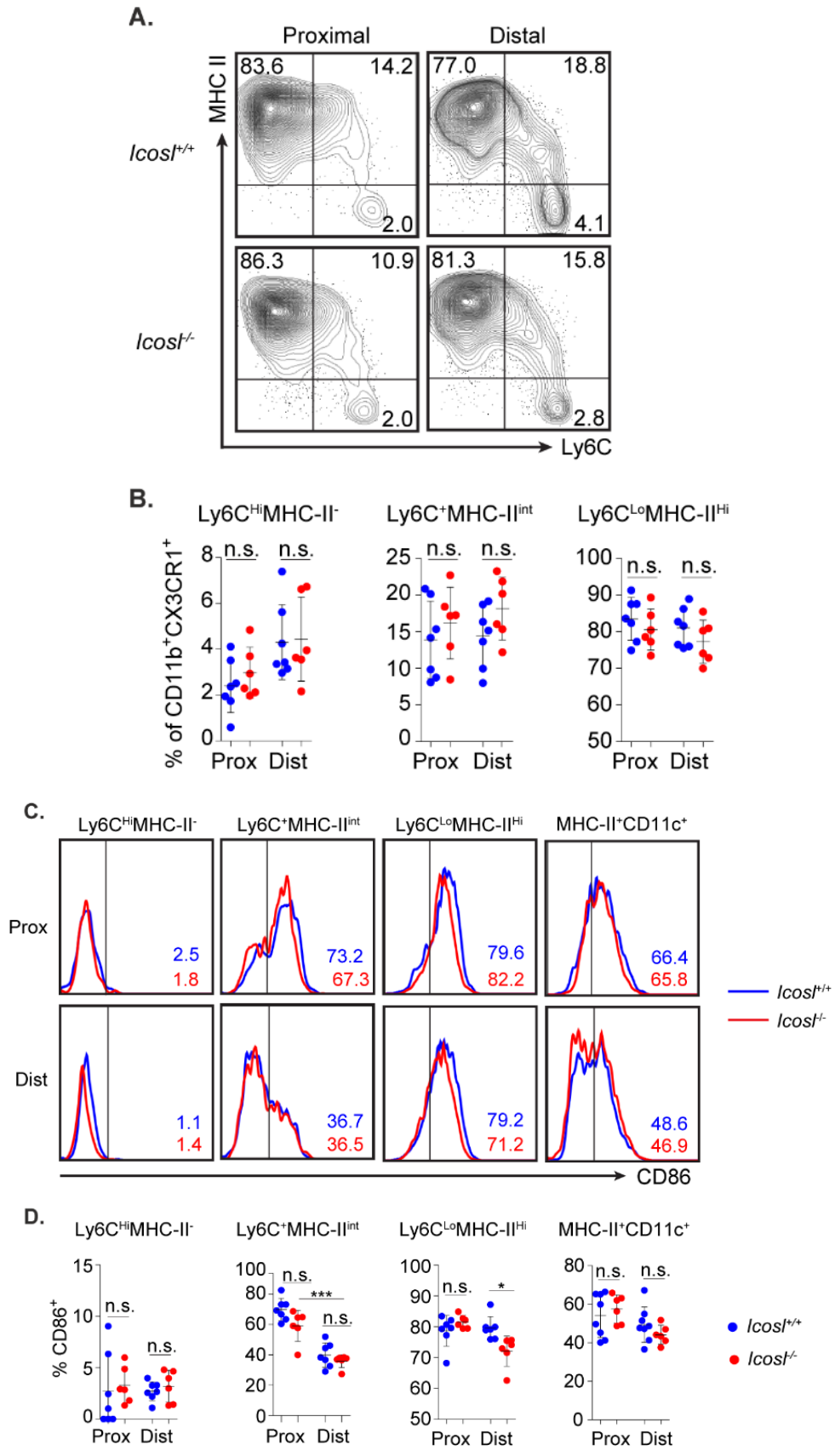
A.



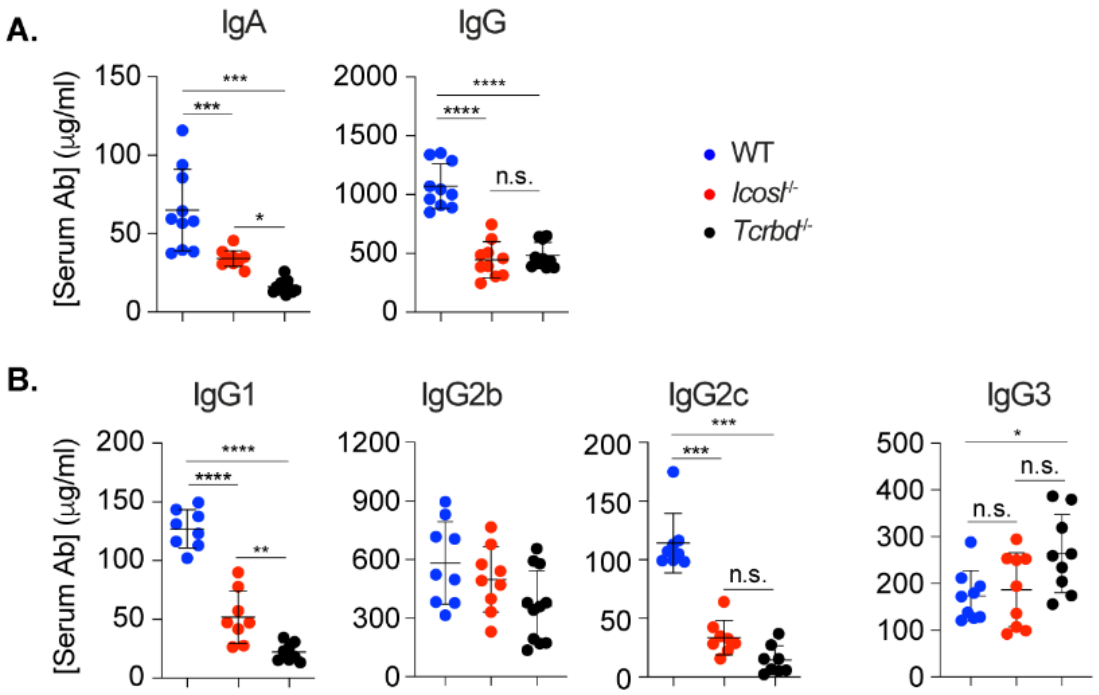
B.

	df	SS	MS	F	R^2	p-value
experiment	2	15302.907	7651.454	10.845	0.319	0.001
experiment:genotype	3	2892.404	964.135	1.367	0.060	0.134
experiment:cage	3	11530.564	3843.521	5.448	0.240	0.001
experiment:cage:genotype	3	2758.205	919.402	1.303	0.057	0.175
Residuals	22	15521.072	705.503		0.323	
Total	33	48005.153				

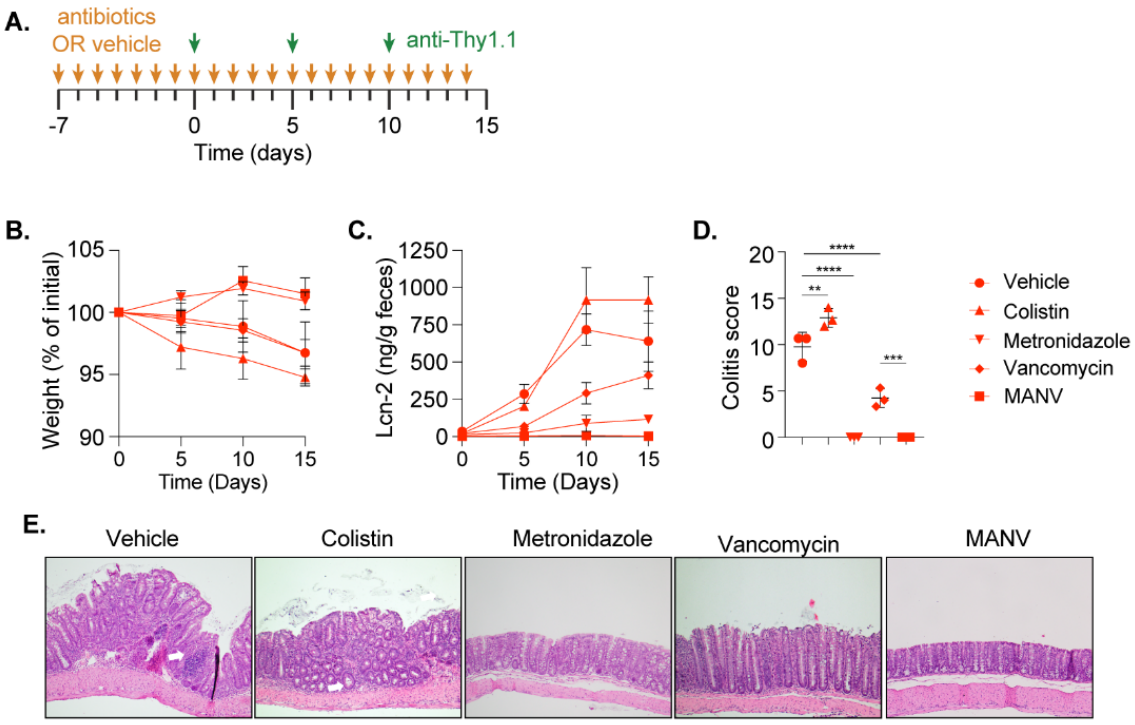
Supplemental Fig. 2: Comparison of fecal microbiota composition of Icosl+/+and Icosl-/- cage mates. (**A**). Principal coordinates analysis (PCoA) of the gut microbiota of Icosl+/+ and Icosl-/-mice from three separate replicate experiments based on the centered log-ratio Aitchison distance. Three replicate experiments were conducted in which Icosl+/+ and Icosl-/-mice were co-housed starting at weaning (day 21 of life). In the PCoA plots, samples that are closer together are more similar in microbial composition. Genotype is signified by color (blue: Icosl+/+; red: Icosl-/-), and samples represented by identical shapes were co-housed. (**B**). Permutational multivariate analysis (PERMANOVA) was used to assess the association between genotype and gut microbial composition while accounting for the variation due to cage effects and stratifying permutations by experiment replicate. Genotype did not significantly affect the composition of the gut microbiota when accounting for covariates (experiment: genotype, PERMANOVA R^2= 0.060, p= 0.1; experiment:cage:genotype, PERMANOVA R^2= 0.057, p= 0.2). Experiment replicate (R2= 0.319, p= 0.001 and the interaction between experimental replicate and cage effects (experiment:cage, PERMANOVA R^2= 0.240, p= 0.001) accounted for a large proportion of the variation in gut microbial community composition.


A.
Proximal
Distal
MHC II
Icosl+/+
Icosl-/-
83.6
14.2
2.0
77.0
18.8
4.1
86.3
10.9
2.0
81.3
15.8
2.8
Ly6C
B.
Ly6CHiMHC-II-
Ly6C+MHC-IIint
Ly6CLoMHC-IIHi
% of CD11b+CX3CR1+
n.s.
Prox
Dist
C.
Ly6CHiMHC-II-
Ly6C+MHC-IIint
Ly6CLoMHC-IIHi
MHC-II+CD11c+
Prox
Dist
2.5
1.8
73.2
67.3
79.6
82.2
66.4
65.8
1.1
1.4
36.7
36.5
79.2
71.2
48.6
46.9
CD86
Icosl+/+
Icosl-/-
D.
Ly6CHiMHC-II-
Ly6C+MHC-IIint
Ly6CLoMHC-IIHi
MHC-II+CD11c+
% CD86+
n.s.
***
*
Prox
Dist
Icosl+/+
Icosl-/-

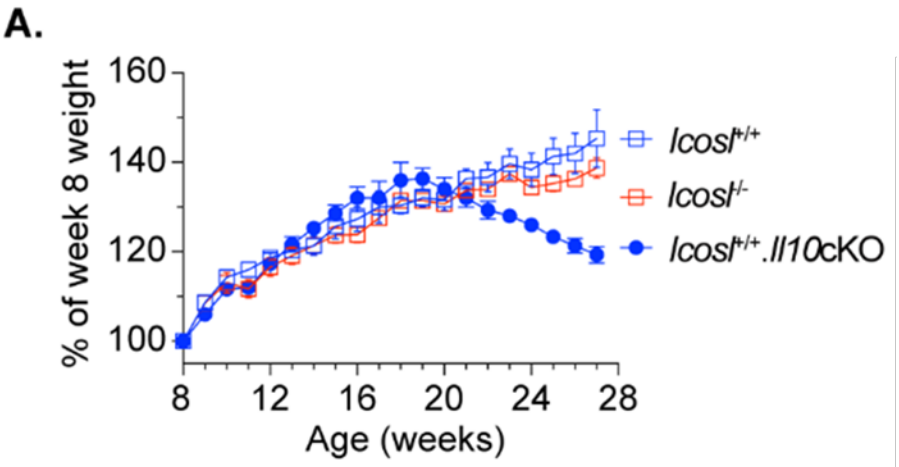
Supplemental Fig. 3: ICOSL deficiency does not result in increased or hyper-activated myeloid cells in the colonic lamina propria. (**A**) Representative flow plots showing colonic monocytes:MHC-II^{int} Ly6C+, newly differentiated inflammatory macrophages: MHC-II^{int}Ly6C+, and mature macrophages: MHC-II+ Ly6Clo;among CD11b+ CX3CR1+ cells in the proximal or distal colon of Icosl+/+ and Icosl-/-mice (**B**) Graphs displaying frequencies of cell subsets as depicted in A. (**C**) CD86 expression by cell subsets defined in A and by dendritic cells (MHC-IIhi CD11c+) in the proximal or distal colon of Icosl+/+and Icosl-/-mice. (**D**) Graphs depicting frequencies of CD86+ cells as shown in C.



Supplemental Fig. 4: Comparable effects of TCR-and ICOSL-deficiency on circulating antibody levels at steady state. (**A**) ELISA of IgA and IgG in serum from WT, Icosl-/-,or Tcrbd-/-mice. n=10 mice per group. (**B**) ELISA of IgG1, IgG2b, IgG2c, or IgG3 in serum of WT, Icosl-/-,or Tcrbd-/-mice. WT, n=9; Icosl-/-, n=10; Tcrbd-/-, n=9.



Supplemental Fig. 5: Colitis induced in ICOSL-deficient mice can be inhibited by pre-treatment with metronidazole. (**A**) Overview of experimental design. 10BiT mice were administered normal drinking water (vehicle) or colistin, metronidazole, vancomycin, or MANV (metronidazole, ampicillin, neomycin, vancomycin) daily from day -7 through day 15. All mice received anti-Thy1.1 on days 0, 5, and 10. (**B**) Relative weight changes, and (**C**) fecal Lcn2 levels, as determined every 5 days. (**D**) Total colitis scores and (**E**) representative histology of H&E-stained colonic tissue sections from one of three independent experiments at day 15, n=3-4 mice/group. Error bars represent mean ± SD; p values were calculated by two-way ANOVA for repeated measures with Bonferroni correction(B and C) or by one-way ANOVA test followed by Tukey's multiple-comparisons test (D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



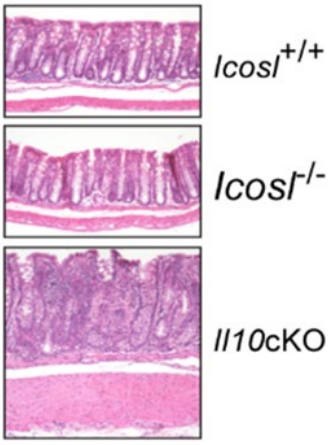
B.

Males:

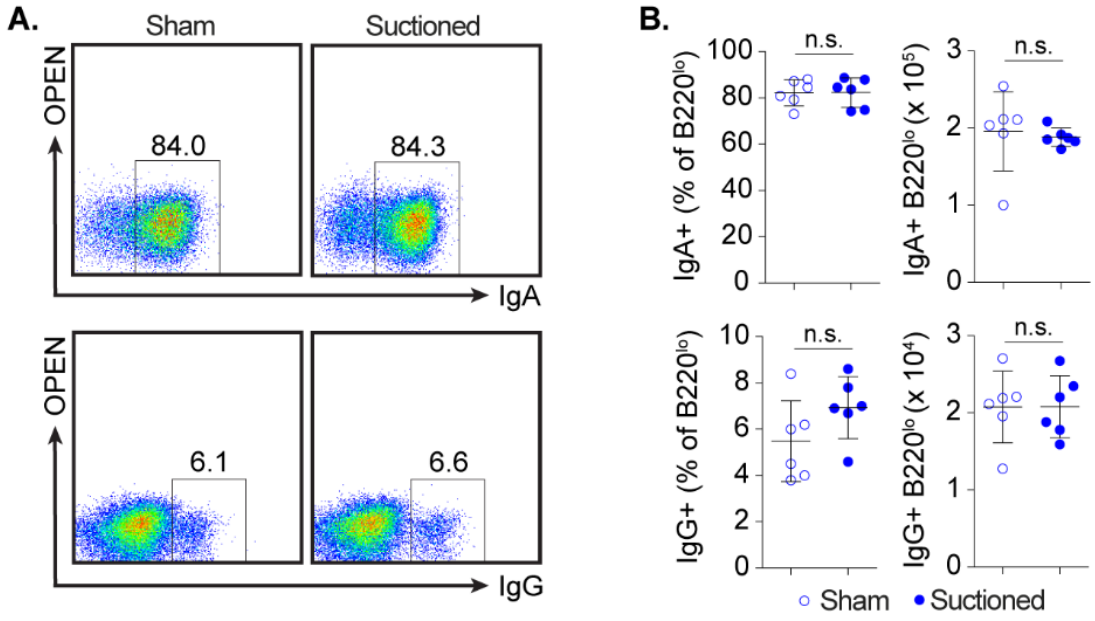
Genotype	*Icosl*+/+	*Icosl*-/-	*Il10*cKO
Colitic	0/6	0/5	5/5

Females:

Genotype	*Icosl*+/+	*Icosl*-/-	*Il10*cKO
Colitic	0/4	0/5	5/5



Supplemental Fig. 6: As expected, Il10cKO mice develop adult-onset colitis. (**A**) Weight charts of Icosl+/+, Icosl-/-, and Icosl+/+.Il10cKO mice monitored weekly from 8 weeks of age and analyzed grossly for presence or absence of severe colitis at 28 weeks (**B**). (**C**) Representative photomicrographs of H&E-stained colon tissue from the 3 groups represented. n=10 mice/group.



Supplemental Fig. 7: Mucus suctioning does not disrupt lamina propria plasma cells. Each colon was bisected longitudinally, and one half underwent sham suction without applied vacuum (Sham) while the other half had mucus collected by gentle suction (Suctioned). Remaining tissue was then enzymatically-digested to release total lamina propria cells. (**A**) Representative flow cytometry plots showing IgA and IgG expression by B220lo cells from the lamina propria of the colon. (**B**) Graphs summarizing frequencies and numbers of B220lo cells expressing IgA or IgG of individual mice, collected as depicted in (A). Error bars represent mean ± SD and p values were calculated by unpaired Student's t test; *p<0.05, **p<0.01, ***p<0.001.

SI References.

1. Kumar R, *et al.* (2014) Getting started with microbiome analysis: sample acquisition to bioinformatics. *Curr Protoc Hum Genet* 82:18 18 11-29.

2. Wang Q, Garrity GM, Tiedje JM, & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73(16):5261-5267.

3. Cole JR, *et al.(*2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* 37(Database issue):D141-145.

4. McDonald D, *et al*. (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 6(3):610-618.

CONCLUSION

IBD and the Role for ICOSL

In the two presented studies, we sought to elucidate the mechanisms underlying the association between GWAS-identified SNP in *ICOSLG* and IBD. This particular SNP in *ICOSLG* is a loss-of-function mutation that manifests as reduced expression of ICOSL, even in heterozygotes. Stimulation of ICOS by ICOSL has been inextricably linked to CD4 T cell production of IL-10 in past literature and defects in the IL-10 signaling pathway have been identified as directly contributive to IBD [1, 2]. As such, the mechanistic explanation behind this SNP has been discounted in the past as a simple reduction of ICOS-ICOSL signaling leading to insufficient IL-10 to prevent intestinal inflammation [3]. However, while IL-10 deficient mice develop spontaneous colitis, ICOS or ICOSL-deficient mice do not. Our findings in the included studies may partially explain this discrepancy through challenging the preconceived notion that ICOS stimulation is requisite for efficient CD4 T cell IL-10 expression. We have determined that ICOS-deficient and ICOSL-deficient mice have equal or elevated amounts of CD4 T cells expressing IL-10 in the colonic lamina propria (cLP) as compared to WT mice. Thus, the influence of the IBD-correlated *ICOSL* SNP may not be simply attributed to reduced intestinal IL-10.

ICOS Controls Treg Cell Stability Through *Foxp3* Demethylation

In addition to IL-10 production, ICOS has been implicated in controlling the balance of central and effector Treg cell populations and prolonging Treg cell survival [4, 5]. Treg cells are a major source of intestinal IL-10 and are highly concentrated in IBD lesions [6, 7]. While ICOS-deficient (*Icos*$^{-/-}$) mice are known to have fewer Treg cells, observations of this have largely been restricted to the thymus and secondary lymphoid tissue [8, 9]. Our first presented study identifies that the disparity in Treg cells between *Icos*$^{-/-}$ and WT mice is even more pronounced in the cLP than in the thymus, spleen, or small intestine. However, as noted, this is not at the expense of overall IL-10 production as *Icos*$^{-/-}$ mice have an increased IL-10 producing Foxp3^{-} CD4 T cell population to offset the diminished Foxp3^{+} contribution. This Foxp3^{-} population may well be compensatory, as IL-10 producing CD4 T cells increase with age in *Icos*$^{-/-}$ mice, much like the documented phenomena of increased Treg cells in periphery of aging WT mice [10, 11]. More Treg cells in the *Icos*$^{-/-}$ cLP expressed Helios, which is commonly used marker that denotes, with exceptions, thymically-derived Treg (tTreg) cells that emerge from the thymus expressing Foxp3 [12, 13]. We also found significantly more T cell receptor excision circles (TRECs) in Foxp3^{+} cells from the spleen of *Icos*$^{-/-}$ mice than WT. As such, Treg cells in the spleen of *Icos*$^{-/-}$ mice were more likely to be of recent thymic origin. Foxp3^{+} cell frequencies within the thymus of *Icos*$^{-/-}$ mice were also not significantly different than those of WT mice. These data strongly suggest that the Treg defect observed in *Icos*$^{-/-}$ mice does not manifest primarily in the tTreg cell population, but rather in T cells that

develop into Foxp3^{+} Treg cells in peripheral tissues, peripherally-derived Treg (pTreg) cells. In fact, the elevated Helios^{+} population in the cLP and increased TREC counts imply that *Icos*$^{-/-}$ mice likely have elevated output of tTreg cells. One mechanism by which this could occur is the documented feedback loop in which mature Treg cells from the periphery return to the thymus to constrain further Treg cell production [14]. As *Icos*$^{-/-}$ mice have fewer Treg cells in other tissues, there are not as many available to suppress thymic Treg cell production. Regardless, peripherally-derived Treg (pTreg) cells are impacted by ICOS-deficiency. We established this using CBir1 TCR transgenic mice which, with other TCR rearrangement prohibited through an additional RAG-1 deficiency, possess only T-cell receptors specific for microbiota derived antigen CBir1 [15]. As such, these mice cannot generate tTreg cells but still make pTreg cells, of which the population was reduced in the absence of ICOS. Peripherally-derived Treg cells are particularly important for suppressing intestinal inflammation [16]. Given the reduced pTreg cell population in *Icos*$^{-/-}$ mice, we sought to determine if ICOS-deficiency affected the ability of Treg cells to restrain colitis. We utilized the CD45RB^{hi} CD4 T cell transfer model, in which transfer of CD45RB^{hi} CD4 T cells into RAG-deficient mice causes colitis 5-8 weeks after transfer [17]. Transfer of WT Treg cells into these CD45RB^{hi} cell recipients can either prevent imminent disease or reverse already ongoing colitis [18]. We examined the comparative functional capacity of *Icos*$^{-/-}$ Treg cells to prevent disease through cotransfer of either WT or *Icos*$^{-/-}$ Treg cells with the CD45RB^{hi} cells. Additionally, we investigated the ability of *Icosl*$^{-/-}$ Treg cells to suppress a pre-existing inflammatory environment through transfer of WT or *Icos*$^{-/-}$ Treg cells into CD45RB^{hi} recipients after colitis onset. While *Icos*$^{-/-}$ Treg were enough to prevent colitis when

cotransferred with the CD45RB^{hi} cells, transfer of *Icos*$^{-/-}$ Treg cells into already colitic mice could not reverse ongoing intestinal inflammation.

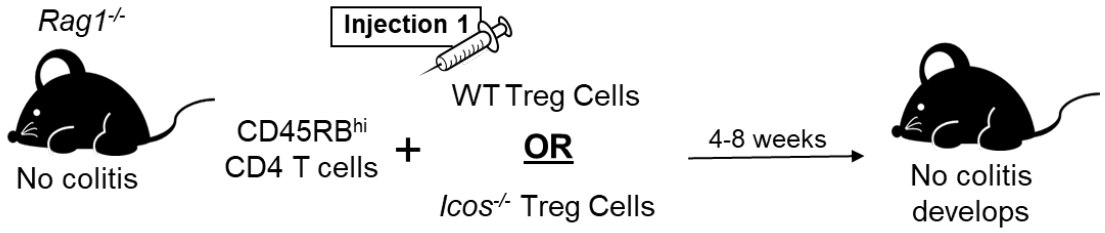


Figure 1: ICOS-deficient Treg cells function to maintain homeostasis. Experimental design and results of co-transferring either WT Treg cells or *Icos*$^{-/-}$ Treg cells with the CD45RB^{hi} cells employed in the CD45RB^{hi} colitis model. Both WT and *Icos*$^{-/-}$ Treg cells were equally capable of preventing intestinal inflammation from developing.

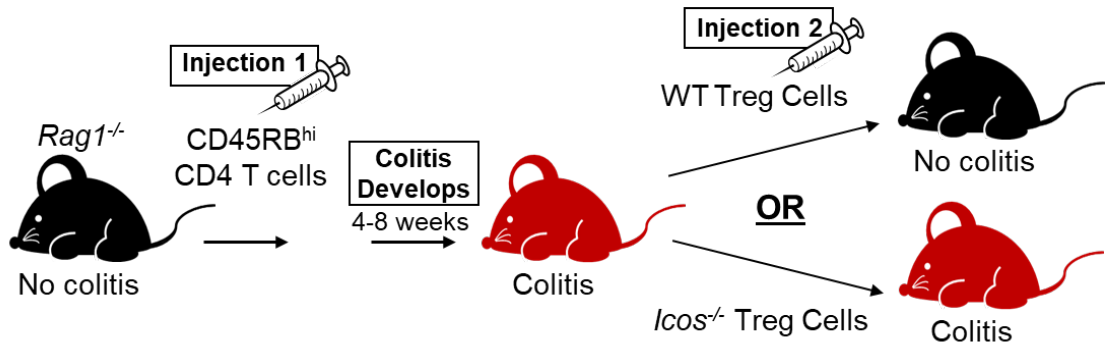


Figure 2: ICOS-deficient Treg cells are incapable of reversing pre-established intestinal inflammation. Experimental design and results of transferring either WT Treg cells or *Icos*$^{-/-}$ Treg cells into colitic hosts from the CD45RB^{hi} colitis model. Transfer of *Icos*$^{-/-}$ Treg cells, unlike that of WT Treg cells, did not mitigate colitis.

Furthermore, upon reisolation of the transferred Treg cells from these mice, we observed equal numbers but massively reduced Foxp3 expression in the *Icos*$^{-/-}$ cells. The similar number of reisolated cells despite the lower frequency of Foxp3^{+} cells led us to hypothesize that, rather than exhibiting impaired survival, ICOS-deficient Treg cells were losing expression of Foxp3. This would explain the reduced functionality of *Icos*$^{-/-}$ Treg cells, as Foxp3 is the "master transcription factor" of Treg cells and plays a major role in maintaining their suppressive capacity. Stability of Foxp3 expression can be approximated by investigating the methylation status of the Treg-specific demethylated region (TSDR) in conserved noncoding sequence 2 (CNS2) of the *Foxp3* locus. Demethylation of CNS2 promotes stable expression of Foxp3 and is so critical to Treg cell function that mice with deletion of CNS2 develop systemic autoimmunity akin to Foxp3-deficient (*Foxp3*$^{-/-}$) mice [17, 18]. In fact, while transfer of WT Treg cells generally prevents the death of *Foxp3*$^{-/-}$ mice from systemic autoimmunity, we observed that transfer of *Icos*$^{-/-}$ Treg cells did not. Bisulfite sequencing performed on Treg cells from WT or *Icos*$^{-/-}$ mice revealed that, while WT Treg cells possessed a properly demethylated CNS2, the CNS2 region in *Icos*$^{-/-}$ Treg cells was methylated to the same degree as in naïve T cells. Collectively, the first study of this dissertation demonstrates a novel role for ICOS stimulation in the epigenetic stability of Treg cells and the functional relevance of this in managing intestinal inflammation.

Significance of Stable Foxp3 Expression by Treg Cells

The stability and number of Treg cells has long been an issue in the fields of autoimmunity and organ transplantation. Transfer of Treg cells can reverse multiple

murine models of autoinflammatory conditions. As such, Treg therapy holds appeal for potential treatment of similar human conditions and graft rejection. However, one of the barriers to such therapy is the production of large amounts of stable autologous Treg cells. Endogenous Treg cells can be expanded and aid in graft survival through low-dose treatments with IL-2 but this, obviously, is a systemic treatment [19]. Expansion of *ex* vivo Treg cells is possible, but their strong reliance on IL-2 can render their replication *in vitro* slower than conventional T cells [20]. In order to efficiently generate antigen-specific Treg cells and control their exposure to various stimuli, *in vitro* development of Treg cells from naïve autologous T cells would be ideal. However, assuring that such Treg cells possess stable expression of Foxp3 before transfer is also a concern. There has been some evidence that *in vitro* Treg (iTreg) cells may lose Foxp3 expression and gain conventional T cell functions to secrete proinflammatory cytokines, which could be disastrous for patient use [21, 22]. However, such results may be attributable to small contaminating populations of uncommitted Treg cells that have not yet demethylated CNS2 and fully committed to a regulatory phenotype [23]. Such loss of Foxp3 expression and insufficient demethylation might be attributable to the common use of only anti-CD3 and anti-CD28 as stimulation sources for the development of iTreg cells [24]. Although eliminating the additional variables that would accompany interfacing with a dendritic cell, additional costimulatory molecules may be required to generate stable iTreg cells that maintain Foxp3 expression and function properly. In this manner, our findings that ICOS contributes to epigenetic stability of Treg cells may be one more step toward the prospect of customized iTreg therapy. Some questions about the role of ICOS stimulation in Treg cell stability remain, however. One of these is the timing with which ICOS

expression and ICOSL provision is required for the demethylation of the CNS2 region. Thymically-derived Treg cells progressively undergo demethylation in the thymus and ICOSL is readily available on MHC Class II -expressing cells at the site. However, *Icos*$^{-/-}$ mice did not appear to have reduced tTreg populations. Additional demethylation is provided outside of the thymus, as evidenced by the difference in CNS2 demethylation between Treg cells from the thymus directly and those from the spleen [24]. ICOSL is also provided by mononuclear phagocytes (MNPs) in the colonic lamina propria (cLP) and could potentially aide the development of stably demethylated pTreg cells there. While ICOS signals could imprint stability during early Treg development, Treg cell demethylation is an actively maintained process. This is evidenced by how mature Treg cells that lose certain factors, like Uhrf1, remethylate Foxp3 and associated Treg genes [25]. As such, ICOS stimulation may be required in more tonic capacity, much like TCR stimulation, for the maintenance of the Treg cell phenotype [26, 27]. The exact mechanism by which ICOS affects methylation of the TSDR is also unknown. ICOS is not yet associated with regulation of the methyltransferases, like DMAT1, or dioxygenases, like Tet2 and Tet3, that modulate Foxp3 expression. To link ICOS directly to TSDR demethylation will require further investigation into Treg cell epigenetic management. Interestingly, given the implied sufficiency of tTreg production but deficit in pTreg populations provided by our work in *Icos*$^{-/-}$ mice, it is possible for methylation to be regulated differently in tTreg versus pTreg populations. Mice deficient in the chromatin-remodeling complex recruiter Mbd2 generate tTreg populations with a stably demethylated TSDR but cannot recruit Tet2 to the TSDR to demethylate pTreg cells [28]. Although avenues for further investigation abound, we have identified a novel role for

ICOS in Treg cell CNS2 demethylation in this study. These findings highlight the involvement of epigenetics in regulation of intestinal inflammation and may eventually contribute to the attainment of stable iTreg cell generation. Furthermore, this study challenged preconceptions about the relationship of ICOS to CD4 T cell IL-10 production. This prompted us to further investigate the link by which the loss-of-function mutation in the loci encoding ICOSL could contribute to IBD incidence.

ICOSL-Deficiency Mimics T Cell Deficiency in Antibody Responses to Commensals

The second study presented in this dissertation also investigates the relation between ICOS-ICOSL signaling and intestinal inflammation, but with a focus on humoral immunity. ICOSL is profoundly important for the production of high-affinity T-dependent antibodies. IBD patients generally have increased levels of antibodies binding the microbiota. ICOSL-deficient (*Icosl*$^{-/-}$) mice hence present an excellent opportunity to examine T-dependent anti-commensal antibodies and how they relate to maintaining intestinal homeostasis. One of the benefits of using *Icosl*$^{-/-}$ mice in such investigations is that *Icosl*$^{-/-}$ mice have T cells, unlike the T cell deficient (*Tcrbd*$^{-/-}$) mice often used to examine effects of T-dependent antibodies. As IBD is commonly thought of as a CD4 T cell driven condition, maintaining the presence of the T cell immune compartment was preferable for our gut-focused inquiries. Notably, unlike their *Icos*$^{-/-}$ counterparts, *Icosl*$^{-/-}$ mice in our colony did not have an observed Treg cell deficiency. The frequency and number of Treg cells in the *Icosl*$^{-/-}$ cLP was not significantly different than WT. Additionally, preliminary bisulfite sequencing results have shown that *Icosl*$^{-/-}$ Treg cells are demethylated at CNS2 to the same or greater degree as WT Treg cells. Because ICOS

and ICOSL are thought to form a monogamous receptor-ligand pair in mice, the reason that the Treg populations differ between our *Icos*$^{-/-}$ and *Icosl*$^{-/-}$ mice remains yet unknown. One potential reason for the difference could be a cell-intrinsic need for ICOS expression to allow TSDR demethylation. ICOS is internalized upon ligation and is recycled or directed to the lysosome depending on whether TCR signals are available or not [29, 30]. Presumably, there is some baseline receptor turnover and ICOS could, in theory, have some activity inside the cell. This remains unlikely since no such role has been described for ICOS or CTLA4, which is internalized with similar mechanisms, to date [31, 32]. The reduced Treg frequencies in *Icos*$^{-/-}$ but not *Icosl*$^{-/-}$ mice may instead indicate a yet undocumented interaction between ICOS and another ligand. Our unpublished observations, in combination with the fact that ICOS is internalized upon interaction with ICOSL, suggest that ICOSL-deficient animals may possess higher levels of surface ICOS. If so, increased availability of the protein may cause interaction artifacts that result in a Treg cell difference between the two genotypes. Nevertheless, although *Icosl*$^{-/-}$ mice do not share the same Treg defect exhibited by *Icos*$^{-/-}$ mice, they do still exhibit deficiencies in T-dependent antibodies.

Icosl$^{-/-}$ mice have similar serum levels of IgA and IgG isotypes to *Tcrbd*$^{-/-}$ mice; despite significant differences in IgA and IgG1, these levels exhibited by *Icosl*$^{-/-}$ mice are still more similar to *Tcrbd*$^{-/-}$ mice than WT. Upon examination of the colon, we found no significant difference in the frequency of cLP IgA^{+} or IgG^{+} B220^{lo} cells, the population which includes plasma cells (PC), between *Icosl*$^{-/-}$ mice and *Tcrbd*$^{-/-}$ mice. Additionally, while *Tcrbd*$^{-/-}$ mice do not have germinal centers by virtue of not having T cells, *Icos*$^{-/-}$ mice are known to have small and poorly organized ones [33]. As such, it was unsurprising

to us that the lymph nodes draining various regions of the colon exhibited far fewer Tfh cells and GC B cells in *Icosl*$^{-/-}$ mice than possessed by WT mice. This disparity between *Icosl*$^{-/-}$ mice and WT was particularly pronounced in the node draining the proximal colon. Focusing even more directly on the host-commensal interface, we assessed antibody content of colonic mucus in *Icosl*$^{-/-}$ mice. ICOSL-deficient mice possessed far less IgG in the mucus than WT mice, akin to *Tcrbd*$^{-/-}$ mouse levels and with even lower IgG3 than those. As such, ICOSL-deficient mice presented a model in with humoral immune deficiencies similar to T cell-deficient mice, including at the interface between the host and gastrointestinal commensals. Since these mice still retain the T cell immune compartment and have no deficit in Treg cells, they presented an excellent opportunity to further examine the effect of T-dependent antibodies on intestinal inflammation.

Although the general level of mucus IgA detected in our second study was similar between *Icosl*$^{-/-}$ and WT mice, our initial assessment of overall IgA content did not include evaluation of the quality of said antibody. Through ELISA using lysates of colonic contents and autologous serum, we determined that the IgA of *Icosl*$^{-/-}$ mice was less capable of binding their own microbiota. This deficit was also shared by *Icosl*$^{-/-}$ IgG and, while shared by binding to luminal bacterial lysates, was especially pronounced when examining antibody affinity for lysates of mucus-associated bacteria. The reduction of anti-commensal antibody quality in *Icosl*$^{-/-}$ mice was confirmed through microarray printed with various antigens associated with gastrointestinal microbes, including those canonically within the mucus layer. Serum from *Icosl*$^{-/-}$ mice had reduced IgA and IgG binding to various commensal antigens, including flagellins linked to CD such as A4Fla2 and Fla-X [34, 35]. Such flagellins compose flagella, whip-like structures that can grant

motility to allow colonization or penetration of the protective colonic mucus layer [36, 37]. Bacteria frequently coated with T-dependent high affinity IgA have also been implicated as colitogenic [38]. Given this, we hypothesized that the inability of antibodies from ICOSL-deficient mice to bind antigens belonging to potentially colitogenic or barrier-encroaching bacteria may render *Icosl*$^{-/-}$ mice more susceptible to colitis.

Elevated IL-10 Producing CD4 T Cell Populations in the ICOSL-Deficient Colon

As previously stated, *Icosl*$^{-/-}$ mice do not develop spontaneous colitis. One potential reason for this may come from the principal difference between *Icosl*$^{-/-}$ and *Tcrbd*$^{-/-}$ mice, the presence of T cells. In *Icosl*$^{-/-}$ mice, investigation into cLP CD4 T cells revealed that, not only do *Icosl*$^{-/-}$ mice have WT levels of Treg cells, but they possess elevated amounts of CD4 T cells producing IL-10. As CD4 T cells are a main source of IL-10 in the colon and IL-10 is a primarily immunosuppressive cytokine that reinforces the intestinal barrier, this increased production of CD4 T cell derived IL-10 compared to WT mice may help compensate for an underlying insufficiency. Interestingly, the IL-10 producing CD4 T cells were most concentrated in the proximal colon, the area for which colon draining lymph nodes in *Icosl*$^{-/-}$ mice had the greatest GC deficits. This led to our hypothesis that *Icosl*$^{-/-}$ mice, with their lack of ICOSL-dependent antibodies, are more reliant on IL-10 to suppress intestinal inflammation.

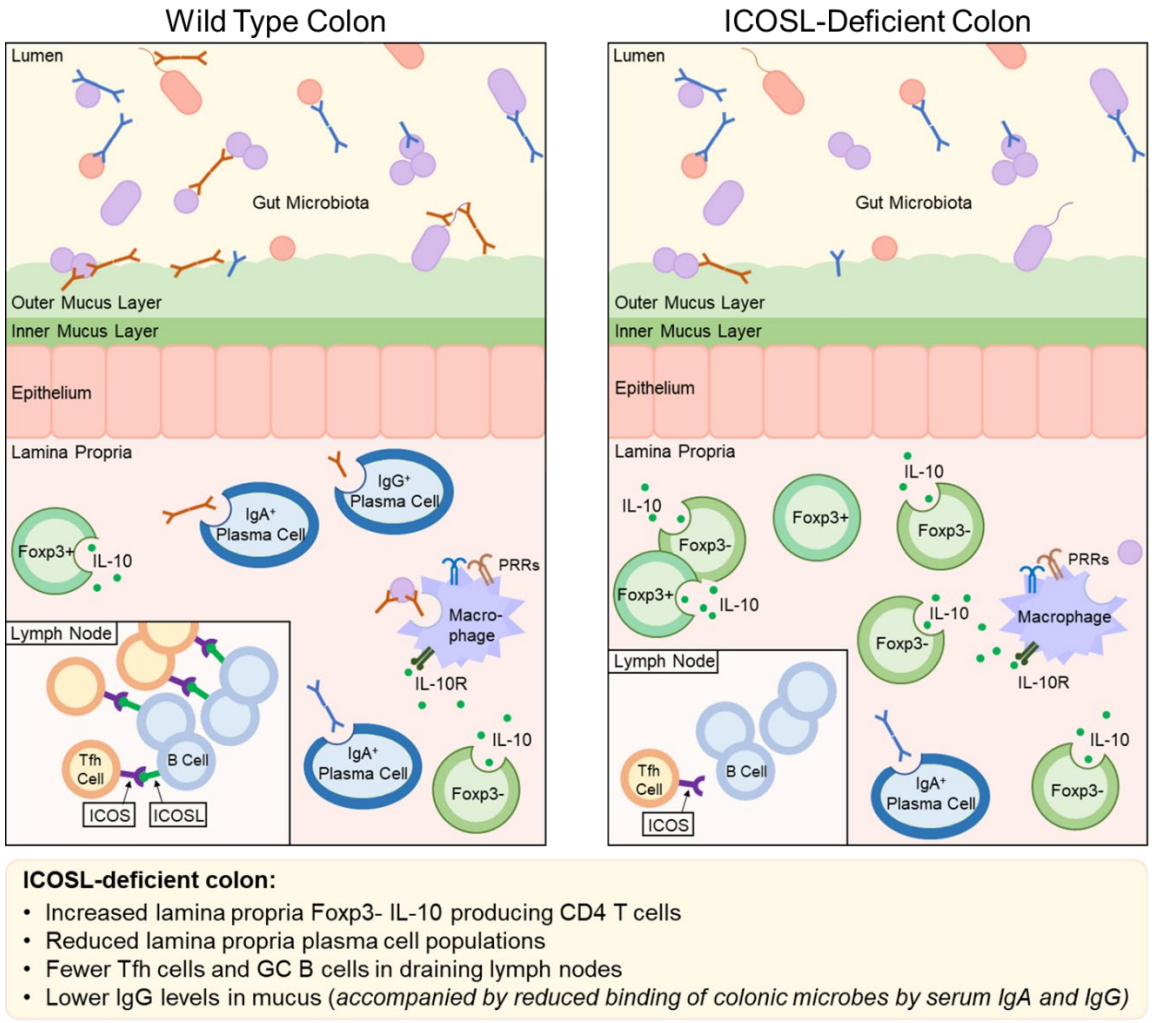


Figure 3: Comparison between the wild-type and ICOSL-deficient colonic environment. Visual representation of uninflamed WT or ICOSL-deficient colon. ICOSL-deficient *(Icosl*$^{-/-}$*)* mice have less IgG in colon mucus. Additionally, the serum IgG and IgA of *Icosl*$^{-/-}$ mice exhibits reduced binding to microbiota-derived antigens. *Icosl*$^{-/-}$ mice have fewer IgA^{+} and IgG^{+} plasma cells in the colonic lamina propria. The Tfh and GC B cell populations in colon draining lymph nodes of *Icosl*$^{-/-}$ mice are also diminished. Altogether, these data indicate *Icosl*$^{-/-}$ mice have impaired high-affinity T-dependent antibody (orange) responses toward the microbiota. The similar or greater total amount of IgA in *Icosl*$^{-/-}$ serum and mucus, however, suggest that T-independent antibody (blue) responses of these mice are intact. *Icosl*$^{-/-}$ mice also have more CD4 T cells producing IL-10 in the colon, most of which do not express Foxp3.

ICOSL Helps Suppress Early-Onset Intestinal Inflammation

To investigate the how this relationship between ICOSL-deficiency and CD4 T cell derived IL-10 influenced intestinal inflammation, we bred mice with a CD4 T cell restricted deficiency of IL-10 (*Il10*cKO), which generally develop spontaneous colitis with time, to also be deficient in ICOSL (*Icosl*$^{-/-}$.*Il10*cKO). We used fecal lipocalin-2 (Lcn-2), an anti-siderophore produced by neutrophils and activated IEC that correlates well with intestinal histology as a longitudinal readout of intestinal inflammation [39]. While IL10cKO mice took longer to develop disease, *Icosl*$^{-/-}$.*Il10*cKO mice exhibited elevated fecal Lcn-2 levels as early as 4 weeks of age. Histological assessment of these mice at 8 weeks of age also revealed significantly more intestinal inflammation than their *Il10*cKO counterparts. This demonstrated a role for ICOSL in suppressing colitis in the absence of CD4 T cell derived IL-10 and confirmed the increased reliance of *Icosl*$^{-/-}$ mice on this cytokine for intestinal health. Given the early age at which inflammation was detectable in *Icosl*$^{-/-}$.*Il10*cKO mice and our interest in whether protection conferred by ICOSL was antibody-mediated, we investigated if the provision of antibodies in early life impacted the observed colitis. Mouse pups that are fostered with different dams take on both the microbiota and milk of the foster dam and breastmilk is a major source of antibodies for nursing animals [40]. We confirmed that provision of milk from WT dams as opposed to *Icosl*$^{-/-}$ dams altered the antibody levels of fostered *Icosl*$^{-/-}$ pups via simply performing cross-fostering, swapping approximately half of the litters from WT and *Icosl*$^{-/-}$ dams to the dam of the opposing genotype within 24 hours of birth. Fostering restored the otherwise deficient mucus and serum IgG content in *Icosl*$^{-/-}$ pups to WT levels. However, mucus and serum IgG were also at normal levels in WT mice fostered

on *Icosl*$^{-/-}$ dams, indicating that host contribution is also a significant antibody source at this stage in life. Regardless, unfostered *Icosl*$^{-/-}$ pups had greatly reduced amounts of IgG in comparison to fostered but significantly higher serum and mucus IgA. This is likely attributable to compensatory production by the host, since neither fostered *Icosl*$^{-/-}$ pups or WT pups receiving *Icosl*$^{-/-}$ milk possessed these IgA levels and there is precedent for antibody isotypes being able to partially compensate for each other [41]. In fact, the capability of *Icosl*$^{-/-}$ mice to enact such compensation may explain the result earlier noted that adult levels of IgA in the colonic mucus were equivalent to that of WT mice. While levels of total IgG were similar in unfostered WT pups and those fostered with *Icosl*$^{-/-}$ dams, isotype distribution was different. Recipients of *Icosl*$^{-/-}$ milk had less serum IgG1, which has been priorly documented as largely T-dependent [42]. The impact that fostering had on colitis onset in *Icosl*$^{-/-}$.*Il10*cKO mice was striking. *Icosl*$^{-/-}$.*Il10*cKO pups that received WT milk had lower fecal Lcn-2 than their unfostered counterparts and presented with lower colitis scores even at 7 weeks of age—indicating that the protection afforded by WT breastmilk lasted long after weaning. Collectively, these findings determined that ICOSL was protective in the absence of IL-10 and that provision of WT milk altered the antibody content of recipient *Icosl*$^{-/-}$ pups and protected from early-onset colitis.

Role for ICOSL in Preventing Intestinal Inflammation in Adulthood

While our prior results indicated that ICOSL helped suppress early-onset intestinal inflammation, we also sought to determine whether ICOSL influenced intestinal health in adulthood. For this, we took further advantage of the *Il10*.Thy1.1 BAC-In Transgenic (10BiT) mouse which expresses Thy1.1 under control of the *Il10*

promoter [43]. Injection of anti-Thy1.1 antibody allowed us to efficiently and temporarily deplete IL-10 producing cells from adult WT or *Icosl*$^{-/-}$ mice. Fascinatingly, while adult WT and *Icosl*$^{-/-}$ mice were both depleted of IL-10 producing cells in the cLP, the population of IL-10 producing CD4 T cells rebounded dramatically faster in *Icosl*$^{-/-}$ mice. This suggests an ongoing demand or signal driving these elevated IL-10 producing populations in *Icosl*$^{-/-}$ mice. In response to short-term depletion of IL-10 expressing cells, WT mice remained uninflamed whereas *Icosl*$^{-/-}$ mice clearly exhibited weight loss, elevated fecal Lcn-2, and histological evidence of colitis. *Icosl*$^{-/-}$ mice also displayed a concomitant increase in populations of CD4 T cells producing IFNγ or IL-17A, both of which are elevated in human CD [44, 45]. Histology revealed that the disease was particularly concentrated in the proximal colon. Notably, this is also the site where the density in IL-10 producing cells was greatest in healthy *Icosl*$^{-/-}$ mice and where corresponding dLN exhibited the most pronounced difference between WT and *Icosl*$^{-/-}$ germinal centers. The rapid disease in response to interruption of IL-10 producing CD4 T cell populations implies that ICOSL continues to help suppress intestinal inflammation in adults. It also highlights the increased reliance compared to WT mice of *Icosl*$^{-/-}$ mice on IL-10 producing CD4 T cell populations to prevent colitis.

Influence of the Microbiota on Colitis Induced in ICOSL-Deficient Mice

As colitis in IL-10 deficient mice is dependent on the presence of microbes and *Icosl*$^{-/-}$ mice exhibit decreased antibody binding to commensals, we hypothesized that the colitis evident in 10BiT.*Icosl*$^{-/-}$ mice upon injection with anti-Thy1.1 was commensal-dependent [46]. To this end we treated *Icosl*$^{-/-}$ mice with various antibiotics to quell

different elements of the gut microbiota and examine the subsequent impact on colitis. Treatment with the antibiotic cocktail MANV (metronidazole, ampicillin, neomycin, vancomycin) or metronidazole alone prevented *Icosl*$^{-/-}$ mice from developing colitis upon the depletion of IL-10 producing cells. This is of particular interest because metronidazole has been used with some limited success as treatment for IBD and, in our model, signifies the colitis-permissive microbe(s) are anaerobic [47]. As most GI commensals are anaerobic, we sought additional specificity through treatment with vancomycin or colistin. Oddly, treatment with vancomycin offered partial protection from colitis. As vancomycin targets Gram-positive organisms, as it cannot penetrate the outer membrane of Gram-negative organisms, it is possible that Gram-positive anaerobes may be driving the colitis witnessed in *Icosl*$^{-/-}$ mice temporarily deprived of IL-10 producing cells. There is precedence for this in the DSS model of colitis, in which depletion of Gram-positive organisms, especially Lachnospiraceae, mitigates disease [48]. Lachnospiraceae antigens were also differentially coated by WT and *Icosl*$^{-/-}$ serum on our microarray and Lachnospiraceae colonizes the mucus layer [49]. Related bacteria *Ruminococcus gnavus* has also been implicated as the main mucolytic bacteria in CD [50]. While this makes it easy to imagine that insufficient antibody-mediated control of such organisms renders *Icosl*$^{-/-}$ mice more susceptible to disease in the absence of IL-10 producing populations, further experimentation with transfers into gnotobiotic mice would be required for confirmation of any one microbe or microbial subset. Treatment with colistin, which primarily targets Gram-negative organisms, actually exacerbated colitis in *Icosl*$^{-/-}$ mice depleted of IL-10 producing cells, perhaps through freeing up space and resources for colistin-resistant microbes.

Altogether, our results indicate that ICOSL allows for effective production of anti-commensal antibodies and helps protect against microbiota-dependent intestinal inflammation throughout the lifespan. The relationship between ICOSL and intestinal IL-10 uncovered in our work is particularly interesting for murine model of IBD. Of the over 230 SNPs identified by GWAS as correlated with IBD, most individually entail only very small increases in odds which can act additively with those of other SNPs [51, 52, 53, 54]. Loss-of-function mutations affecting either ICOSL expression or IL-10 signaling individually contribute to incidence of IBD [1]. In *Icosl*$^{-/-}$ mice, we have witnessed how the intestinal impact of ICOSL-deficiency is masked by compensatory responses from IL-10 producing populations. As such, we have demonstrated that the intestinal impact of one protein encoded by an IBD susceptibility locus is concealed by the product another susceptibility locus. Only through the disruption of both is the full influence on intestinal inflammation revealed, much like the additive effects documented in IBD SNPs. The involvement of the microbiota as well renders the interruption of CD4 T cell provided IL-10 in *Icosl*$^{-/-}$ mice a multi-hit murine model of a condition with multifactorial etiology. As such, this opens new questions about potential interplay between IBD-related susceptibility loci and subtle deficiencies in microbial control being thusly concealed until a triggering event.

Forces Driving IL-10 Producing Colonic CD4 T Cells in ICOSL-Deficient Mice

Although our work highlights roles for ICOSL in management of the microbiota and subsequent protection from intestinal inflammation, further investigation is always warranted. One unknown that necessitates additional study is the factor driving the elevated IL-10 producing CD4 T cell population in the cLP of *Icosl*$^{-/-}$ mice. We have established in the first presented study that, contrary to expectations, ICOS stimulation is not required for CD4 T cell production of IL-10 in the large intestine. However, *Icosl*$^{-/-}$ mice possess an increased Foxp3^{-} CD4 T cell population that results in levels of CD4 T cell derived IL-10 that surpass even that of WT mice. Demand for this population is continual, as IL-10 producing cells rebound quickly upon depletion from *Icosl*$^{-/-}$ mice with anti-Thy1.1 injection. This indicates the continual presence of a factor driving IL-10 producing cell development or expansion that is not exclusively autocrine, as such signals would be eliminated with the cell population. Although the increase belongs to the Foxp3^{-} subset, we did not confirm the population was T regulatory type 1 (Tr1) cells because we did not stain for traditional markers like LAG-3 and CD49b [55]. Still, the lack of Foxp3 expression in the IL-10 producing CD4 T cells may merit some attention paid to factors that normally propel Tr1 populations. One of these factors is the cytokine IL-27, produced primarily by activated APCs. IL-27 can promote Tr1 cell development, suppress Th17 development through inhibiting RORγt, and even elicit IL-10 production from conventional T cells [56, 57, 58]. It also is important to Tfh function in the germinal center and may maintain Treg cell demethylation through Blimp1 [59, 60, 61]. SNPs in IL-27 have been linked to IBD and treatment with IL-27 has been successful in mitigating multiple models of colitis [62, 63, 64]. Results of manipulating IL-27 signaling in the intestine have yielded mixed results however, with IL-27 receptor deficient (*Il27ra*$^{-/-}$) mice

experiencing reduced colitis in the IL-10 deficient mouse model [65]. This is perhaps unsurprising as the model lacks one of the principal regulatory tools elicited by IL-27, IL-10. One reason to probe if IL-27 promotes the expanded IL-10 producing CD4 T cell population in *Icosl*$^{-/-}$ mice, however, stems from its relationship with ICOS. IL-27 induces ICOS expression and *in vitro* IL-27 induced Tr1s are reduced in the absence of ICOS [66]. With the absence of ICOS stimulation in *Icosl*$^{-/-}$ mice, IL-27 signals to upregulate ICOS would still not achieve the usual result of increasing ICOS-ICOSL signaling. It is possible that the lack of success eliminates some sort of negative feedback and causes perpetual attempts in the form of increased IL-27 production *in vivo*. IL-27 is just one possible way by which IL-10 producing CD4 T cells might be promoted in *Icosl*$^{-/-}$ mice, however, and there are many more possibilities. As to the roles the large IL-10 producing CD4 T cell population in the cLP of *Icosl*$^{-/-}$ mice plays, they may contribute to barrier integrity through traditional means. IL-10 aids in the production of colonic mucus by reducing protein misfolding in goblet cells [67]. It also reinforces tight junctions between IECs and promotes mucosal wound healing [68, 69]. Perhaps most importantly, IL-10 restrains myeloid cell function through several means such as inhibiting TLR-promoted MyD88, encouraging MHC Class II and CD86 disposal, and reducing proinflammatory cytokine production [70, 71, 72, 73, 74, 75, 76]. Such tasks are essential in managing what would otherwise be a strong inflammatory response to the ever present stimulation provided by gastrointestinal commensals [77]. As such, elevated IL-10 may merely perform canonical functions to aid *Icosl*$^{-/-}$ mice in maintaining host-microbiota relations in the face of deficient antibody-mediated commensal control. The immunosuppressive cytokine may also be an attempt to bolster the deficient antibody production in *Icosl*$^{-/-}$ mice, as IL-10

helps induce PC production of IgA and IgG and may enhance affinity maturation [78, 79, 80]. In that case, some feedback signaling associated with low antibody production or commensal-binding may be eliciting enhanced T cell-derived IL-10. However, we have observed no evidence of elevated CD4 T cell production of IL-10 in the cLP of B cell-deficient (μMT) mice. The cause of the increased IL-10 producing CD4 T cell population in *Icosl*$^{-/-}$ relative to WT mice is currently unknown and worth further study to define the means by how this population that conceals an underlying predisposition to intestinal inflammation is regulated.

Entry of IgG Into the Gastrointestinal Tract

Another route of additional investigation to be pursued involves the exact mechanisms by which microbiota-binding IgG encounters antigens from colonic commensals. During our work we detected low concentrations of IgG in mucus, largely reflecting the serum distribution of isotypes. As there was no significant change to B220^{lo} proportions in the underlying colonic lamina propria (cLP), we can conclude that this is not attributable to contamination by cLP plasma cells. While means by which IgA may enter the mucus and lumen are readily apparent, such as unidirectional translocation through the polymeric Ig receptor (pIgR), the method by which the observed IgG enters mucus is less clear. The neonatal Fc receptor (FcRn) is expressed by rodent and human intestinal epithelial cells and antigen presenting cells throughout the lifespan [81]. It is generally stored in vesicles near the apical side of IECs and is capable of moving IgG to either side of the IEC barrier through transcytosis [82, 83]. As such, systemic IgG may be capable of moving across the epithelium and thusly end up in mucus to interact with

microbiota antigens. The compromised response of FcRn-deficient mice to the intestinal pathogen *Citrobacter rodentium* provides evidence for the role of FcRn routed IgG in at least the disposal of epithelial-effacing pathogens [84]. In fact, as FcRn binds better at low pH and inflammation generally lowers pH of the surrounding environment, inflammatory reactions to such adherent organisms may augment its activity. The relationship between IgG and FcRn also provides an interesting linkage between antibody repertoire and how antigen is processed. FcRn allows recycling of lone antibody or immune complexes (IC) with monomeric or minimal IgG binding. However, high IgG content in IC allows for FcRn crosslinking and routing of the IC for lysosomal degradation and antigen presentation [85]. As such, potential exists that altered IgG targeting and concentration, like that observed in ICOSL-deficient mice, may affect how antigens are treated by APCs. In addition to FcRn, simple mechanical damage to the epithelium may result in leakiness that would allow for the presence of IgG in gastrointestinal mucus. Systemic IgG also enters the GI tract to some extent through its natural disposal process in the bile [86]. The initially intact IgG is degraded into Fab' fragments as it passes through the intestine, but the Fab' fragments may retain binding capability [87]. In this manner, systemic IgG would be able to directly interact with commensals, although it would evade detection by assays utilizing the Fc region. However, while systemic IgG does enter the GI tract, the presence of plasma cells in the lamina propria also implies local production is present. In addition to potential routes by which IgG could enter the colon, anti-commensal IgG may merely bind from serum to microbes that have penetrated the otherwise sterile lamina propria. The opsonization of microbial products would then function as a secondary barrier beyond the mucosal interface, allowing for the quick disposal of microbes before they

can disseminate. Similar action can be seen in the ability of anti-commensal antibodies to contain systemic infection by pathogens, as witnessed with anti-murein lipoprotein IgG and *Salmonella* [88]. The significance of anti-commensal IgG, specifically, to the gut and the primary route by which IgG enters the intestine remain mysteries for further investigation. Only once those are fully elucidated can the potential impact of modulating IgG amount and affinity, such as through ICOSL-deficiency, be defined.

Summary and Outstanding Questions

The presented studies collectively reveal roles for ICOS-ICOSL dependent factors in control of the microbiota and subsequent protection from intestinal inflammation. These roles manifest in both the epigenetic regulation of Treg stability by ICOS and the cooperation between ICOSL and IL-10 in suppressing intestinal inflammation. Collectively, our work has highlighted the contributions of ICOS to Treg cell stability in a manner that may contribute to eventual production of iTreg cell therapy. It also has challenged the paradigm that ICOS stimulation is requisite for efficient IL-10 production by CD4 T cells. Through our studies of ICOSL-deficient mice we have underlined a role for ICOSL-dependent antibodies in binding colonic commensals. Additionally, we uncovered a previously unknown interaction between two IBD susceptibility loci in the large intestine and the potential of compensatory activity from one to mask the colitis-predisposing deficit in another. In the course of this, we employed a novel multi-hit microbiota-dependent murine model of IBD. As ever, there is room for many additional inquiries, including the exact mechanism by which ICOS impacts CNS2 demethylation and the means by which IL-10 producing CD4 T cells are strongly elicited in the cLP of

ICOSL-deficient mice. Further work may also focus on the identity of particular microbes and microbial antigens that elude antibody-mediated control in the absence of ICOSL and their impact upon colitis susceptibility. Through understanding the influence of ICOS-ICOSL signals upon intestinal health, we may one day be able to manipulate their mechanisms to mitigate intestinal inflammation.

LIST OF REFERENCES

1. Dahlhamer, J.M., Zammitti, E.P., Ward, B.W., Wheaton, A.G. & Croft, J.B. Prevalence of Inflammatory Bowel Disease Among Adults Aged ≥18 Years - United States, 2015. *MMWR Morb Mortal Wkly Rep* **65**, 1166-1169 (2016).

2. Nguyen, G.C., Chong, C.A. & Chong, R.Y. National estimates of the burden of inflammatory bowel disease among racial and ethnic groups in the United States. *J Crohns Colitis* **8**, 288-295 (2014).

3. M'Koma, A.E. Inflammatory bowel disease: an expanding global health problem. *Clin Med Insights Gastroenterol* **6**, 33-47 (2013).

4. Schwartz, D.A. *et al.* The natural history of fistulizing Crohn's disease in Olmsted County, Minnesota. *Gastroenterology* **122**, 875-880 (2002).

5. Lee, M.J. *et al.* Efficacy of Medical Therapies for Fistulizing Crohn's Disease: Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* **16**, 1879-1892 (2018).

6. Burke, J.P. *et al.* Fibrogenesis in Crohn's disease. *Am J Gastroenterol* **102**, 439-448 (2007).

7. BORLEY, N.R., MORTENSEN, N.J. & JEWELL, D.P. Preventing postoperative recurrence of Crohn's disease. *BJS (British Journal of Surgery)* **84**, 1493-1502 (1997).

8. Mekhjian, H.S. *et al.* National Cooperative Crohn's Disease Study: factors determining recurrence of Crohn's disease after surgery. *Gastroenterology* **77**, 907-913 (1979).

9. Leijonmarck, C.E., Persson, P.G. & Hellers, G. Factors affecting colectomy rate in ulcerative colitis: an epidemiologic study. *Gut* **31**, 329-333 (1990).

10. Langholz, E., Munkholm, P., Davidsen, M. & Binder, V. Colorectal cancer risk and mortality in patients with ulcerative colitis. *Gastroenterology* **103**, 1444-1451 (1992).

11. Bohl, J.L. & Sobba, K. Indications and Options for Surgery in Ulcerative Colitis. *Surg Clin North Am* **95**, 1211-1232, vi (2015).

12. Vavricka, S.R. *et al.* Frequency and risk factors for extraintestinal manifestations in the Swiss inflammatory bowel disease cohort. *Am J Gastroenterol* **106**, 110-119 (2011).

13. Lönnfors, S., Vermeire, S. & Avedano, L. IBD and health-related quality of life — Discovering the true impact ☆. *Journal of Crohn's and Colitis* **8**, 1281-1286 (2014).

14. Olén, O. *et al.* Mortality in adult-onset and elderly-onset IBD: a nationwide register-based cohort study 1964–2014. *Gut* **69**, 453-461 (2020).

15. Olen, O. *et al.* Increased Mortality of Patients With Childhood-Onset Inflammatory Bowel Diseases, Compared With the General Population. *Gastroenterology* **156**, 614-622 (2019).

16. Neurath, M.F. Current and emerging therapeutic targets for IBD. *Nat Rev Gastroenterol Hepatol* **14**, 269-278 (2017).

17. Roda, G., Jharap, B., Neeraj, N. & Colombel, J.F. Loss of Response to Anti-TNFs: Definition, Epidemiology, and Management. *Clin Transl Gastroenterol* **7**, e135 (2016).

18. Yu, H. *et al.* Market share and costs of biologic therapies for inflammatory bowel disease in the USA. *Alimentary Pharmacology & Therapeutics* **47**, 364-370 (2018).

19. Stronkhorst, A. *et al.* CD4 antibody treatment in patients with active Crohn's disease: a phase 1 dose finding study. *Gut* **40**, 320-327 (1997).

20. Emmrich, J., Seyfarth, M., Fleig, W.E. & Emmrich, F. Treatment of inflammatory bowel disease with anti-CD4 monoclonal antibody. *Lancet* **338**, 570-571 (1991).

21. Monteleone, G. *et al.* Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* **112**, 1169-1178 (1997).

22. Parronchi, P. *et al.* Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol* **150**, 823-832 (1997).

23. Fuss, I.J. *et al.* Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* **157**, 1261-1270 (1996).

24. Gonsky, R. *et al.* IFNG rs1861494 polymorphism is associated with IBD disease severity and functional changes in both IFNG methylation and protein secretion. *Inflamm Bowel Dis* **20**, 1794-1801 (2014).

25. Jostins, L. *et al.* Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119-124 (2012).

26. Ito, R. *et al.* Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol* **146**, 330-338 (2006).

27. Powrie, F. *et al.* Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553-562 (1994).

28. Reinisch, W. *et al.* Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study. *Inflamm Bowel Dis* **16**, 233-242 (2010).

29. Fujino, S. *et al.* Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* **52**, 65-70 (2003).

30. Nurieva, R. *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* **448**, 480-483 (2007).

31. Monteleone, G. *et al.* Interleukin-21 enhances T-helper cell type I signaling and interferon-γ production in Crohn’s disease. *Gastroenterology* **128**, 687-694 (2005).

32. Aggarwal, S., Ghilardi, N., Xie, M.-H., de Sauvage, F.J. & Gurney, A.L. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *Journal of Biological Chemistry* **278**, 1910-1914 (2003).

33. Stritesky, G.L., Yeh, N. & Kaplan, M.H. IL-23 promotes maintenance but not commitment to the Th17 lineage. *Journal of immunology (Baltimore, Md. : 1950)* **181**, 5948-5955 (2008).

34. Duerr, R.H. *et al.* A Genome-Wide Association Study Identifies IL23R as an Inflammatory Bowel Disease Gene. *Science* **314**, 1461-1463 (2006).

35. Pidasheva, S. *et al.* Functional studies on the IBD susceptibility gene IL23R implicate reduced receptor function in the protective genetic variant R381Q. *PLoS One* **6**, e25038 (2011).

36. Zwiers, A. *et al.* Cutting edge: a variant of the IL-23R gene associated with inflammatory bowel disease induces loss of microRNA regulation and enhanced protein production. *J Immunol* **188**, 1573-1577 (2012).

37. Kobayashi, T. *et al.* IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* **57**, 1682 (2008).

38. Liu, Z. *et al.* The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity. *Journal of Leukocyte Biology* **89**, 597-606 (2011).

39. Kamada, N. *et al.* Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest* **118**, 2269-2280 (2008).

40. Ogawa, A., Andoh, A., Araki, Y., Bamba, T. & Fujiyama, Y. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clinical Immunology* **110**, 55-62 (2004).

41. O'Connor, W., Jr. *et al.* A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol* **10**, 603-609 (2009).

42. Tachibana, M. *et al.* Ablation of IL-17A leads to severe colitis in IL-10-deficient mice: implications of myeloid-derived suppressor cells and NO production. *International Immunology* **32**, 187-201 (2020).

43. Wan, C.-K. *et al.* The cytokines IL-21 and GM-CSF have opposing regulatory roles in the apoptosis of conventional dendritic cells. *Immunity* **38**, 514-527 (2013).

44. Wang, Y. *et al.* IL-21/IL-21R signaling suppresses intestinal inflammation induced by DSS through regulation of Th responses in lamina propria in mice. *Sci Rep* **6**, 31881-31881 (2016).

45. Hueber, W. *et al.* Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* **61**, 1693 (2012).

46. Yen, D. *et al.* IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *The Journal of clinical investigation* **116**, 1310-1316 (2006).

47. Hue, S. *et al.* Interleukin-23 drives innate and T cell–mediated intestinal inflammation. *Journal of Experimental Medicine* **203**, 2473-2483 (2006).

48. Cox, J.H. *et al.* Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. *Mucosal Immunology* **5**, 99-109 (2012).

49. Franchi, L., Eigenbrod, T., Muñoz-Planillo, R. & Nuñez, G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol* **10**, 241-247 (2009).

50. Ranson, N. *et al.* NLRP3-dependent and-independent processing of interleukin (IL)-1β in active ulcerative colitis. *International Journal of Molecular Sciences* **20**, 57 (2019).

51. Mao, L., Kitani, A., Strober, W. & Fuss, I.J. The Role of NLRP3 and IL-1β in the Pathogenesis of Inflammatory Bowel Disease. *Frontiers in immunology* **9**, 2566-2566 (2018).

52. Braegger, C.P., Nicholls, S., Murch, S.H., MacDonald, T.T. & Stephens, S. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *The Lancet* **339**, 89-91 (1992).

53. Breese, E.J. *et al.* Tumor necrosis factor α-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology* **106**, 1455-1466 (1994).

54. Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F. & Kollias, G. Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. *Immunity* **10**, 387-398 (1999).

55. Birkl, D. *et al.* TNFα promotes mucosal wound repair through enhanced platelet activating factor receptor signaling in the epithelium. *Mucosal immunology* **12**, 909-918 (2019).

56. Liu, J.Z. *et al.* Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet* **47**, 979-986 (2015).

57. de Lange, K.M. *et al.* Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. *Nat Genet* **49**, 256-261 (2017).

58. Cho, J.H. & Brant, S.R. Recent Insights Into the Genetics of Inflammatory Bowel Disease. *Gastroenterology* **140**, 1704-1712.e1702 (2011).

59. Festen, E.A.M. *et al.* Genetic Analysis in A Dutch Study Sample Identifies More Ulcerative Colitis Susceptibility Loci and Shows Their Additive Role in Disease Risk. *Official journal of the American College of Gastroenterology | ACG* **105** (2010).

60. Lin, Z. *et al.* Genetic association and epistatic interaction of the interleukin-10 signaling pathway in pediatric inflammatory bowel disease. *World J Gastroenterol* **23**, 4897-4909 (2017).

61. Nimmo, E.R. *et al.* Genome-wide Methylation Profiling in Crohn's Disease Identifies Altered Epigenetic Regulation of Key Host Defense Mechanisms Including the Th17 Pathway. *Inflammatory Bowel Diseases* **18**, 889-899 (2012).

62. Cleynen, I. *et al.* Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. *The Lancet* **387**, 156-167 (2016).

63. Abreu, M.T. *et al.* Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* **123**, 679-688 (2002).

64. Lewis, A. *et al.* Low Serum Levels of MicroRNA-19 Are Associated with a Stricturing Crohn's Disease Phenotype. *Inflammatory Bowel Diseases* **21**, 1926-1934 (2015).

65. Latiano, A. *et al.* Polymorphism of theIRGMGene Might Predispose to Fistulizing Behavior in Crohn's Disease. *American Journal of Gastroenterology* **104**, 110-116 (2009).

66. Sidiq, T., Yoshihama, S., Downs, I. & Kobayashi, K.S. Nod2: A Critical Regulator of Ileal Microbiota and Crohn's Disease. *Frontiers in immunology* **7**, 367-367 (2016).

67. Petnicki-Ocwieja, T. *et al.* Nod2 is required for the regulation of commensal microbiota in the intestine. *Proceedings of the National Academy of Sciences* **106**, 15813-15818 (2009).

68. Rehman, A. *et al.* Nod2 is essential for temporal development of intestinal microbial communities. *Gut* **60**, 1354-1362 (2011).

69. Li, E. *et al.* Inflammatory Bowel Diseases Phenotype, C. difficile and NOD2 Genotype Are Associated with Shifts in Human Ileum Associated Microbial Composition. *PLOS ONE* **7**, e26284 (2012).

70. Halfvarson, J., Bodin, L., Tysk, C., Lindberg, E. & Järnerot, G. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. *Gastroenterology* **124**, 1767-1773 (2003).

71. Orholm, M., Binder, V., Sørensen, T.I., Rasmussen, L.P. & Kyvik, K.O. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand J Gastroenterol* **35**, 1075-1081 (2000).

72. Halme, L. *et al.* Family and twin studies in inflammatory bowel disease. *World J Gastroenterol* **12**, 3668-3672 (2006).

73. Manichanh, C. *et al.* Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* **55**, 205-211 (2006).

74. Clooney, A.G. *et al.* Ranking microbiome variance in inflammatory bowel disease: a large longitudinal intercontinental study. *Gut*, gutjnl-2020-321106 (2020).

75. Martinez, C. *et al.* Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission. *Am J Gastroenterol* **103**, 643-648 (2008).

76. Frank, D.N. *et al.* Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A* **104**, 13780-13785 (2007).

77. Sokol, H. *et al.* Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* **105**, 16731-16736 (2008).

78. Sutherland, L. *et al.* Double blind, placebo controlled trial of metronidazole in Crohn's disease. *Gut* **32**, 1071-1075 (1991).

79. Sellon, R.K. *et al.* Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infection and immunity* **66**, 5224-5231 (1998).

80. Seregin, S.S. *et al.* NLRP6 Protects Il10(-/-) Mice from Colitis by Limiting Colonization of Akkermansia muciniphila. *Cell Rep* **19**, 733-745 (2017).

81. Aranda, R. *et al.* Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. *The Journal of Immunology* **158**, 3464-3473 (1997).

82. Garrett, W.S. *et al.* Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell* **131**, 33-45 (2007).

83. Allais, L. *et al.* Chronic cigarette smoke exposure induces microbial and inflammatory shifts and mucin changes in the murine gut. *Environ Microbiol* **18**, 1352-1363 (2016).

84. Li, H. *et al.* Increased oxidative stress and disrupted small intestinal tight junctions in cigarette smoke-exposed rats. *Molecular medicine reports* **11**, 4639-4644 (2015).

85. Danese, S. Inflammation and the mucosal microcirculation in inflammatory bowel disease: the ebb and flow. *Current Opinion in Gastroenterology* **23** (2007).

86. Ananthakrishnan, A.N. *et al.* A Prospective Study of Long-term Intake of Dietary Fiber and Risk of Crohn's Disease and Ulcerative Colitis. *Gastroenterology* **145**, 970-977 (2013).

87. Ananthakrishnan, A.N. *et al.* Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease. *Gut* **63**, 776-784 (2014).

88. Chassaing, B. *et al.* Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature* **519**, 92-96 (2015).

89. Biedermann, L. *et al.* Smoking cessation alters intestinal microbiota: insights from quantitative investigations on human fecal samples using FISH. *Inflamm Bowel Dis* **20**, 1496-1501 (2014).

90. Makki, K., Deehan, E.C., Walter, J. & Bäckhed, F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. *Cell Host & Microbe* **23**, 705-715 (2018).

91. Ge, Y. *et al.* Effect of industrial trans-fatty acids-enriched diet on gut microbiota of C57BL/6 mice. *Eur J Nutr* **58**, 2625-2638 (2019).

92. Liu, S. *et al.* The Host Shapes the Gut Microbiota via Fecal MicroRNA. *Cell Host Microbe* **19**, 32-43 (2016).

93. Jørgensen, S.F. *et al.* Altered gut microbiota profile in common variable immunodeficiency associates with levels of lipopolysaccharide and markers of systemic immune activation. *Mucosal Immunology* **9**, 1455-1465 (2016).

94. Fadlallah, J. *et al.* Microbial ecology perturbation in human IgA deficiency. *Science Translational Medicine* **10**, eaan1217 (2018).

95. Elinav, E. *et al.* NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* **145**, 745-757 (2011).

96. Ivanov, I.I. *et al.* Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* **139**, 485-498 (2009).

97. Atarashi, K. *et al.* Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell* **163**, 367-380 (2015).

98. Roediger, W.E. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* **21**, 793-798 (1980).

99. Donohoe, Dallas R. *et al.* The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon. *Cell Metabolism* **13**, 517-526 (2011).

100. Kelly, C.J. *et al.* Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell host & microbe* **17**, 662-671 (2015).

101. Karhausen, J. *et al.* Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *The Journal of clinical investigation* **114**, 1098-1106 (2004).

102. Singh, N. *et al.* Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* **40**, 128-139 (2014).

103. Smith, P.M. *et al.* The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science (New York, N.Y.)* **341**, 569-573 (2013).

104. Silva, Y.P., Bernardi, A. & Frozza, R.L. The role of short-chain fatty acids from gut microbiota in gut-brain communication. *Frontiers in Endocrinology* **11**, 25 (2020).

105. Pascal, V. *et al.* A microbial signature for Crohn's disease. *Gut* **66**, 813-822 (2017).

106. Wang, W. *et al.* Increased proportions of Bifidobacterium and the Lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease. *J Clin Microbiol* **52**, 398-406 (2014).

107. Wang, Q. *et al.* A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. *J Exp Med* **203**, 2853-2863 (2006).

108. Duan, J., Avci, F.Y. & Kasper, D.L. Microbial carbohydrate depolymerization by antigen-presenting cells: deamination prior to presentation by the MHCII pathway. *Proc Natl Acad Sci U S A* **105**, 5183-5188 (2008).

109. Mazmanian, S.K., Round, J.L. & Kasper, D.L. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**, 620-625 (2008).

110. Prindiville, T.P. *et al.* Bacteroides fragilis enterotoxin gene sequences in patients with inflammatory bowel disease. *Emerg Infect Dis* **6**, 171-174 (2000).

111. Zamani, S. *et al.* Detection of enterotoxigenic Bacteroides fragilis in patients with ulcerative colitis. *Gut Pathog* **9**, 53-53 (2017).

112. Blandford, L.E., Johnston, E.L., Sanderson, J.D., Wade, W.G. & Lax, A.J. Promoter orientation of the immunomodulatory Bacteroides fragilis capsular polysaccharide A (PSA) is off in individuals with inflammatory bowel disease (IBD). *Gut Microbes* **10**, 569-577 (2019).

113. Mitsuyama, K. *et al.* Antibody markers in the diagnosis of inflammatory bowel disease. *World journal of gastroenterology* **22**, 1304-1310 (2016).

114. Saxon, A., Shanahan, F., Landers, C., Ganz, T. & Targan, S. A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. *J Allergy Clin Immunol* **86**, 202-210 (1990).

115. Hardarson, S., Labrecque, D.R., Mitros, F.A., Neil, G.A. & Goeken, J.A. Antineutrophil cytoplasmic antibody in inflammatory bowel and hepatobiliary diseases. High prevalence in ulcerative colitis, primary sclerosing cholangitis, and autoimmune hepatitis. *Am J Clin Pathol* **99**, 277-281 (1993).

116. Reese, G.E. *et al.* Diagnostic precision of anti-Saccharomyces cerevisiae antibodies and perinuclear antineutrophil cytoplasmic antibodies in inflammatory bowel disease. *Am J Gastroenterol* **101**, 2410-2422 (2006).

117. Landers, C.J. *et al.* Selected loss of tolerance evidenced by Crohn's disease–associated immune responses to auto-and microbial antigens. *Gastroenterology* **123**, 689-699 (2002).

118. Wei, B. *et al.* Pseudomonas fluorescens encodes the Crohn's disease-associated I2 sequence and T-cell superantigen. *Infection and immunity* **70**, 6567-6575 (2002).

119. Sutton, C.L. *et al.* Identification of a novel bacterial sequence associated with Crohn's disease. *Gastroenterology* **119**, 23-31 (2000).

120. Lodes, M.J. *et al.* Bacterial flagellin is a dominant antigen in Crohn disease. *The Journal of clinical investigation* **113**, 1296-1306 (2004).

121. Targan, S.R. *et al.* Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology* **128**, 2020-2028 (2005).

122. Schoepfer, A.M. *et al.* Phenotypic associations of Crohn's disease with antibodies to flagellins A4-Fla2 and Fla-X, ASCA, p-ANCA, PAB, and NOD2 mutations in a Swiss Cohort. *Inflammatory bowel diseases* **15**, 1358-1367 (2009).

123. Kobayashi, K. *et al.* An FcRn-dependent role for anti-flagellin immunoglobulin G in pathogenesis of colitis in mice. *Gastroenterology* **137**, 1746-1756.e1741 (2009).

124. Leiper, K. *et al.* Randomised placebo-controlled trial of rituximab (anti-CD20) in active ulcerative colitis. *Gut* **60**, 1520-1526 (2011).

125. Christmann, B.S. *et al.* Human seroreactivity to gut microbiota antigens. *Journal of Allergy and Clinical Immunology* **136**, 1378-1386.e1375 (2015).

126. Brunkow, M.E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature genetics* **27**, 68-73 (2001).

127. Ochs, H.D., Ziegler, S.F. & Torgerson, T.R. FOXP3 acts as a rheostat of the immune response. *Immunological Reviews* **203**, 156-164 (2005).

128. Lee, H.-M., Bautista, J.L. & Hsieh, C.-S. Chapter 2 - Thymic and Peripheral Differentiation of Regulatory T Cells. In: Alexander, R. & Shimon, S. (eds). *Advances in Immunology*, vol. 112. Academic Press, 2011, pp 25-71.

129. Zheng, Y. *et al.* Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* **463**, 808-812 (2010).

130. Li, X., Liang, Y., LeBlanc, M., Benner, C. & Zheng, Y. Function of a Foxp3 cis-element in protecting regulatory T cell identity. *Cell* **158**, 734-748 (2014).

131. Toker, A. *et al.* Active demethylation of the Foxp3 locus leads to the generation of stable regulatory T cells within the thymus. *The Journal of Immunology* **190**, 3180-3188 (2013).

132. Feng, Y. *et al.* A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. *Nature* **528**, 132-136 (2015).

133. Kitagawa, Y. *et al.* Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat Immunol* **18**, 173-183 (2017).

134. Rubtsov, Y.P. *et al.* Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. *Immunity* **28**, 546-558 (2008).

135. Olson, B., Sullivan, J. & Burlingham, W. Interleukin 35: A Key Mediator of Suppression and the Propagation of Infectious Tolerance. *Frontiers in Immunology* **4** (2013).

136. Nakamura, K., Kitani, A. & Strober, W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* **194**, 629-644 (2001).

137. Li, M.O., Sanjabi, S. & Flavell, Richard A. Transforming Growth Factor-β Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms. *Immunity* **25**, 455-471 (2006).

138. Chen, W. *et al.* Conversion of Peripheral CD4+CD25− Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. *Journal of Experimental Medicine* **198**, 1875-1886 (2003).

139. Fahlén, L. *et al.* T cells that cannot respond to TGF-β escape control by CD4+ CD25+ regulatory T cells. *The Journal of experimental medicine* **201**, 737-746 (2005).

140. Sun, L., Jin, H. & Li, H. GARP: a surface molecule of regulatory T cells that is involved in the regulatory function and TGF-β releasing. *Oncotarget* **7**, 42826-42836 (2016).

141. Niedbala, W. *et al.* IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* **37**, 3021-3029 (2007).

142. Wirtz, S., Billmeier, U., McHedlidze, T., Blumberg, R.S. & Neurath, M.F. Interleukin-35 mediates mucosal immune responses that protect against T-cell-dependent colitis. *Gastroenterology* **141**, 1875-1886 (2011).

143. Collins, A.V. *et al.* The Interaction Properties of Costimulatory Molecules Revisited. *Immunity* **17**, 201-210 (2002).

144. Yokosuka, T. *et al.* Spatiotemporal basis of CTLA-4 costimulatory molecule-mediated negative regulation of T cell activation. *Immunity* **33**, 326-339 (2010).

145. Qureshi, O.S. *et al.* Trans-Endocytosis of CD80 and CD86: A Molecular Basis for the Cell-Extrinsic Function of CTLA-4. *Science* **332**, 600 (2011).

146. Gearing, A.J.H., Wadhwa, M. & Perris, A.D. Interleukin 2 stimulates T cell proliferation using a calcium flux. *Immunology Letters* **10**, 297-302 (1985).

147. Fontenot, J.D., Rasmussen, J.P., Gavin, M.A. & Rudensky, A.Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature Immunology* **6**, 1142-1151 (2005).

148. Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M.J. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* **8**, 1353-1362 (2007).

149. Labasi, J.M. *et al.* Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *The Journal of Immunology* **168**, 6436-6445 (2002).

150. Cauwels, A., Rogge, E., Vandendriessche, B., Shiva, S. & Brouckaert, P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death & Disease* **5**, e1102-e1102 (2014).

151. Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* **204**, 1257-1265 (2007).

152. Grossman, W.J. *et al.* Human T Regulatory Cells Can Use the Perforin Pathway to Cause Autologous Target Cell Death. *Immunity* **21**, 589-601 (2004).

153. Boissonnas, A. *et al.* Foxp3+ T Cells Induce Perforin-Dependent Dendritic Cell Death in Tumor-Draining Lymph Nodes. *Immunity* **32**, 266-278 (2010).

154. Park, O., Grishina, I., Leung, P.S., Gershwin, M.E. & Prindiville, T. Analysis of the Foxp3/scurfin gene in Crohn's disease. *Ann N Y Acad Sci* **1051**, 218-228 (2005).

155. Zhang, M. *et al.* Association of CTLA-4 variants with susceptibility to inflammatory bowel disease: A meta-analysis. *Human Immunology* **75**, 227-233 (2014).

156. Friedman, D.J. *et al.* CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. *Proceedings of the National Academy of Sciences* **106**, 16788 (2009).

157. Franke, A. *et al.* Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet* **40**, 1319-1323 (2008).

158. Lv, H. *et al.* Association between polymorphisms in the promoter region of interleukin-10 and susceptibility to inflammatory bowel disease. *Molecular Biology Reports* **41**, 1299-1310 (2014).

159. Mottet, C., Uhlig, H.H. & Powrie, F. Cutting Edge: Cure of Colitis by CD4⁺CD25⁺ Regulatory T Cells. *The Journal of Immunology* **170**, 3939-3943 (2003).

160. Boschetti, G. *et al.* Gut Inflammation in Mice Triggers Proliferation and Function of Mucosal Foxp3+ Regulatory T Cells but Impairs Their Conversion from CD4+ T Cells. *Journal of Crohn's and Colitis* **11**, 105-117 (2017).

161. Maul, J. *et al.* Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* **128**, 1868-1878 (2005).

162. Holmén, N. *et al.* Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflamm Bowel Dis* **12**, 447-456 (2006).

163. Hedl, M., Lahiri, A., Ning, K., Cho, J.H. & Abraham, C. Pattern recognition receptor signaling in human dendritic cells is enhanced by ICOS ligand and modulated by the Crohn's disease ICOSLG risk allele. *Immunity* **40**, 734-746 (2014).

164. Sato, T. *et al.* Hyperexpression of inducible costimulator and its contribution on lamina propria T cells in inflammatory bowel disease. *Gastroenterology* **126**, 829-839 (2004).

165. Smigiel, K.S. *et al.* CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *Journal of Experimental Medicine* **211**, 121-136 (2013).

166. Cretney, E. *et al.* The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* **12**, 304-311 (2011).

167. Kornete, M., Sgouroudis, E. & Piccirillo, C.A. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. *J Immunol* **188**, 1064-1074 (2012).

168. Pierson, W. *et al.* Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3+ regulatory T cells. *Nature Immunology* **14**, 959-965 (2013).

169. Burmeister, Y. *et al.* ICOS Controls the Pool Size of Effector-Memory and Regulatory T Cells. *The Journal of Immunology* **180**, 774-782 (2008).

170. Toomer, K.H. *et al.* Essential and non-overlapping IL-2Rα-dependent processes for thymic development and peripheral homeostasis of regulatory T cells. *Nature Communications* **10**, 1037 (2019).

171. Löhning, M. *et al.* Expression of ICOS In Vivo Defines CD4+ Effector T Cells with High Inflammatory Potential and a Strong Bias for Secretion of Interleukin 10. *Journal of Experimental Medicine* **197**, 181-193 (2003).

172. Hutloff, A. *et al.* ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397**, 263-266 (1999).

173. Fos, C. *et al.* ICOS ligation recruits the p50alpha PI3K regulatory subunit to the immunological synapse. *J Immunol* **181**, 1969-1977 (2008).

174. Stone, E.L. *et al.* ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. *Immunity* **42**, 239-251 (2015).

175. Weber, J.P. *et al.* ICOS maintains the T follicular helper cell phenotype by down-regulating Kruppel-like factor 2. *J Exp Med* **212**, 217-233 (2015).

176. Leavenworth, J.W., Verbinnen, B., Yin, J., Huang, H. & Cantor, H. A p85α-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nature immunology* **16**, 96-106 (2015).

177. Choi, Y.S. *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* **34**, 932-946 (2011).

178. Vu, F., Dianzani, U., Ware, C.F., Mak, T. & Gommerman, J.L. ICOS, CD40, and Lymphotoxin β Receptors Signal Sequentially and Interdependently to Initiate a Germinal Center Reaction. *The Journal of Immunology* **180**, 2284-2293 (2008).

179. Dong, C., Temann, U.-A. & Flavell, R.A. Cutting Edge: Critical Role of Inducible Costimulator in Germinal Center Reactions. *The Journal of Immunology* **166**, 3659-3662 (2001).

180. Mahajan, S. *et al.* The role of ICOS in the development of CD4 T cell help and the reactivation of memory T cells. *European Journal of Immunology* **37**, 1796-1808 (2007).

181. Dong, C. *et al.* ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* **409**, 97-101 (2001).

182. Gigoux, M. *et al.* Inducible costimulator promotes helper T-cell differentiation through phosphoinositide 3-kinase. *Proceedings of the National Academy of Sciences* **106**, 20371-20376 (2009).

183. Chen, Q. *et al.* A Novel ICOS-Independent, but CD28- and SAP-Dependent, Pathway of T Cell-Dependent, Polysaccharide-Specific Humoral Immunity in Response to Intact Streptococcus pneumoniae versus Pneumococcal Conjugate Vaccine. *The Journal of Immunology* **181**, 8258-8266 (2008).

184. Inamine, A. *et al.* Two waves of memory B-cell generation in the primary immune response. *Int Immunol* **17**, 581-589 (2005).

185. Toyama, H. *et al.* Memory B Cells without Somatic Hypermutation Are Generated from Bcl6-Deficient B Cells. *Immunity* **17**, 329-339 (2002).

186. Liu, D. *et al.* T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. *Nature* **517**, 214-218 (2015).

187. Hendrickx, A.P. *et al.* Antibiotic-driven dysbiosis mediates intraluminal agglutination and alternative segregation of Enterococcus faecium from the intestinal epithelium. *MBio* **6** (2015).

188. Moor, K. *et al.* High-avidity IgA protects the intestine by enchaining growing bacteria. *Nature* **544**, 498-502 (2017).

189. Boullier, S. *et al.* Secretory IgA-mediated neutralization of Shigella flexneri prevents intestinal tissue destruction by down-regulating inflammatory circuits. *J Immunol* **183**, 5879-5885 (2009).

190. Murthy, A.K. *et al.* Mannose-Containing Oligosaccharides of Non-Specific Human Secretory Immunoglobulin A Mediate Inhibition of Vibrio cholerae Biofilm Formation. *PLOS ONE* **6**, e16847 (2011).

191. Mathias, A. & Corthésy, B. Recognition of gram-positive intestinal bacteria by hybridoma- and colostrum-derived secretory immunoglobulin A is mediated by carbohydrates. *J Biol Chem* **286**, 17239-17247 (2011).

192. Bunker, J.J. *et al.* Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science (New York, N.Y.)* **358**, eaan6619 (2017).

193. Macpherson, A.J. *et al.* A Primitive T Cell-Independent Mechanism of Intestinal Mucosal IgA Responses to Commensal Bacteria. *Science* **288**, 2222 (2000).

194. Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* **11**, 34-46 (2011).

195. Cerutti, A. The regulation of IgA class switching. *Nature Reviews Immunology* **8**, 421-434 (2008).

196. He, B. *et al.* Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* **26**, 812-826 (2007).

197. Cullender, Tyler C. *et al.* Innate and Adaptive Immunity Interact to Quench Microbiome Flagellar Motility in the Gut. *Cell Host & Microbe* **14**, 571-581 (2013).

198. Roco, J.A. *et al.* Class-Switch Recombination Occurs Infrequently in Germinal Centers. *Immunity* **51**, 337-350.e337 (2019).

199. Bunker, J.J. *et al.* Innate and Adaptive Humoral Responses Coat Distinct Commensal Bacteria with Immunoglobulin A. *Immunity* **43**, 541-553 (2015).

200. Palm, N.W. *et al.* Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* **158**, 1000-1010 (2014).

201. Feng, T., Elson, C.O. & Cong, Y. Treg cell–IgA axis in maintenance of host immune homeostasis with microbiota. *International Immunopharmacology* **11**, 589-592 (2011).

202. Wei, M. *et al.* Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. *Nature Immunology* **12**, 264-270 (2011).

203. Shin, N.-R., Whon, T.W. & Bae, J.-W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends in Biotechnology* **33**, 496-503 (2015).

204. Kawamoto, S. *et al.* The Inhibitory Receptor PD-1 Regulates IgA Selection and Bacterial Composition in the Gut. *Science* **336**, 485-489 (2012).

205. Slack, E. *et al.* Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science (New York, N.Y.)* **325**, 617-620 (2009).

206. Zeng, M.Y. *et al.* Gut Microbiota-Induced Immunoglobulin G Controls Systemic Infection by Symbiotic Bacteria and Pathogens. *Immunity* **44**, 647-658 (2016).

207. Fadlallah, J. *et al.* Synergistic convergence of microbiota-specific systemic IgG and secretory IgA. *Journal of Allergy and Clinical Immunology* **143**, 1575-1585.e1574 (2019).

208. Bai, X. *et al.* T Follicular Helper Cells Regulate Humoral Response for Host Protection against Intestinal Citrobacter rodentium Infection. *The Journal of Immunology* **204**, 2754 (2020).

209. Brandtzaeg, P. *et al.* Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. *Gastroenterology* **97**, 1562-1584 (1989).

210. Castro-Dopico, T. *et al.* Anti-commensal IgG Drives Intestinal Inflammation and Type 17 Immunity in Ulcerative Colitis. *Immunity* **50**, 1099-1114.e1010 (2019).

211. Rengarajan, S. *et al.* Dynamic immunoglobulin responses to gut bacteria during inflammatory bowel disease. *Gut microbes* **11**, 405-420 (2020).

212. Kamada, N. *et al.* Humoral Immunity in the Gut Selectively Targets Phenotypically Virulent Attaching-and-Effacing Bacteria for Intraluminal Elimination. *Cell Host & Microbe* **17**, 617-627 (2015).

213. Johansson, M.E. *et al.* Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut* **63**, 281-291 (2014).

214. Johansson, M.E., Thomsson, K.A. & Hansson, G.C. Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. *J Proteome Res* **8**, 3549-3557 (2009).

215. Harada, N. *et al.* Human IgGFc binding protein (FcgammaBP) in colonic epithelial cells exhibits mucin-like structure. *J Biol Chem* **272**, 15232-15241 (1997).

216. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-274 (1993).

217. Spencer, S.D. *et al.* The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J Exp Med* **187**, 571-578 (1998).

218. Xu, M. *et al.* c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* **554**, 373-377 (2018).

219. Roers, A. *et al.* T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *The Journal of experimental medicine* **200**, 1289-1297 (2004).

220. Madan, R. *et al.* Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *Journal of immunology (Baltimore, Md. : 1950)* **183**, 2312-2320 (2009).

221. Zigmond, E. *et al.* Macrophage-Restricted Interleukin-10 Receptor Deficiency, but Not IL-10 Deficiency, Causes Severe Spontaneous Colitis. *Immunity* **40**, 720-733 (2014).

222. Krause, P. *et al.* IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nature Communications* **6**, 7055 (2015).

223. Moran, C.J. *et al.* IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis* **19**, 115-123 (2013).

224. Zheng, L. *et al.* Microbial-Derived Butyrate Promotes Epithelial Barrier Function through IL-10 Receptor–Dependent Repression of Claudin-2. *The Journal of Immunology* **199**, 2976 (2017).

225. Quiros, M. *et al.* Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. *The Journal of Clinical Investigation* **127**, 3510-3520 (2017).

226. Hasnain, S.Z. *et al.* IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. *Gastroenterology* **144**, 357-368 e359 (2013).

227. Madsen, K.L. *et al.* Interleukin-10 Gene-Deficient Mice Develop a Primary Intestinal Permeability Defect in Response to Enteric Microflora. *Inflammatory Bowel Diseases* **5**, 262-270 (1999).

228. Chang, J., Kunkel, S.L. & Chang, C.H. Negative regulation of MyD88-dependent signaling by IL-10 in dendritic cells. *Proc Natl Acad Sci U S A* **106**, 18327-18332 (2009).

229. Hoshi, N. *et al.* MyD88 signalling in colonic mononuclear phagocytes drives colitis in IL-10-deficient mice. *Nat Commun* **3**, 1120 (2012).

230. Furuta, K., Walseng, E. & Roche, P.A. Internalizing MHC class II-peptide complexes are ubiquitinated in early endosomes and targeted for lysosomal degradation. *Proc Natl Acad Sci U S A* **110**, 20188-20193 (2013).

231. Thibodeau, J. *et al.* Interleukin-10-induced MARCH1 mediates intracellular sequestration of MHC class II in monocytes. *Eur J Immunol* **38**, 1225-1230 (2008).

232. Tze, L.E. *et al.* CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. *J Exp Med* **208**, 149-165 (2011).

233. Shouval, D.S. *et al.* Interleukin 1β Mediates Intestinal Inflammation in Mice and Patients With Interleukin 10 Receptor Deficiency. *Gastroenterology* **151**, 1100-1104 (2016).

234. Coccia, M. *et al.* IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4+ Th17 cells. *Journal of Experimental Medicine* **209**, 1595-1609 (2012).

235. Ludwiczek, O. *et al.* Imbalance between interleukin-1 agonists and antagonists: relationship to severity of inflammatory bowel disease. *Clin Exp Immunol* **138**, 323-329 (2004).

236. Clarke, C.J., Hales, A., Hunt, A. & Foxwell, B.M. IL-10-mediated suppression of TNF-alpha production is independent of its ability to inhibit NF kappa B activity. *Eur J Immunol* **28**, 1719-1726 (1998).

237. Snijders, A. *et al.* Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. *The Journal of Immunology* **156**, 1207 (1996).

238. Li, B. *et al.* IL-10 engages macrophages to shift Th17 cytokine dependency and pathogenicity during T-cell-mediated colitis. *Nature Communications* **6**, 6131 (2015).

239. Wadwa, M. *et al.* IL-10 downregulates CXCR3 expression on Th1 cells and interferes with their migration to intestinal inflammatory sites. *Mucosal Immunology* **9**, 1263-1277 (2016).

240. Kamanaka, M. *et al.* Memory/effector (CD45RBlo) CD4 T cells are controlled directly by IL-10 and cause IL-22–dependent intestinal pathology. *Journal of Experimental Medicine* **208**, 1027-1040 (2011).

241. Huber, S. *et al.* Th17 cells express interleukin-10 receptor and are controlled by $Foxp3^{-}$ and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* **34**, 554-565 (2011).

242. Chaudhry, A. *et al.* Interleukin-10 Signaling in Regulatory T Cells Is Required for Suppression of Th17 Cell-Mediated Inflammation. *Immunity* **34**, 566-578 (2011).

243. Brockmann, L. *et al.* IL-10 Receptor Signaling Is Essential for TR1 Cell Function In Vivo. *J Immunol* **198**, 1130-1141 (2017).

244. Fluckiger, A.C., Garrone, P., Durand, I., Galizzi, J.P. & Banchereau, J. Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *Journal of Experimental Medicine* **178**, 1473-1481 (1993).

245. Rousset, F. *et al.* Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proceedings of the National Academy of Sciences* **89**, 1890-1893 (1992).

246. Laidlaw, B.J. *et al.* Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol* **2**, eaan4767 (2017).

247. He, B. *et al.* The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88. *Nat Immunol* **11**, 836-845 (2010).

248. Pazmandi, J., Kalinichenko, A., Ardy, R.C. & Boztug, K. Early-onset inflammatory bowel disease as a model disease to identify key regulators of immune homeostasis mechanisms. *Immunological Reviews* **287**, 162-185 (2019).

249. Nishioka, T., Shimizu, J., Iida, R., Yamazaki, S. & Sakaguchi, S. CD4+ CD25+ Foxp3+ T cells and CD4+ CD25− Foxp3+ T cells in aged mice. *The Journal of Immunology* **176**, 6586-6593 (2006).

250. Gregg, R. *et al.* The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. *Clinical & Experimental Immunology* **140**, 540-546 (2005).

251. Thornton, A.M. *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* **184**, 3433-3441 (2010).

252. Szurek, E. *et al.* Differences in Expression Level of Helios and Neuropilin-1 Do Not Distinguish Thymus-Derived from Extrathymically-Induced CD4+Foxp3+ Regulatory T Cells. *PLoS One* **10**, e0141161 (2015).

253. Thiault, N. *et al.* Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. *Nat Immunol* **16**, 628-634 (2015).

254. Cong, Y., Feng, T., Fujihashi, K., Schoeb, T.R. & Elson, C.O. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 19256-19261 (2009).

255. Josefowicz, S.Z. *et al.* Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* **482**, 395-399 (2012).

256. Tahvildari, M. *et al.* In Vivo Expansion of Regulatory T Cells by Low-Dose Interleukin-2 Treatment Increases Allograft Survival in Corneal Transplantation. *Transplantation* **100**, 525-532 (2016).

257. Benoist, C. & Mathis, D. Treg Cells, Life History, and Diversity. *Cold Spring Harbor Perspectives in Biology* **4** (2012).

258. Koenen, H.J. *et al.* Human CD25highFoxp3pos regulatory T cells differentiate into IL-17–producing cells. *Blood, The Journal of the American Society of Hematology* **112**, 2340-2352 (2008).

259. Hoffmann, P. *et al.* Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. *European Journal of Immunology* **39**, 1088-1097 (2009).

260. Miyao, T. *et al.* Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* **36**, 262-275 (2012).

261. Ohkura, N. *et al.* T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* **37**, 785-799 (2012).

262. Helmin, K.A. *et al.* Maintenance DNA methylation is essential for regulatory T cell development and stability of suppressive function. *J Clin Invest* **130**, 6571-6587 (2020).

263. Levine, A.G., Arvey, A., Jin, W. & Rudensky, A.Y. Continuous requirement for the TCR in regulatory T cell function. *Nat Immunol* **15**, 1070-1078 (2014).

264. Vahl, J.C. *et al.* Continuous T cell receptor signals maintain a functional regulatory T cell pool. *Immunity* **41**, 722-736 (2014).

265. Wang, L. *et al.* Mbd2 promotes foxp3 demethylation and T-regulatory-cell function. *Mol Cell Biol* **33**, 4106-4115 (2013).

266. Lownik, J.C. *et al.* ADAM10-Mediated ICOS Ligand Shedding on B Cells Is Necessary for Proper T Cell ICOS Regulation and T Follicular Helper Responses. *J Immunol* **199**, 2305-2315 (2017).

267. Lownik, J.C., Conrad, D.H. & Martin, R.K. T cell receptor signaling defines the fate and pathway of ICOS internalization. *Biochem Biophys Rep* **24**, 100803 (2020).

268. Rudd, C.E., Taylor, A. & Schneider, H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* **229**, 12-26 (2009).

269. Qureshi, O.S. *et al.* Constitutive clathrin-mediated endocytosis of CTLA-4 persists during T cell activation. *J Biol Chem* **287**, 9429-9440 (2012).

270. Lodes, M.J. *et al.* Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* **113**, 1296-1306 (2004).

271. Hamilton, A.L. *et al.* Serologic antibodies in relation to outcome in postoperative Crohn's disease. *J Gastroenterol Hepatol* **32**, 1195-1203 (2017).

272. Furter, M., Sellin, M.E., Hansson, G.C. & Hardt, W.-D. Mucus Architecture and Near-Surface Swimming Affect Distinct Salmonella Typhimurium Infection Patterns along the Murine Intestinal Tract. *Cell Rep* **27**, 2665-2678.e2663 (2019).

273. Chassaing, B. *et al.* Fecal Lipocalin 2, a Sensitive and Broadly Dynamic Non-Invasive Biomarker for Intestinal Inflammation. *PLOS ONE* **7**, e44328 (2012).

274. Daft, J.G., Ptacek, T., Kumar, R., Morrow, C. & Lorenz, R.G. Cross-fostering immediately after birth induces a permanent microbiota shift that is shaped by the nursing mother. *Microbiome* **3**, 17 (2015).

275. Catanzaro, J.R. *et al.* IgA-deficient humans exhibit gut microbiota dysbiosis despite secretion of compensatory IgM. *Sci Rep* **9**, 13574 (2019).

276. Seppälä, I.J., Hurme, M. & Mäkelä, O. Antibody responses to hapten in thymectomized mice: extraordinarily pronounced deficiency in IgG1 production. *Scand J Immunol* **29**, 469-475 (1989).

277. Maynard, C.L. *et al.* Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* **8**, 931-941 (2007).

278. Nakanishi, Y., Sato, T. & Ohteki, T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. *Mucosal Immunol* **8**, 152-160 (2015).

279. Paone, P. & Cani, P.D. Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut* **69**, 2232 (2020).

280. Png, C.W. *et al.* Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *Am J Gastroenterol* **105**, 2420-2428 (2010).

281. Gagliani, N. *et al.* Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nature medicine* **19**, 739 (2013).

282. Diveu, C. *et al.* IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells. *J Immunol* **182**, 5748-5756 (2009).

283. Murugaiyan, G. *et al.* IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *The Journal of Immunology* **183**, 2435-2443 (2009).

284. Stumhofer, J.S. *et al.* Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* **8**, 1363-1371 (2007).

285. Batten, M. *et al.* IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells. *Journal of Experimental Medicine* **207**, 2895-2906 (2010).

286. Heinemann, C. *et al.* IL-27 and IL-12 oppose pro-inflammatory IL-23 in CD4+ T cells by inducing Blimp1. *Nature Communications* **5**, 3770 (2014).

287. Garg, G. *et al.* Blimp1 Prevents Methylation of Foxp3 and Loss of Regulatory T Cell Identity at Sites of Inflammation. *Cell Rep* **26**, 1854-1868.e1855 (2019).

288. Troy, A.E. *et al.* IL-27 regulates homeostasis of the intestinal CD4+ effector T cell pool and limits intestinal inflammation in a murine model of colitis. *J Immunol* **183**, 2037-2044 (2009).

289. Franke, A. *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nature genetics* **42**, 1118-1125 (2010).

290. Villarino, A.V. *et al.* IL-27R deficiency delays the onset of colitis and protects from helminth-induced pathology in a model of chronic IBD. *Int Immunol* **20**, 739-752 (2008).

291. Pot, C. *et al.* Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *Journal of immunology (Baltimore, Md. : 1950)* **183**, 797-801 (2009).

292. Qiao, S.-W., Lencer, W.I. & Blumberg, R.S. How the controller is controlled - neonatal Fc receptor expression and immunoglobulin G homeostasis. *Immunology* **120**, 145-147 (2007).

293. Claypool, S.M. *et al.* Bidirectional Transepithelial IgG Transport by a Strongly Polarized Basolateral Membrane Fcγ-Receptor. *Molecular Biology of the Cell* **15**, 1746-1759 (2004).

294. Dickinson, B.L. *et al.* Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. *The Journal of clinical investigation* **104**, 903-911 (1999).

295. Weflen, A.W. *et al.* Multivalent immune complexes divert FcRn to lysosomes by exclusion from recycling sorting tubules. *Molecular biology of the cell* **24**, 2398-2405 (2013).

296. Reynoso-Paz, S. *et al.* The immunobiology of bile and biliary epithelium. *Hepatology* **30**, 351-357 (1999).

297. Quan, C.P., Ruffet, E., Arihiro, K., Pires, R. & Bouvet, J.P. High affinity serum-derived Fab fragments as another source of antibodies in the gut lumen of both neonates and adults. *Scand J Immunol* **44**, 108-114 (1996).

APPENDIX A

PERMISSION FOR REPRINTING

CUTTING EDGE: ICOS-DEFICIENT REGULATORY T CELLS DISPLAY NORMAL INDUCTION OF IL10 BUT READILY DOWNREGULATE EXPRESSION OF FOXP3

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