Interleukin-10 in the Control of Tumor Immunity and Autoimmunity

by

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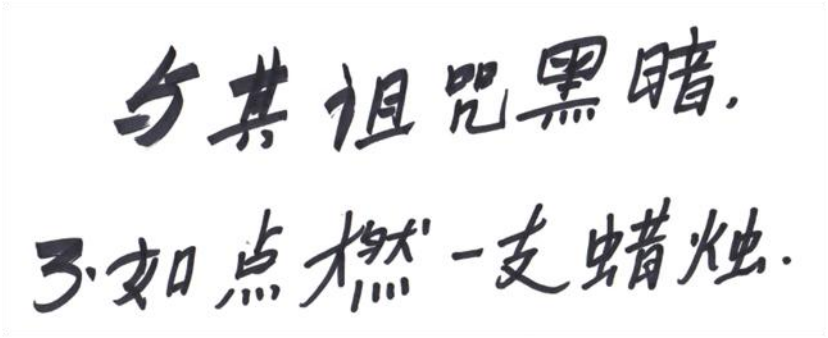

“It is better to light a candle than to curse the darkness.”

(Ancient Chinese Proverb)

“Make of yourself a light.” (Buddha)

This is dedicated to my grandmother,

Alice Marie Dewhirst Olsen,

in honor of her indefatigable spirit,

her easy laughter,

and her belief in the infinite possibility of things.

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Chapter 1
Introduction

Inflammation.

Inflammation is the term given to a mixture of biological processes set in motion by vascular tissues to combat invading pathogens or physiological insult. These processes include increased blood flow, accelerated cellular metabolism, vasodilatation, fluid and cell extravasation, infiltration of cells into the damaged tissue, and release of soluble mediators such as cytokines, chemokines, prostaglandins, leukotrienes, and various other proteins, peptides and enzymes. The purpose of inflammation is to protect the host, eliminate trespassing organisms or objects, and begin the healing process. Classical signs of inflammation include rubor (redness), tumor (swelling), calor (heat), dolor (pain), and functio laesa (loss or disturbance of function)[1]. Based upon pathological progression, there are two types of inflammation: acute and chronic. Acute inflammation is most typically experienced as a result of tissue injury or infection and resolves after the pathogen has been removed or the wound healed. Inflammation is typically self-limiting: it requires constant stimulation to be maintained. Cellular infiltrates in acute inflammation generally include neutrophils and macrophages, whose presence allows for the elimination of cellular detritus via phagocytosis. Macrophages and neutrophils also secrete proinflammatory cytokines (notably tumor necrosis factor-alpha (TNFα) and interleukin(IL)-1) and chemokines that increase leukocyte trafficking to the affected area, as well as proteins responsible for collagen deposition, fibroblast proliferation, and tissue remodeling [2]. In situations of incomplete healing or where the offending agent persists, high numbers of macrophages and neutrophils remain in the affected area, secreting proinflammatory mediators and other tissue-remodeling agents, leading to bystander tissue damage and in some

cases, fibrosis. Monocytes, macrophages, and dendritic cells (DC), functioning as antigen-presenting cells (APCs), serve as the bridge between the innate and adaptive immune systems, and play a central role in polarization of T-helper-cell (Th; $CD4^+$ T cell) subsets: macrophages can control skewing away from a T helper-1(Th1)-mediated environment to a T helper-17(Th17)-controlled setting. Th1 cells, whose development is controlled by the cytokines interleukin(IL)-12 and interferon-gamma (IFNγ) and the transcription factor T-box expressed in T cells (Tbet/Tbx21), are useful in combating infection. Th17 cells, first discovered in 2005 [3-4], are controlled by the transcription factor RAR-related orphan receptor gamma T (RORγt) and the cytokines transforming growth factor-beta (TGFβ), IL-6, IL-1, and IL-23. Although beneficial in certain cases of bacterial invasion, this cell population is more often associated with the development of chronic inflammation and autoimmunity.

Our laboratory has examined some of the cellular and molecular events that might happen in a paradigm shift from acute to chronic inflammation. IFNγ is a signature pro-inflammatory cytokine produced predominantly by immune cells in conditions of viral or bacterial infection. IFNγ can inhibit viral replication directly, activate the lysosome, and increase antibacterial activity in macrophages [5]. It serves as a chemoattractant or repellant for leukocytes [6], controls isotype switching [7] and antibody production in B cells, and directs the growth and differentiation of several cell types [8-9]. IFNγ is a well-known product of T-helper-1 (Th1) cells and plays a key role in skewing of naïve T cells to a Th1 phenotype, both through inhibition of IL-4 production [10] and induction of IL-12 secretion [11]. We found that Th1-derived IFNγ could rapidly induce elevated B7-H1 expression on APCs and stimulate their production of IL-1 and IL-23. B7-H1 signaling resulted in abrogation of the Th1-polarizing capacity of APCs, while APC secretion of IL-1 and IL-23 directed T cells towards a memory Th17-expanding phenotype [12]. In the course of inflammation, then, we believe that the acute Th1-mediated response is attenuated by IFNγ-induced B7-H1 on APCs and is subsequently evolved toward Th17-mediated chronic inflammation by APC-derived IL-1 and IL-23. In addition to challenging the dogma that IFNγ

suppresses Th17 and enhances Th1 development, this data reinforces the notion that T cell subset kinetics depend strongly on the context of the ongoing immune response and the constituents of the cytokine milieu, both of which are influenced by disease progression.

The human body possesses multiple control systems whereby situations of chronic inflammation are averted. These include immunosuppressive cell subsets, like regulatory T cells (Tregs), and the major immunoregulatory cytokines, transforming growth factor-beta (TGFβ) and interleukin(IL)-10, that serve to control immune responses and inhibit the development of autoimmunity. IL-10, the object of this thesis work, has been studied for more than two decades.

Interleukin-10.

IL-10 was first identified by Fiorentino, *et al* in 1988 as a product of T-helper-type 2 (Th2) cells that inhibited cytokine production from Th1 cells [13]. Subsequent early reports investigated effects of IL-10 on T cell development, while later studies established IL-10's reputation as a modulator of APC capacity. In more recent years, several groups have explored the immune-stimulating potential of IL-10.

Much of what we know about the regulatory roles of IL-10 we have learned from experiments investigating anti-viral responses. IL-10 is produced by many types of cells, including macrophages and dendritic cells (DCs), B cells [14], keratinocytes, mast cells [15-16], and several subsets of T cells, including Tregs, Th2 cells, and interestingly, a population of Th17 cells [17]. It has recently been shown that IL-10 has direct effects on Tregs, which secrete IL-10 as one of their many immunosuppressive activities. Murai, *et al* demonstrated that myeloid-derived IL-10 maintains Forkhead box P3 (FoxP3) expression and suppressive function in mice with colitis [18]. Several studies in the last decade have confirmed the importance of IL-10 in mediating intestinal homeostasis [19-23]. In addition to effects upon and via Tregs, IL-10 is a key modulator of antigen presentation.

The IL-10 receptor (IL-10R) is a heterodimer, made up of two subunits termed α and β. IL-10Rα exists on most hematopoetic cells. Activation of T cells

leads to a decrease in T cell expression levels of IL-10Rα, while the receptor is upregulated on monocytes after their activation. IL-10Rα expression can be induced in fibroblasts, epidermal cells, and keratinocytes via various stimuli. Interestingly, but perhaps not surprisingly, this receptor is constitutively expressed on the colonic epithelium, where IL-10 signaling plays a key role in maintaining an immunosuppressive environment. IL-10Rβ, however, is constitutively expressed on most cells and tissues, but its expression does not appear to be regulated by cell activation. It is hypothesized, therefore, that presence of the IL-10Rα should render a given cell responsive to environmental IL-10 [24].

IL-10 enacts most of its immunosuppressive activity indirectly, via effects on APCs such as monocytes, DCs, and macrophages. IL-10 downregulates major histocompatibility molecule (MHC) and B7 co-stimulatory molecule expression on APCs. It limits proinflammatory cytokine and chemokine expression in APCs, but can also directly affect $CD4^+$ T cells by limiting their activation, proliferation, and antigen-dependent production of cytokines such as IFNγ, IL-2, IL-4, IL-5, IL-13, and TNFα [24-27]. Interestingly, a study with *mycobacterium* showed that autocrine IL-10 signaling in DCs can prevent their trafficking to lymph nodes [28]. This impedes the recruitment of naïve T cells to draining lymph nodes, as well as polarization of these same T cells to a Th1 phenotype.

Splenic B cells from mice upregulate their expression of MHC II upon treatment with either human or mouse recombinant (r)IL-10 [29]. IL-10 also serves as a survival factor for B cells and increases their antibody production. Early experiments on human B cells demonstrated that IL-10 served as a co-stimulatory factor for B cell proliferation and synergized with IL-4 to expand B cell cultures even further [30]. Additionally, IL-10 treatment stimulated B cell production of immunoglobulin (Ig)M, IgG, and IgA. Because IL-10 augmented the number of antibody-producing B cells in culture, it is of course possible that this increased Ig resulted from increased cell viability and not from a direct effect of IL-10 on Ig production itself. A subsequent study showed that IL-10 supported

viability of human germinal center B cells and induced synthesis of B-cell lymphoma 2(bcl-2) [31], a protein which was already known to play a key role in the rescue of germinal center B cells from apoptosis [32].

MacNeil, *et al* first showed in 1990 that IL-10, along with IL-2 and/or IL-4, supported the growth and proliferation of mature and immature T cells [33]. Shortly thereafter, more specific effects of IL-10 on $CD8^+$ T cells were explored. Interestingly, IL-10 was found to be capable of inducing cytotoxic lymphocyte (CTL) precursors (in cell populations cultured in the presence of IL-2) and augmenting cytotoxic function [34]. IL-10 is therefore a growth and differentiation factor for $CD8^+$ T cells.

As we mentioned above, much of our current knowledge regarding the functions of IL-10 arises from studies of viral immunity and *in vitro* studies. Many reports present evidence for IL-10's involvement in systemic or chronic/ non-healing leishmaniasis [35]. Investigation of a mouse model of influenza infection showed that recovered mice were much more susceptible to secondary infection by *pneumococcal pneumonia*. Part of this susceptibility was due to excessive IL-10 production in the lungs, quite possibly a consequence of immune response resolution [36]. The laboratory of Joshua Fierer has demonstrated that IL-10 levels correlate directly to murine susceptibility to *Coccidioides immitis* peritonitis at least in part because IL-10 downregulates nitric oxide synthesis [37-38]. Other groups have shown that IL-10 abolishes host resistance to *Listeria monocytogenes* [39]. One study investigated the effects of increased IL-10 in autoimmune disease, viral infection, and tumor immunity. As might be hypothesized, mice expressing higher levels of IL-10 had impaired immune responses to transferred tumors [40].

Interleukin-10 in malignancy.

In the past few years, a few laboratories have documented new roles for IL-10, especially in the context of tumor immunity. Some of the perhaps surprisingly contradictory effects of this cytokine in a malignant setting are reviewed in [41]. Suzuki, *et al* showed that tumor cells transfected with murine IL-10 grew more slowly *in vivo* and were frequently rejected by the host animals

[42]. The following year, IL-10 was discovered to inhibit tumor metastasis in both experimental and spontaneous tumor models via effects on natural killer (NK) cells [43]. Although the precise mechanisms involved were not explored in this paper, a subsequent study from the Fulton group demonstrated that IL-10 downregulated MHC I expression on tumor cells and in doing so, supported NK cell-mediated tumor cell lysis [44]; without expression of self-MHC on target cells, the inhibitory signal to NK cells is abolished and lysis ensues [45]. A more recent paper examining the effect of IL-10 on antitumor CTLs found that recombinant human (rh)IL-10 treatment in immunized mice after tumor challenge significantly enhanced antitumor immunity and vaccine efficacy. Three weeks after IL-10 administration, the investigators found that splenic $CD8^+CD44^{hi}CD122^+$ (activated memory) T cell numbers had increased and antigen-specific proliferation *in vitro* was enhanced. Additionally, antigen-specific IFNγ production at the single-cell level was increased in animals challenged with tumor and then treated with IL-10 when compared to animals only given tumor challenge. Interestingly, IL-10's effect on CTL function could be enhanced by $CD4^+$ T cell depletion, supporting the notion that IL-10 can have opposing effects on $CD8^+$ and $CD4^+$ T cells, at least in a tumor model [46]. In the first part of this thesis, we more closely examine the mechanisms whereby IL-10 supports the antitumor immune response.

Of course, numerous publications have also documented functions of IL-10 that interrupt or suppress immunity in tumor-bearing hosts. In several preclinical models, blockade of IL-10 signaling by tumor or T cells via several mechanisms has been shown to enhance antitumor immunity [47-49]. In 2002, Seo, *et al* observed that $\gamma\delta$ T cells and intermediate $\alpha\beta$ T cells could produce TGFβ and IL-10, which immediately suppresses NK and Natural Killer T (NKT) cells, and ultimately impedes Th1 cell and CTL activation [50]. Other studies have demonstrated that IL-10 (from tumor cells themselves or elsewhere) can downregulate MHC molecule expression on tumor cells *in vitro*, thus hindering CTL-mediated killing [51-52]. Tumor cell-derived IL-10 has been associated with increased expression of the nonclassical human leukocyte antigen (HLA) class

Ib molecule HLA-G; this also obstructs host lymphocyte cytolytic activity [53]. The tumor microenvironment has been characterized as IL-10-rich. Tumor-infiltrating DC are capable of producing large amounts of IL-10 [54], which can anergize CTL towards melanoma-associated antigens [55]. Perhaps not surprisingly, T cell proliferation-inhibiting IL-10-producing monocytes have been isolated from ascites of ovarian cancer patients [56]. Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), another molecule of interest in the study of tumor immunity, seems to act downstream of IL-10 signaling: blockade of IL-10 abrogates IFNγ production induced by CTLA-4 signaling, and interruption of either CTLA-4 or IL-10 induces anti-tumor responses of comparable efficacy [57]. It has also been established that some of the immunosuppressive effects of tumor cell-derived cyclooxygenase(COX)-2 activity are due to upregulation of IL-10 [58-60]. It is of note, then, to recognize that the functions of IL-10 are many and context-dependent. As investigators, we may see simply the net result of IL-10's individual effects on several molecules and cell types in a given system.

Interleukin-10 in autoimmunity.

IL-10 is an interesting cytokine in the context of autoimmunity. Primarily, it is well-known for limiting the inflammatory response and preventing unneeded tissue damage. Conversely, because of its capability to temper the responses of innate and adaptive immune cell subsets, it can interfere with pathogen clearance and contribute to sustained infection [25,61] [and discussed above]. As a product of regulatory T cells, IL-10 has been implicated in the susceptibility to and development of low-level chronic infection by some parasites and fungal pathogens [62-63]. IL-10 serves as the master regulator of homeostasis and maintainer of tolerance to resident flora in the gut. Because of its strong immunomodulatory functions, IL-10 has often been explored as a means of treatment in several autoimmune diseases, such as psoriasis and inflammatory bowel disease [64]. Many of these treatments have experienced limited success, so it appears necessary to further investigate the molecules and cells under the control of IL-10. Perhaps one or more of these targets will be a more appropriate candidate for treatment of autoimmune responses. In this dissertation, we have

investigated the relationship between IL-10 and Th17 cells in IL-10-deficient mice and patients with Crohn's Disease (CD).

Inflammation and cancer.

Perhaps not surprisingly, inflammation serves multiple functions in settings of malignancy and can initiate or facilitate conditions of autoimmunity. For more than a century, physicians and researchers have noted that patients with chronic inflammatory conditions were more likely to develop tumors in the affected organs than healthy patients. Recent findings have substantiated this relationship in a number of ways. Ongoing cell proliferation in an area already subject to chronic inflammation exposes the cells to growth and survival factors, various inflammatory mediators, activated stroma, and agents that are capable of damaging DNA, such as reactive oxygen species. Additionally, in an environment where cancer is already growing, the process of "smoldering inflammation" contributes to the longevity of the tumor—certain pro-inflammatory cytokines are also pro-angiogenic and support the growth of blood vessels or lymphatics that serve as delivery systems to feed malignancies. Additionally, many cellular subsets, traditionally categorized as "pro-inflammatory," such as immature myeloid cells and fibroblasts, secrete chemokines and cytokines that serve as mitogens for neoplastic cells [65]. In the current view, then, inflammation and tumor growth are quite intricately linked: in select pathological instances, one cannot examine the causality of malignancy without postulating an inflamed environment or preceding infection (such as in hepatocellular carcinoma), and cannot treat a chronic inflammatory condition without evaluating the likelihood of future tumor development (colitis, etc.). It is with this in mind that our laboratory has further explored the relationship between an immune cell subset now classically linked with chronic inflammation—Th17—and its role in cancer development.

Interleukin-17-producing cells and receptors.

IL-17 is a proinflammatory cytokine which can profoundly induce the recruitment of neutrophils [66] and the production of other proinflammatory cytokines, chemokines, and prostaglandins. It has six family members (IL-17A

through F) which are expressed by a variety of innate and adaptive immune cell types, including mast cells, epithelial cells, smooth muscle cells, invariant natural killer T (iNKT) cells, NK cells, paneth cells, lymphoid-tissue inducer (LTi)-like cells, neutrophils, and finally, gamma-delta (γδ) and alpha-beta (αβ) T cells (both $CD4^+$ and $CD8^+$) [67]. The most well-studied of these cytokines are IL-17A and IL-17F; this dissertation focuses on IL-17A. As for the IL-17 receptor, there are at least three variants, termed IL-17 receptor (IL-17R) A, B, and C. Earlier studies demonstrated the ubiquity of IL-17R expression (on hematopoetic and non-hematopoetic tissue, including tumor cell lines and primary tumors [68-71]), but current knowledge suggests that these reports be re-examined to determine which receptor subunits are involved. Fibroblasts, epithelial cells, macrophages, and endothelial cells express both IL-17RA and IL-17RC, whereas T cells express only IL-17RA homodimers [72]. The IL-17RA-IL-17RC heterodimer has a much higher affinity for IL-17 than the IL-17RA homodimer. T cells may thus have a lower affinity for IL-17 than those cells that express heterodimeric receptor complexes [72-73]. Functional IL-17RA has also been found on glial cells in the central nervous system [74].

Interleukin-1-expressing cells and receptors.

There are two IL-1 agonists, termed IL-1α and IL-1β, and an antagonist, termed IL-1 receptor antagonist (IL-1Ra). While both agonists bind the same receptors and induce the same cellular effects, they are active in predominantly different ways. The rarely-secreted agonist IL-1α is active in both cytoplasmic and membrane-bound forms, more well-known as an autocrine regulator of cell homeostasis. Its expression is upregulated in conditions of inflammation. In contrast, IL-1β is secreted only upon cellular reception of inflammatory signals; its secreted form is its only active form. IL-1β is present within the cell as an inactive precursor protein, pro-IL-1β. Cleavage of pro-IL-1β to its active form requires the enzymatic activity of caspase-1 (also termed Interleukin-1β-converting enzyme, or ICE) on an inflammasome scaffold. The NALP3 inflammasome is well-studied in this context. Signals such as endotoxin exposure induce the activation of caspase-1 and subsequent cleavage of pro-IL-

1β, while a second signal, like exposure to ATP, maximizes IL-1β secretion [75]. The primary sources of IL-1 are antigen-presenting cells, such as monocytes, macrophages, and myeloid dendritic cells. IL-1 can also be produced by endothelium, stromal and epidermal cells, fibroblasts, granulocytes, mast cells, platelets, and various lymphocytes. Interestingly, necrotizing cells can also release IL-1 [76].

There are multiple receptors for IL-1. IL-1 receptor (IL-1R) I is the receptor that propagates cellular signals upon IL-1α or IL-1β binding. IL-1RII serves as a decoy receptor, and binding to it induces no signal. IL-1RII likely serves as a scavenger for surplus IL-1 and protects the host from excessive inflammation. Binding of IL-1RI by its ligand induces the recruitment of the IL-1R accessory protein (IL-1RAcP), which forms a heterodimer with IL-1RI. This receptor transmits the IL-1 signal to the nucleus, where it effects the production of many other proinflammatory mediators, often via activation of the transcription factor nuclear factor kappa beta (NFκβ). Both the binding of IL-1 agonists to IL-1RII and IL-Ra binding to IL-1RI fail to recruit IL-1RAcP, so no signal is propagated [76-77]. The IL-1RI is present on a variety of cells, including B and T lymphocytes, monocytes and macrophages, endothelial and epithelial cells, and mesenchymal cells [78].

It is now commonly accepted that IL-1 signaling plays a crucial role in Th17 lineage commitment and expansion. As Chung and colleagues demonstrated in 2009, IL-1 is required for early programming of the Th17 lineage, and IL-1R expression on T cells is induced by IL-6. IL-1 signaling is also required for dendritic cell-mediated Th17 differentiation from naïve or regulatory T cell precursors. Cytokine expression in Th17 cells is maintained by IL-1, IL-6, and IL-23. Moreover, IL-1 regulated the expression of transcription factors IRF4 and RORγt during Th17 cell differentiation [79]. Interestingly, Gulen *et al* recently observed that single Ig IL-1R-related molecule (SIGIRR) negatively regulates the expression of IRF4 and RORγt; SIGIRR is induced during Th17 lineage commitment and governs differentiation. T cells lacking SIGIRR can more easily be polarized to a Th17 phenotype, and this polarization is even stronger in the

presence of exogenous IL-1. SIGIRR controls the IL-1-mediated phosphorylation of JNK and mTOR kinase. In mTOR-deficient Th17 cells, IL-1 cannot induce expansion as it normally does, demonstrating an essential role for mTOR activation in Th17 proliferation [80].

Th17 and cancer.

Since their discovery only five years ago, Th17 cells have risen to prominence in studies of virology, autoimmune disease, inflammation, and immune responses to various parasites and fungi. While their role in the pathogenesis of many of these conditions is rather well-defined, their function(s) in the context of tumor immunology remains controversial. These cells have been examined in cancer patients by a few laboratories, including our own. We have shown that human tumor-associated Th17 cells express minimal levels of HLA-DR, CD25, and granzyme B, suggesting that they are not a “conventional” effector cell population. Moreover, these cells also do not express programmed cell death 1 (PD-1) or FoxP3, making it unlikely that they enact immune suppression through either pathway. As for cytokine products, Th17 cells in cancer patients produce high levels of granulocyte-macrophage colony stimulating factor (GM-CSF), TNFα, IL-2, and IFNγ, but no IL-10 [81]. Tumor-associated Th17 cytokine products mimic those found in some instances of viral infection [82-83]; we believe that tumor-associated Th17 cells have the ability to influence immune responses through the action of these proteins.

Many laboratories have studied Th17 populations in the blood and (occasionally) tissues of patients with various cancers. Our group has extensively examined Th17 distribution and function in ovarian cancer patients. We have made several key observations: firstly, that the prevalence of Th17 in the tumor-draining lymph nodes (TDLN) and blood of these patients is comparable to that of healthy donors. Secondly, although Th17 cells constitute a small population within the tumor microenvironment, they are found in proportionally higher numbers here in comparison to other immune cell subsets. Tumor-associated Th17 levels correlate positively with microenvironmental Th1 cells, cytotoxic $CD8^+$ T cells, and NK cells, and inversely with Tregs [81,84]. Su,

et al also found significantly higher numbers of Th17 cells expanded or induced from TIL populations in cancer patients than in lymphocyte populations from non-tumor tissue [85]. In ovarian cancer patients, Th17 cells were the sole source of IL-17 in ascites, and the level of IL-17 in this fluid correlated positively with patient survival. Even after controlling for surgical debulking and other parameters, tumor-associated IL-17 was a negative predictor of death hazard. In the tumor microenvironment, IL-17 synergized with IFNγ to induce CXCL9 and CXCL10 production. These Th1-type chemokines recruit effector populations to the tumor itself: we found that ascites levels of CXCL9 and CXCL10 correlated directly with tumor-infiltrating NK and $CD8^+$ T cells [81]. In agreement with our finding that Th17 cells are protective, Sfanos, *et al* found an inverse correlation between the differentiation stage of Th17 cells in prostate glands of cancer patients and their tumor progression [86]. However, in another study examining patients with hormone-resistant prostate cancer, Derhovanessian, *et al* demonstrated an inverse correlation between pre-treatment circulating levels of Th17 cells and time to disease progression[87]. Recall that the levels of Th17 cells are usually limited in cancer patients [81,84]. A larger population of Th17 in the blood may indicate an underlying infection or inflammatory state, which would influence the efficacy of immunotherapy and speed of tumor development. It would be interesting to further evaluate these patient samples and try to determine the initial cause of the expanded blood Th17 populations. Finally, Ye and colleagues investigated Th17 cells from 30 patients with lung adenocarcinoma or squamous cell carcinoma. Malignant pleural effusion (MPE) from these patients was chemotactic for Th17 cells, and this activity was partially abrogated by CCL20 and/or CCL22 blockade. Interestingly, higher accumulation of Th17 cells in MPE predicted improved patient survival [88].

Our laboratory has also studied Th17 cells in murine cancer. Similar to humans, Th17 populations are limited in healthy mice, but relatively expanded in the blood, bone marrow, and spleens of mice bearing the aggressive B16 melanoma. Interestingly, the largest populations of Th17 cells occurred within the tumor. We also observed expanded Th17 populations in mouse melanoma,

prostate cancer, fibrosarcoma, and advanced head and neck cancer [89]. The laboratory of Nicholas Restifo published a study in 2008 investigating the effect of a tumor antigen-specific T cell clone on the eradication of murine melanoma. Interestingly, Th17-polarized (via IL-6 and TGFβ) T cell clones were better than Th1-polarized clones in destroying advanced B16 tumors, although their effect seemed to depend largely on their production of IFNγ. Soon after these experiments were published, Sharma, *et al* treated B16-bearing mice with an indoleamine 2,3-dioxygenase (IDO) inhibitor and antitumor vaccine which increased the frequency of IL-6 production by plasmacytoid DCs (pDC). This treatment also caused a conversion of many Tregs in TDLNs to Th17 cells, and the investigators observed an increase in activated $CD8^+$ T cells along with augmented antitumor efficacy [90]. Several other groups have investigated Th17 cells in murine cancer, with controversial results [91-94].

In the more exhaustive studies of patients with established epithelial cancer, Th17 presence and function have correlated with reduced tumor progression and improved patient survival. In mice with established tumors, studies have documented potent antitumor efficacy for both Th17 and IL-17^+CD8^+ T cell (Tc17) populations. However, it is possible that Th17 function may vary according to cancer cause, type, and location [95], as well as stage of disease. Although human studies are technically challenging, it is now essential to investigate the roles of Th17 cells and IL-17 in the very early phases of human tumor growth to better understand how these roles may change during disease progression.

Th17 in autoimmunity.

Autoimmunity develops when the body turns against itself: when cells of the host immune system begin attacking host tissues. In tumor-bearing patients, induction of immunity of this sort—to “altered” host tissues—is desirable. Autoreactive immune responses that occur under homeostatic conditions are both necessary and regulatory. However, when pathological autoimmunity develops it is quite harmful. Over the past several years, it has been established that Th17 cells play significant roles in the development and pathogenesis of

many autoimmune diseases, where once Th1 cells were thought to be key mediators. Several of these conditions include psoriasis, rheumatoid arthritis (RA), multiple sclerosis (MS), and the family of inflammatory bowel diseases (IBD).

Psoriasis is a chronic inflammatory disease of the skin involving epidermal infiltration of T cells, DC, and monocytes. The condition is characterized by epidermal hyperplasia and angiogenesis in the dermis. Both Th1-type and Th17-type cytokines are over-expressed in lesional skin and serum of patients, but more convincing proof that Th17 cells have a key role is the up-regulation of RAR-related orphan receptor C (RORC), IL-1β, IL-6 and IL-23 in psoriatic skin when compared with healthy skin samples [96-97]. Our laboratory has recently documented expanded populations of both Tc17 cells and Th17 cells in psoriatic lesions, and that myeloid APC from psoriasis samples support induction of these populations. We also found that IFNγ, which is increased in psoriatic blood and skin, programs myeloid APCs to induce human IL-17$^+$ T cells via IL-1 and IL-23 [98]. IFNγ also stimulates APC production of CCL20, a chemokine which supports IL-17$^+$ T cell migration. It is possible that Th1 cells and IL-17$^+$ T cells may collaborate in the pathogenesis of human psoriasis. Interestingly, treatments that target the p40 subunit of IL-12 and IL-23 have been shown to be effective in psoriasis patients [99], and that patient improvement is associated with a decrease in multiple proinflammatory cytokines and chemokines that may further mediate disease pathogenesis [100]. A recent study investigating etanercept (the soluble TNF receptor) showed that treatment downregulated many Th17-polarizing cytokines, as well as CCL20 and certain anti-microbial peptides [101]. Some of the same investigators then found that psoriatic lesions are characterized by an accumulation of immature CD11c$^+$blood dendritic cell antigen (BDCA)$^-$ DC that secrete inflammatory cytokines [102]. These “psoriatic dermal DCs” induced a population of activated T cells that produced both IL-17 and IFNγ: a population of T cells not induced by normal (BDCA$^+$) dermal DCs. It remains to be determined if these IL-17$^+$ IFNγ$^+$ double-positive cells are

pathogenic. It appears that treatments targeting Th17 cells or factors that support Th17 development are promising in the management of psoriasis.

Another rheumatic autoimmune disease mediated by Th17 cells is RA. RA patients suffer from chronic inflammation in multiple joints, and this is associated with bone and cartilage destruction [96]. Mouse studies of collagen-induced arthritis (CIA) have demonstrated that IL-23, a cytokine crucial in Th17 polarization, is necessary for disease; mice deficient in the p19-subunit of IL-23 do not develop arthritis [103]. IL-12p35-deficient mice actually develop more severe disease, which suggests a protective role for IL-12 and/or IFNγ. Mice lacking IL-17 develop less severe arthritis, and joint inflammation in wild-type mice has been ameliorated via administration of anti-IL-17 antibody or soluble IL-17 receptor (IL-17R) [104-106]. IL-17 also has demonstrated involvement in human RA. Multiple studies have shown increases in IL-17 in sera and synovial fluid of RA patients when compared to healthy controls, and there is also evidence for IL-17 in the T cell-rich areas of the joint [107-110]. Recent research has established that the development of a cytokine environment favoring Th17 cell development is an early event in RA pathogenesis [111]. Studies of disease severity in RA patients have linked higher amounts of IL-17 and TNFα in the synovium with more severe joint damage over time [112]. Investigators recently showed that inhibition of IL-17 by an anti-IL-17 ribonucleic acid (RNA) aptamer slowed onset of arthritic and neurological symptoms in mouse models of RA and MS, respectively [113]. Apart from IL-17's role as the signature cytokine of Th17 cells, it can induce the production of a host of other proinflammatory mediators from myeloid cells and synovial fibroblasts, such as IL-1β, TNFα, IL-6 and IL-23, therefore perpetuating the existence of an inflammatory environment and positively feeding back into Th17 development and maintenance [114-115]. Studies from David Fox's lab have shown that cytokine-activated T cells can adhere to RA synovial fibroblasts and induce production of the prototypical inflammatory cytokines IL-6 and IL-8; this production was increased upon addition of IL-17 [116]. Interestingly, blockade of membrane-bound TNFα abrogated this cytokine production. Not surprisingly, then, a very recent report

showed that Th17 presence in the joints of arthritis patients correlated positively with several other synovial and systemic markers of inflammation [117]. Th17 cells are also capable of upregulating receptor activator of nuclear factor κβ (RANK) ligand and effecting downstream bone destruction [118-119]. It seems that IL-17, through both direct and indirect means, contributes to the maintenance of the chronically inflamed environment observed in joints affected by RA. Regulation of IL-17 expression by multiple factors [120] will no doubt serve as a likely mechanism for future treatment options.

Th17 cells do not only play central roles in chronic rheumatic diseases; they also serve to initiate and support various other conditions of autoimmunity. Two of these are MS and IBD. MS, and its induced mouse model, experimental autoimmune encephalomyelitis (EAE), are characterized by damage to the myelin sheaths surrounding the axons of the nerves in the brain and spinal cord. In 1999, it was discovered that there were increased levels of IL-17 message in MS patients' blood and cerebrospinal fluid (CSF) [121]. Higher levels of IL-17 and IL-8 were present in the CSF of Asian patients with the more severe opticospinal form of disease when compared to patients with conventional MS [96,122]. Du and colleagues reported that expression of mir-326—a Th17-associated micro RNA—correlated significantly with disease severity in MS patients and mice with EAE [123]. Abrogation of miR-326 expression resulted in fewer Th17 cells and mild EAE, and its upregulation increased Th17 cell numbers and was associated with severe EAE. Although EAE was once thought to be a Th1-mediated condition, subsequent experiments with IL-12 receptor β2-deficient and IL-23p19-deficient mice proved that theory incorrect. Whereas IL-12Rβ2$^{-/-}$ mice developed more severe disease, disease in IL-23p19$^{-/-}$ mice was completely abolished [4,124]. The CD4^{+} T cells that infiltrated the central nervous system (CNS) in the IL-23p19$^{-/-}$ mice lacked IL-17, TNFα, and IL-6 expression. Investigators found that antigen-activated CD4^{+} T cells displayed increased IL-17 production upon the addition of exogenous IL-23, and that adoptive transfer of these Th17 cells was sufficient to induce EAE in mice predisposed to disease [4]. Moreover, Kebir and colleagues demonstrated in

2007 that *in vitro*-polarized Th17 cells could more easily invade a layer of blood brain barrier endothelial cells (BBB-EC) than Th1-polarized cells. They also showed that treatment of BBB-EC with IL-17 or IL-22 made it easier for human PBMC CD4+ T cells to travel through the monolayer [125]. It is possible then, that Th17 in MS serve to weaken the blood brain barrier, facilitating the influx of cells into the CNS. More recently, McGeachy *et al* found that stimulation of myelin-reactive T cells with TGFβ and IL-6 eliminated their pathogenic function, even though they up-regulated expression of IL-17 [17]. These cells failed to express the chemokines crucial for CNS inflammation but instead produced IL-10. In contrast, stimulation of these same myelin-reactive T cells with IL-23 induced IL-17 and proinflammatory chemokine expression. It seems that TGFβ and IL-6 are required for Th17 lineage commitment but are instrumental in curbing the pathogenic functions of these cells; rather, IL-23 exposure stimulates pathogenicity. Intriguingly, CNS-resident NK cells may play a role in Th17 control and therefore extent of disease in MS models, since NK enrichment in mice with EAE resulted in disease amelioration, whereas disease worsened when NK cells were prevented from migrating to the CNS [126]. CNS-resident NK cells interacted with microglia and enacted functional suppression of myelin-reactive Th17 cells. Melton and colleagues recently demonstrated that the integrin αvβ8, which can activate TGFβ, plays a critical role in Th17 cell development [127]. Th17 cells were nearly absent in the colons of mice lacking αvβ8 expression on DCs, and cells from these mice were defective in Th17 induction. Strikingly, these mice were almost completely refractory to induction of EAE. In the future, DC αvβ8 may serve as a therapeutic target for the treatment of Th17-driven autoimmune disease.

An additional family of chronic inflammatory diseases in which Th17 play a role is the group of inflammatory bowel diseases, which includes colitis and Crohn's Disease (CD). Multiple studies of murine intestinal inflammation have established that IL-23 is requisite in both spontaneous and infection-induced disease [128-130]. Perhaps not surprisingly, then, antibodies and inhibitors that target the p40 subunit shared by IL-12 and IL-23 have demonstrated clinical

efficacy in ameliorating CD patient symptoms [131-132]. Human lamina propria monocyte and macrophage-derived IL-1β and IL-6, cytokines required for early T cell commitment to the Th17 lineage, have long been regarded as key mediators of intestinal inflammation [133-134]. Recent experiments by Huff *et al* have established that IL-1β and IL-6 in CD stromal-conditioned media promoted T cell proliferation, and that IL-1β alone could promote IL-17 and IFNγ expression. Examination of tissues from colitis and CD patients revealed that IL-17 was expressed in the inflamed colon, and that Th17 cells were clustered in the lamina propria [135-137]. In multiple studies, restimulated T cells isolated from CD patients were capable of producing high levels of IL-17 [138-140]. Interestingly, IL-17 has also been found to inhibit the proliferation of intestinal epithelial cells, a phenomenon that may contribute to the maintenance of the chronic inflammatory environment in IBD by preventing damaged tissue from healing [141]. It seems then that IL-17 itself, the mediators that induce its expression, and the downstream targets of IL-17 are all functionally relevant in IBD. Animal and early clinical trials targeting many of these molecules are ongoing [142-144], and it is possible that several will prove useful for patient management of these multifaceted diseases. We have accordingly explored the relationship of IL-10 and IL-1 signaling to Th17 development in an inflammatory setting in the second part of this dissertation.

Dendritic cells in cancer and autoimmunity.

Myeloid dendritic cells (mDC) may be the most often-studied of the antigen presenting cell subsets. They play a key role in the adaptive arm of the immune system by stimulating the activation of naïve T cells [145]. Pulsing of DC with killed ovarian tumor cells has been shown to effectively stimulate tumor-specific blood-derived T cells, and these MHC-I-restricted T cells can produce IFNγ upon encountering autologous tumor cells [146]. However, the tumor or tumor environment often produces factors that suppress the development and stimulatory function of DC [147-148], which in turn undermines antitumor immunity and leads to accelerated tumor growth. Our laboratory's studies of ovarian cancer have demonstrated this suppression in numerous ways. In 2003,

we documented low expression levels of the inhibitory molecule B7-H1 on blood- and lymph node(LN)-derived mDC in healthy individuals, but observed a striking upregulation of B7-H1 on mDC from tumor-draining lymph nodes (TDLN) and tumors [149] from ovarian cancer patients. In this study, B7-H1 expression on these cells was upregulated via interleukin(IL)-10 and vascular endothelial growth factor (VEGF). Interestingly, IL-10 had previously been shown to decrease costimulatory molecule expression on DC [150], while VEGF could inhibit DC differentiation from hematopoetic precursors [147]. B7-H1 blockade enhanced mDC-mediated T-cell activation, and was associated with downregulation of IL-10 and upregulation of IL-2 and IFNγ production by T cells. Interestingly, this treatment also downregulated IL-10 in mDC and stimulated an increase in IL-12 expression. Finally, T cells conditioned with the B7-H1−blocked mDC were more potent inhibitors of autologous human ovarian carcinoma growth in non-obese diabetic−severe combined immunodeficient (NOD-SCID) mice. In 2008, Huarte, *et al* demonstrated that $CD11c^{+}DEC205^{+}$ DCs coexpressing alpha-smooth muscle actin and VE-cadherin migrate to perivascular areas in ovarian carcinoma and are essential in maintaining intratumoral tumor vasculature [151]. Perhaps not surprisingly, subsequent experiments involving DC depletion in mice bearing various established ovarian cancers delayed tumor growth and enhanced chemotherapeutic effects. Altogether, mDC are thought to be the major functional DC subsets in tumor environments. However, in patients with ovarian cancer, functional mature mDC exist in limited numbers within the tumor. This fact, along with data demonstrating that mDC are phenotypically and functionally altered by tumor environments and are either dysfunctional or mediate immune suppression, support the heretofore unsatisfying clinical outcomes of DC vaccine trials.

DC in Crohn's Disease have been studied extensively. There are imbalances in population distribution and cytokine expression in diseased intestinal tissue. DCs coexpressing $CD11c^{+}CD83^{+}$DC-$SIGN^{+}$ are significantly reduced in inflamed lamina propria and submucosa. Interestingly, myeloid DC (mDC) levels are elevated in the omentum of CD patients, while there is a

significant decrease of mDC and plasmacytoid (pDC) in CD blood. Myeloid DC expression of CD1a is increased in the lamina propria and ileum of CD patients; this surface molecule both designates “conventional” DC that can skew T cells to a Th1 phenotype and is a receptor for self or foreign lipid antigens; when lipids are bound to CD1a, it can stimulate T cell activation [152-153]. The higher incidence of $CD1a^+$ DC in CD tissues may indicate an increased sensitivity to immune activation by foreign or self antigens. A high percentage of DC isolated from CD patients produce IL-12 and IL-6, in contrast to a very low percentage of DC isolated from healthy donors [154]. Toll-like receptor (TLR) distribution is also radically changed on DC from CD patients. A subset of those with CD have mutations in their NOD2 receptors. Certain mutations prevent TLR2 signaling from downregulating NFκβ, which, while active, induces proinflammatory cytokine production. Patients who are homozygous for the NOD2fs mutation (which predisposes the carrier to CD) have normal DC TLR receptor expression but fail to upregulate CD80/86 in response to muramyl dipeptide (MDP; a NOD2 ligand). MDP is also capable of inducing TNFα, IL-12 and IL-10 from normal DC, but cannot elicit the same cytokine response in DC with the NOD2fs mutation. These DC therefore have a loss-of-function phenotype, and may contribute to the reduced IL-10 seen in some CD patients [155]. Interestingly, Correa and colleagues documented that monocyte-derived DC from patients with severe CD produce significantly less IL-10 upon LPS stimulation than those from healthy donors [156]. Although mutations in the IL-10 promoter region have been observed, this phenomenon was not associated with them. A possible link between the decreased IL-10 production and mutations in the IL-10RA and B genes previously seen in some patients with early-onset colitis [157] was not investigated.

Other irregularities in immune cell subsets and their products have been observed in settings of IBD: there is increased recruitment and retention of macrophages, neutrophils, and T cells in the gut of affected patients. This phenomenon contributes to the increased levels of proinflammatory cytokines (IL-12, IL-17, IL-21, IL-23, and IL-27 are selectively upregulated in CD, while IL-

1β, IL-6, IL-8, IL-22, and TNFα are broadly observed), chemokines, and adhesion (ICAM1) and costimulatory molecules (CD40, CD80/86, ICOS). Increased endothelial expression of VCAM1, VLA4, and ICAM1 causes higher percentages of circulating monocytes and neutrophils to adhere to inflamed vessel walls in and near the gut. B cell responses to enteric flora are enhanced, while intestinal T cells both execute inappropriately aggressive responses to enteric flora and appear to be less susceptible to apoptosis [158-161]. Additionally, constitutive activation of STAT3 (required in Th17 differentiation [162]) and STAT4 has been observed in intestinal T cells from CD patients [163]. Taken together, it is not hard to comprehend the massively proinflammatory, activated milieu of cells and intercellular mediators present in tissues affected by IBD.

Suppressive cell subsets in cancer and autoimmunity: regulatory T cells.

T regulatory (Treg) cells are a subpopulation of $CD4^+$ T cells with suppressive functionality. In healthy individuals, perhaps the most important role of T regulatory cells is to maintain immune tolerance to self-antigens, which prevents development of autoimmune disease. Treg cells are also responsible for limiting tissue damage during ongoing and resolving immune responses, maintaining oral and feto-maternal tolerance, and restraining asthma and allergy. In settings of organ transplant and cancer, the suppressive function of Treg cells is currently being manipulated in order to improve patient health and survival. Investigators of transplantation biology are exploring ways to increase the number of alloantigen-reactive T regulatory cells in transplant recipients to minimize grafted tissue damage and prevent organ rejection[164]. In cancer patients, where T regulatory cells contribute to the dampening of the anti-tumor immune response, combination therapies that include the inhibition of T regulatory cell function have been explored. Although few stage III trials of Treg inhibition have reached their clinical endpoints, analysis of Tregs in tumor environments can still yield useful information about patient prognosis and tumor growth, and may eventually lead to new, more successful treatment regimes.

T regulatory cells, originally termed suppressive T cells, were first

described by Gershon *et al.* [165-166] in the early 1970s as thymus-derived lymphocytes that tolerized bone marrow-derived lymphocytes to antigenic challenge. Research in the laboratory of R. J. North subsequently demonstrated that T cells expressing CD4 and CD25 from tumor-bearing mice abrogated tumor rejection; this suggested the existence of a tumor-suppressor T cell population [167-169] . Many years later, after more than a decade of intense skepticism regarding the suppressive cells' existence, Sakaguchi, *et al* ascertained that the interleukin-2 (IL-2) receptor α-chain (also called CD25) could be used to identify them [170]. Later studies in the same laboratory, as well as studies from Rudensky *et al*, established the transcription factor forkhead box P3 (FoxP3) as both a key intracellular marker of $CD4^+CD25^+$ T regulatory cells and necessary factor for development and proper function of these cells [171-173], which was described early on as prevention of autoimmune conditions (e.g. colitis [21]) and suppression of $CD8^+$ T cell homeostatic proliferation [174]. Beginning with these reports, the field of T regulatory cells has expanded and progressed rapidly. In fact, several distinct regulatory T cell populations have been proposed, including $CD8^+$ subsets. These include thymically-derived $CD8^+CD25^+$ T cells that utilize cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) and transforming growth factor β (TGFβ) to suppress cell proliferation and activation [175], as well as a $CD8^+CD28^-$ T cell population from the periphery that targets immunoglobulin-like transcripts 3 (ILT3) and 4 (ILT4) on dendritic cells (DCs) [176]. Our group has identified $CD8^+$ T cells [148,177] in human ovarian cancer that secrete the suppressive cytokine interleukin-10 (IL-10). Interestingly, a $CD8^+$ regulatory T cell population specific for heme oxygenase-1 (HO1) has recently been identified [178]. This population, isolated from the peripheral blood of cancer patients, inhibited proliferation, cytotoxicity, and cytokine production of other cell immune cells. Groux, *et al* identified a $FoxP3^-$ $CD4^+$ population (termed T_R1 cells) which may also suppress through IL-10 *in vitro* [179]. Weiner characterized a $CD4^+$ $TGFβ^+$ population (T_H3) that exerts suppressive action *in vivo* through TGFβ [180]. Both aforementioned populations are likely derived from the periphery. Classic T regulatory cells (Treg), $CD4^+CD25^+FoxP3^+$ T cells, differentiate in the

thymus and migrate to the periphery [181-182]. They constitutively express leukocyte common antigen isoform RO (CD45RO), glucocorticoid-induced tumour-necrosis factor receptor-related protein (GITR), and CTLA-4 [183-187]. Finally, an excellent recent paper from the laboratory of Shimon Sakaguchi presents the possibility of further categorizing naturally-occurring T_{Regs} into three subgroups: $CD45RA^{+}$ $FoxP3^{lo}$ resting Treg, termed "rTreg" by the authors, $CD45RA^{-}$ $FoxP3^{hi}$ activated Treg (aTreg) cells, and cytokine-secreting $CD45RA^{-}$ $FoxP3^{lo}$ non-suppressive T cells [188]. Ongoing investigations into phenotype, function, and associations with disease states will likely contribute to knowledge of an even wider range of regulatory T cell populations in the future. Regardless, it is important to emphasize that regulatory T cells must be defined not only by phenotypic markers, but also by their suppressive activity *in vivo*.

In healthy mice and humans, Treg cells are found primarily in the thymus, peripheral blood, lymph nodes, and spleen. They constitute 5-10% of the resident CD4+ T cells in each of these organs [189-191]. In bone marrow, however, Treg cells account for a remarkable 25% of $CD4^{+}$ T cells [192]. Bone marrow is the preferential site of metastasis for some cancers (such as breast, lung, and prostate), suggesting that the suppressive environment here is conducive to tumor growth. In tumors themselves, however, there are a number of ways that Treg cells might accumulate: trafficking to the tumor under the influence of chemokine ligand 22 (CCL22) [193], differentiation [148,177,194-197] or expansion [198-200] within the tumor stroma, and conversion from normal T cells [201-204]. Many tumors express tumor-associated antigens (TAAs), molecules found on tumor cells but also on certain populations of normal cells. The work of several groups has identified multiple mechanisms of suppression by TAA-specific Treg cells. These may include induction of IL-10, which can drastically suppress APC and T cell function [205], induction of TGFβ, which may suppress natural killer (NK) cell function [206], competitive consumption of interleukin-2 (IL-2), which is a survival factor for conventional T cells [189,207-208], perforin and granzyme-dependent killing of T cells and APCs [209-210], CTLA-4 induction of indolamine 2,3-dioxygenase (IDO)-expressing

APCs, which suppress T cell activation and promote tolerance [211-212], and finally, induction of B7-H4 expression on APCs, which renders them immunosuppressive [213-214]. Thus, Treg cells target both T cells and APCs to create a generally tolerant tumor microenvironment.

Tumor-associated Treg cells have been studied largely with reagents that target them in tumor-bearing mice. Treatment with CD25-specific antibody (PC61) *in vivo* suppressed growth of several tumor types [215-216]. These early studies demonstrated a correlation between reduced Treg numbers and reduced tumor volume. Interestingly, depletion of total $CD4^+$ T cells corroborated these data and lead to improved tumor immunity and rejection of tumors [217-219]. Several groups confirmed these data with CD25-depletion alone or in concert with other treatments, such as anti-CTLA4 antibody [218], anti-B7H1 antibodies (WZ *et al*, unpublished observations), exogenous interferon-α (IFNα) [220] or interleukin-12 (IL-12) [221], adoptive transfer of DCs [220,222], and irradiated tumor cells [223]. Adoptive transfer of human [193] or mouse [224-225] Treg cells into mice have also provided a direct functional connection between Treg cell presence and reduced tumor immunity. One study examined B16 melanoma-bearing mice that received tumor-specific $CD8^+$ T cells with either classic Treg cells or with $CD25^-CD4^+$ T cells [225]. $CD8^+$ T-cell-mediated tumor immunity was abrogated in mice receiving classic Treg cells, but not $CD25^-CD4^+$ T cells. These studies demonstrate that Treg cells inhibit murine TAA-specific immunity.

As for human cancer, June, *et al* observed increased numbers of Treg cells in patients with non-small-cell lung cancer and ovarian carcinoma when compared to healthy patients [226]. Since this study in 2001, several other groups have made similar observations in the peripheral blood of patients with various types of cancer, including pancreatic and breast cancer [227], colorectal cancer [228-229], gastric and esophageal cancer [230-231], leukemia and lymphoma [232-233], melanoma [234-235], lung and ovarian cancer [226,229], and hepatocellular carcinoma [236]. In many cancers, increased Treg populations correlate inversely with patient disease stage and survival. However,

this is not always the case [237]. In studies of gastric, colorectal, and anal cancer, increased Treg populations within the tumor tissue seem to be beneficial. It is interesting to note that all of these cancers are localized to the gastrointestinal tract, portions of which are sites of the most rapid cell turnover in the human body. It is possible that Treg cells in this environment are more crucial for restraining inflammation (and thus preventing angiogenesis and other developments beneficial to tumor growth and survival) than for shutting off the host's response to the tumor [65,238].

Interestingly, Tregs have also been found in significant numbers in mice and humans with IBD. We have already mentioned that IL-10 is essential for their suppressor function [21]. In patients with active Crohn's Disease. Treg populations are expanded in areas affected by inflammation—namely, in the mucosal lymphoid tissues, including the lamina propria and mesenteric lymph nodes [239-240]. In these same patients, Treg numbers in the blood are decreased. This observation begs the question of how many of these cells are needed and what functionalities are required to actively control a chronic inflammatory response. Fantini and colleagues have demonstrated that effector T cells in CD are more resistant to Treg-mediated suppression in patients with CD, and that this phenomenon is Smad7-dependent [241]. Intriguingly, Tregs isolated from IBD patients can suppress T cell proliferation equally as well as those from healthy donors. Takahashi *et al* have established that higher numbers of blood Tregs indicate less severe ulcerative colitis (UC) in patients, and vice versa [242]. Recall that certain polymorphisms in the NOD2 gene are associated with CD; patients with these mutations have significantly fewer Tregs than those who do not. The NOD2 ligand, muramyl dipeptide (MDP), can activate NFκβ in human Tregs. Interestingly, MDP-stimulated Tregs are protected from apoptosis mediated by Fas; this protection was not observed in Tregs isolated from Crohn's Disease patients possessing the disease-associated polymorphisms in NOD2. Because the Crohn's Disease environment is rich in Fas ligand, it is possible that Tregs in these patients may not live long enough to mediate suppression of some of the inflammatory mediators in the intestine

[243]. Making the situation even more intriguing is the recent observation of populations of T cells demonstrating characteristics specific to both Tregs and Th17 cells in settings of chronic inflammation. These “double feature” cells can suppress T cell activation and proliferation, but also stimulate proinflammatory cytokines UC tissue [244]. These cells may contribute to UC pathogenesis via inflammatory cytokine induction and inhibition of local T cell immunity. Further research may reveal the existence and functionality of these cells in other pathologies.

Suppressive cell subsets in cancer and autoimmunity: myeloid-derived suppressor cells.

Myeloid-derived suppressor cells (MDSC) are a heterogeous population of immature myeloid cells that are expanded in a number of pathologies, including inflammation and cancer. Although their existence has been noted for decades, the functionalities of MDSC have only recently begun to be elucidated. MDSC can be divided into two main subpopulations, granulocytic (polymorphonuclear) and monocytic (mononuclear, and in mice definable by their expression of the surface marker CD115). The MDSC population as a whole expresses CD11b and GR-1, but is predominantly characterized by its suppressor activity *in vitro* and *in vivo*. During homeostasis, immature myeloid cells reside in the bone marrow and are not suppressive; in pathological situations, however, they are found in lymphoid organs and certain tissues (including tumor). Activated MDSC display increased production of reactive oxygen and nitrogen species, and arginase. Via these molecules, MDSC can strongly suppress T cell activation, proliferation, and other functions [245].

In the blood of patients with various cancers, MDSC are expanded [246-249]. In tumor-bearing mice, MDSC populations are increased in the spleen, tumor-draining lymph nodes, and are present in tumor tissue itself. In addition to negatively regulating the actions of T helper cells and CTLs in tumor-bearing hosts, MDSC are also capable of Treg induction from bystander $CD4^+$ T cells [250-251]. Research is ongoing to determine whether MDSC execute their suppressive effects in an antigen-specific or broad, non-specific manner (or

both). MDSC traffic to the tumor environment, where their suppressive activities are more profound than in the periphery. Upon entering the tumor microenvironment, they upregulate arginase and nitric oxide species, downregulate their expression of reactive oxygen species, and can differentiate into tumor-associated macrophages (TAMs), which nonspecifically suppress antitumor T cell responses [252-253].

MDSC have also been observed in autoimmune disorders. The laboratory of Tim Greten has explored this cell population in both murine and human settings of IBD. In a mouse model of colitis induced by hemagglutinin (HA)-specific T cells, investigators found an increase in $NOS2^{+}Arg^{+}$ $CD11b^{+}$ $GR\text{-}1^{+}$ myeloid cells in the spleen and intestines. These cells demonstrated *ex vivo* suppressive capacity. Additionally, concurrent adoptive transfer of the myeloid cells with HA-specific $CD8^{+}$ T cells into naïve VILLIN-HA mice abrogated disease development, demonstrating that MDSC have an immunoregulatory effect on T cell induction of IBD. In the same report, Haile and colleagues observed an increase in suppressive MDSC in the blood of patients with IBD [254]. Beatty *et al* also documented increases in splenic MDSC in another mouse model of colitis [255]. MDSC are present in settings of IBD, but it may be that they are not powerful enough or do not exist in great enough numbers to inhibit ongoing, self-perpetuating inflammation.

Chapter 2

Interleukin-10 and antitumor immunity

Because our current knowledge of IL-10 biology arises largely from infectious disease studies, the role of IL-10 in cancer is often thought of as analogous to that observed in chronic infectious diseases. Although the underlying mechanisms are poorly understood, early studies have documented an immune stimulatory role for exogenous IL-10 in the tumor microenvironment. Transfection of tumor cells with IL-10 or systemic IL-10 administration significantly suppressed tumor growth and led to tumor rejection [42-43,46,256]. These data suggest that the biological activities of IL-10 (particularly endogenous IL-10) in tumor immunity may be highly context-dependent.

Over the past few years, we and others have achieved important insights into tumor immunopathogenesis in patients with cancer: the tumor microenvironment contains Treg cells [257-258], MDSCs [195,251,253,259-264] and dysfunctional APCs [149,213,265-266] that form a suppressive network to defeat tumor-specific immunity and efficaciously promote tumor. Because IL-10 is functionally linked to this immunosuppressive network, and Treg cells and MDSCs have not been examined in the previous studies which investigated the role of endogenous IL-10 in tumor immunity [43,46,256], we revisited the role of IL-10 in tumor immunity in this study and examined how endogenous IL-10 is involved in regulating MDSCs, Tregs, and effector T cells in the tumor microenvironment.

IL-10 deficiency increases tumor incidence, growth, and foci formation.

The immune-inhibitory role of IL-10 has been well defined in numerous experimental settings. However, the *in vivo* effects of endogenous IL-10 on tumorigenesis and tumor immunity are poorly understood. We compared tumor incidence, growth, and foci formation in IL-10-deficient ($IL\text{-}10^{-/-}$) and wild-type (IL-

$10^{+/+}$) mice. The mice were subjected to administration of dextran sodium sulfate (DSS) and/or azoxymethane (AOM) as previously described [267]. IL-$10^{-/-}$ mice—but not IL-$10^{+/+}$ mice—treated with DSS developed numerous colon polyps. In the presence of DSS and AOM, colon polyps developed in both IL-$10^{-/-}$ and IL-$10^{+/+}$ mice. However, there were more polyps in IL-$10^{-/-}$ mice than IL-$10^{+/+}$ mice (Figure 2.1A). We next subcutaneously injected a colon cancer cell line, MC38, into mice and monitored tumor growth over a period of two weeks. MC38 had accelerated growth in IL-$10^{-/-}$ mice as compared to IL-$10^{+/+}$ mice (Figure 2.1B, C). We further examined the effect of endogenous IL-10 on the development of mouse lung foci. To this end, MCA310, a methylcholanthrene-induced sarcoma, was intravenously injected into IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. IL-$10^{-/-}$ mice had more tumor foci in the lungs than IL-$10^{+/+}$ mice (Figure 2.2). Thus, IL-10 deficiency increases tumor incidence, growth, and foci formation.

Fig. 2.1A

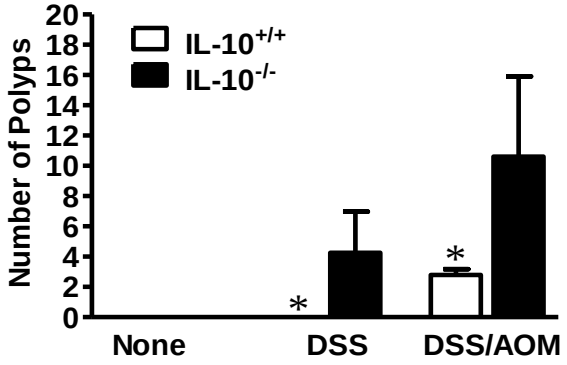


Fig. 2.1B

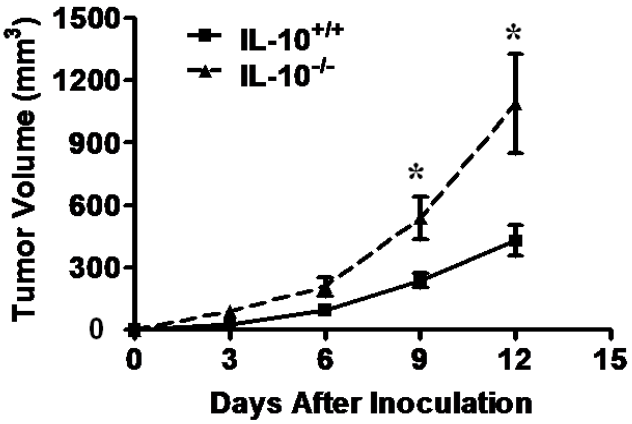


Fig. 2.1C

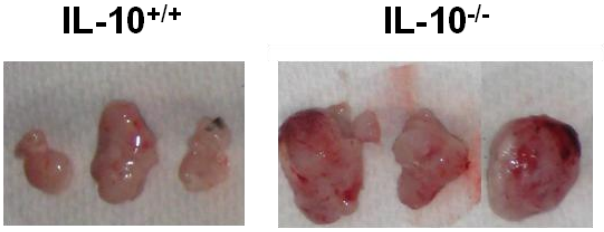


Figure 2.1: IL-10 deficiency increased tumor incidence and growth.
A. Chemically-induced tumor incidence in IL-10$^{+/+}$ and IL-10$^{-/-}$ mice. IL-10$^{+/+}$ and IL-10$^{-/-}$ mice were given no treatment, DSS, or DSS and AOM as described in Material and Methods. Numbers of colon polyps were recorded. Results are expressed as the mean of colon polyps +/- SEM. 6 mice per genotype.
B, C. Tumor growth in IL-10$^{+/+}$ and IL-10$^{-/-}$ mice. MC38 cells were inoculated subcutaneously into the left flank of IL-10$^{+/+}$ and IL-10$^{-/-}$ mice. B. Tumor volume was monitored and recorded. C. Six actual tumors are shown. 8 mice per genotype. *, $P < 0.05$.

Fig. 2.2

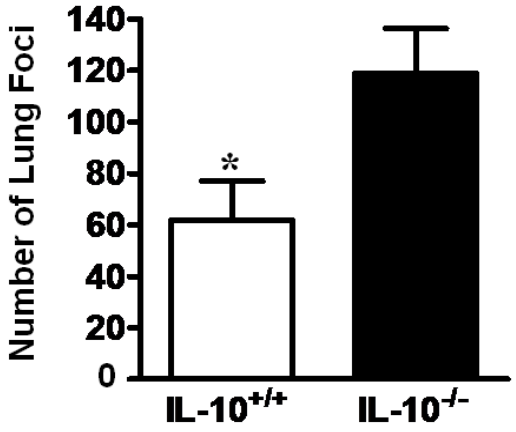


Figure 2.2: IL-10 deficiency increased tumor foci formation.
Tumor lung foci in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. MCA310 cells were injected intravenously into IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. The numbers of lung tumor foci were counted two weeks after tumor inoculation. 6 mice per genotype. *, $P < 0.05$.

IL-10 deficiency decreases immune surveillance in the tumor.

We then investigated the phenotype and cytokine profile of key innate and adaptive immune cells in the tumor and tumor-draining lymph nodes (TDLN) in these mice. Not surprisingly, MHC I expression was increased on both DCs (Figure 2.3A) and MDSCs (Figure 2.3B) in IL-$10^{-/-}$ mice. Interestingly, the percentage (Figure 2.4A, B) and absolute numbers (Figure 2.4 C) of NK cells (both $NK1.1^{+}CD49b^{+}$ and $NK1.1^{-}CD49b^{+}$ populations) were lower in tumors and TDLNs in IL-$10^{-/-}$ mice than in IL-$10^{+/+}$ mice bearing MC38. We further quantified the numbers of tumor-infiltrating $CD8^{+}$ T cells. Intriguingly, there were also fewer tumor-infiltrating $CD8^{+}$ T cells in IL-$10^{-/-}$ mice than in IL-$10^{+/+}$ mice (Figure 2.5A). Furthermore, the expression levels of effector cytokines, including IFNγ and TNFα, were reduced in $CD4^{+}$ and $CD8^{+}$ T cells in the tumors and TDLNs of IL-$10^{-/-}$ mice (Figure 2.5B). This suggests that IL-10 may influence either the development or function of effector T cells. In support of this possibility, we demonstrated that addition of IL-10 directly stimulated basal expression of IFNγ, TNFα, and IL-2 in $CD4^{+}$ and $CD8^{+}$ T cells from IL-$10^{-/-}$ mice (Figure 2.6A), but not from IL-$10^{+/+}$ mice (not shown). This suggests that biological levels of endogenous IL-10 may support effector T cell function. To further support this possibility, we stimulated IL-$10^{+/+}$ $CD4^{+}$ and $CD8^{+}$ T cells with optimal concentrations of anti-CD3 and anti-CD28 in culture, and blocked endogenous levels of IL-10. We observed that blockade of IL-10 reduced IFNγ expression in $CD4^{+}$ and $CD8^{+}$ T cells (Figure 2.6B). These data provide strong evidence that endogenous IL-10 can support T cell function, and that IL-10 deficiency decreases immune surveillance in the tumor.

Figure 2.3A

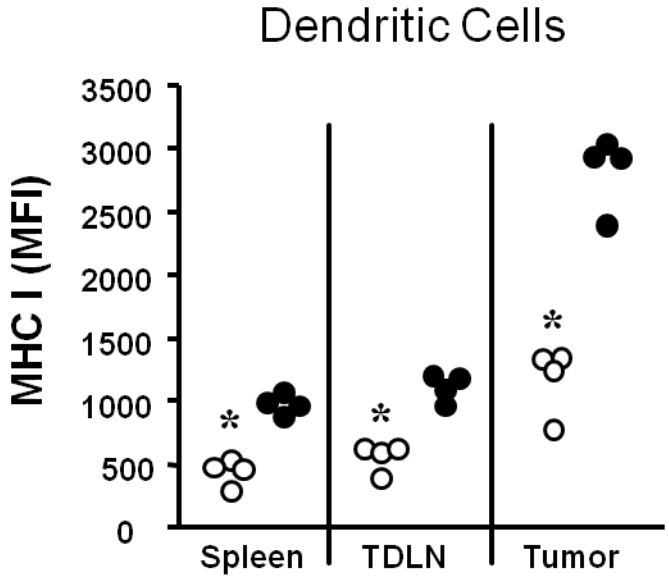


Figure 2.3B

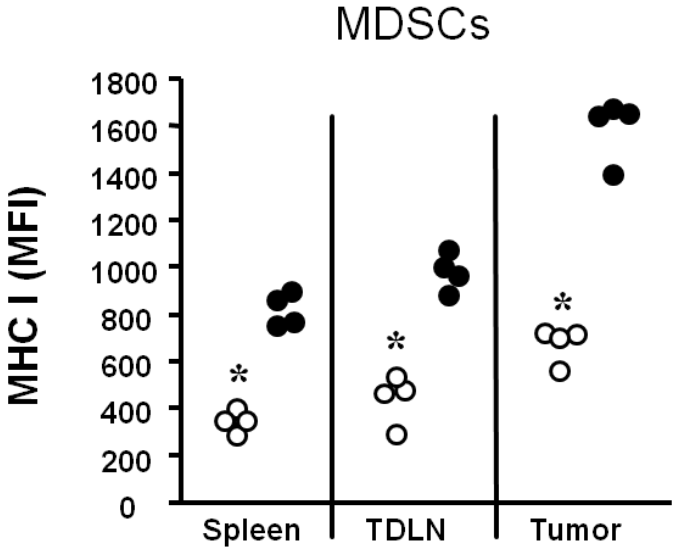


Figure 2.3: MHC I expression in APC subsets in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. Single-cell suspensions were made from tumor tissues, spleen, and TDLN, and stained with anti-CD11b, CD11c, CD19, CD45, CD90, and MHC I. MHC I expression was analyzed by FACS. A. DCs: CD19^{-}CD11c^{+}MHC^{+} dendritic cells. B. MDSCs: CD19^{-}CD11b^{+}CD11c^{-}MHC^{+} cells. Results are expressed as the mean fluorescence intensity (MFI) of MHC expression in APC subsets +/- SEM. White circles are IL-10$^{+/+}$ mice, filled circles are IL-10$^{-/-}$ mice. 4-8 mice per group. *, $P < 0.05$, IL-10$^{-/-}$ versus IL-10$^{+/+}$ mice.

Fig. 2.4A

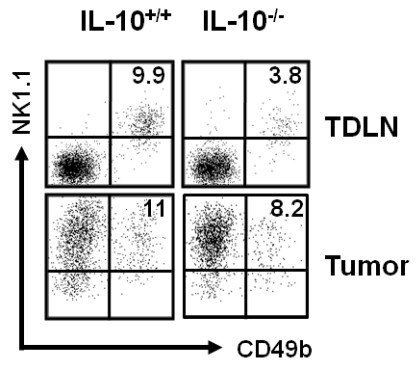


Fig. 2.4B

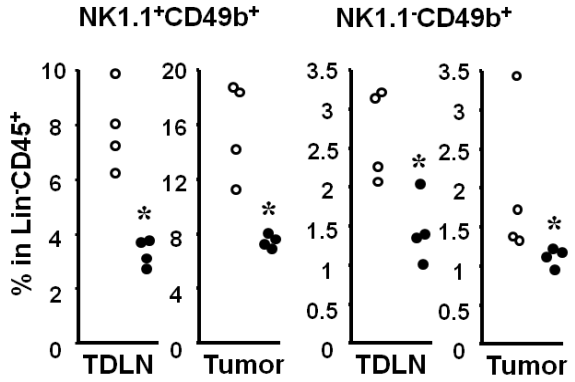


Fig. 2.4C

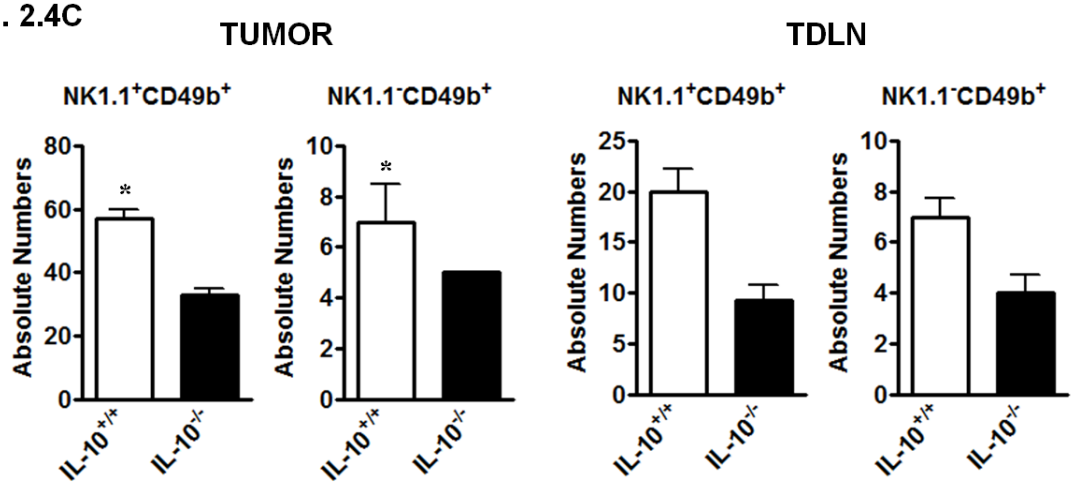


Figure 2.4: IL-10 deficiency reduced tumor surveillance by NK cells. Single-cell suspensions were made from tumor tissues and TDLN. These cells were stained with anti-CD90, CD45, CD19, NK1.1 and CD49b for membrane antigen expression. Antigen expression was analyzed by FACS. NK cells were determined as $Lin^-CD45^+NK1.1^+CD49b^+$ or $Lin^-CD45^+NK1.1^-CD49b^+$ cells. A. Representative FACS plot of NK data. B. Graphed results are expressed as the percent of $NK1.1^+CD49b^+$ or $NK1.1^-CD49b^+$ cells in Lin^-CD45^+ cells. *, $P < 0.05$, $IL\text{-}10^{-/-}$ mice (filled circles) versus $IL\text{-}10^{+/+}$ mice (unfilled circles). C. Absolute numbers of NK cells were quantified by flow cytometry in $1x10^4$ tumor-derived and $1x10^5$ TDLN $CD45^+$ mononuclear cells. *, $P > 0.05$, $IL\text{-}10^{-/-}$ versus $IL\text{-}10^{+/+}$ mice. 4-8 mice per group.

Fig 2.5A

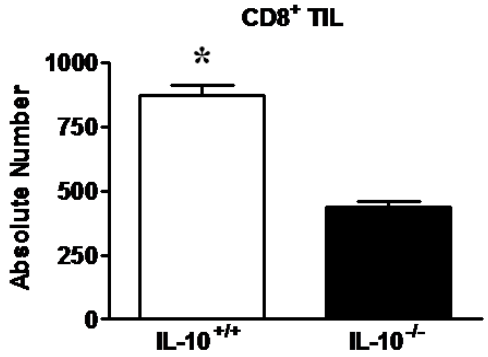


Fig 2.5B

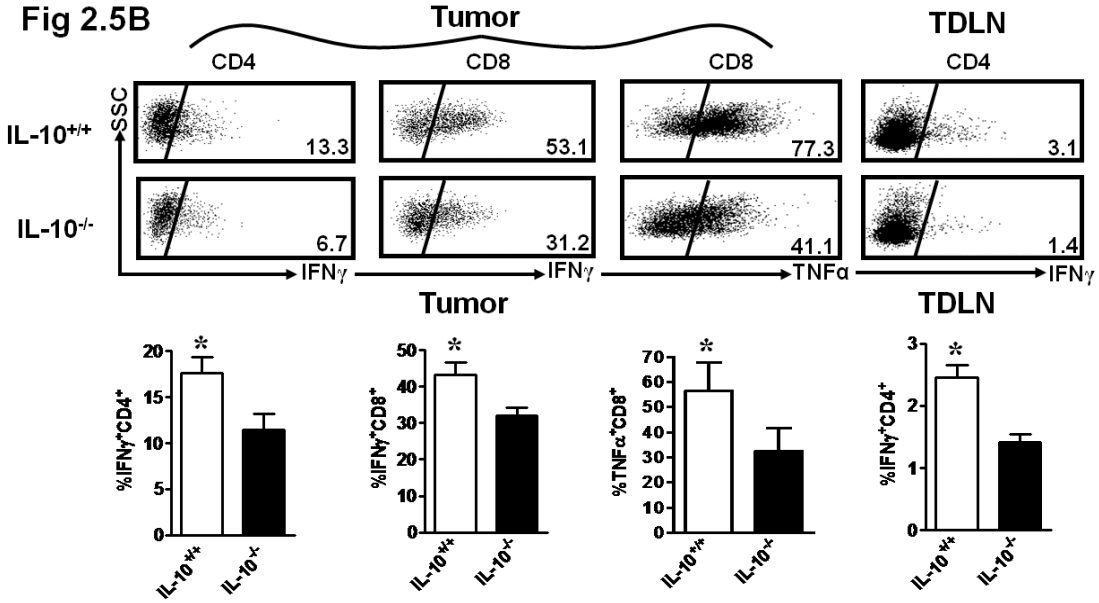


Figure 2.5: IL-10 deficiency reduced tumor surveillance by CD8$^+$ T cells. Single-cell suspensions were made from tumor tissues and TDLN. These cells were stained with anti-CD90, CD45, CD4, and CD8, for membrane antigen expression, and with anti-IL-2, TNFα and IFNγ for intracellular cytokine expression. Antigen and cytokine expression was analyzed by FACS. A. Absolute numbers of CD8$^+$ T cells quantified in 3 x10^3 tumor-infiltrating CD45$^+$ cells in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. 4-8 mice per group. *, $P < 0.05$. B. Effector cytokine expression in tumor-infiltrating T cells and TDLN T cells in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. Results are expressed as the percentage of a given cytokine-expressing T cell population in CD4$^+$ and CD8$^+$ T cell parent populations. 4-8 mice per group. *, $P < 0.05$, IL-10$^{-/-}$ mice versus IL-10$^{+/+}$ mice.

Fig. 2.6A

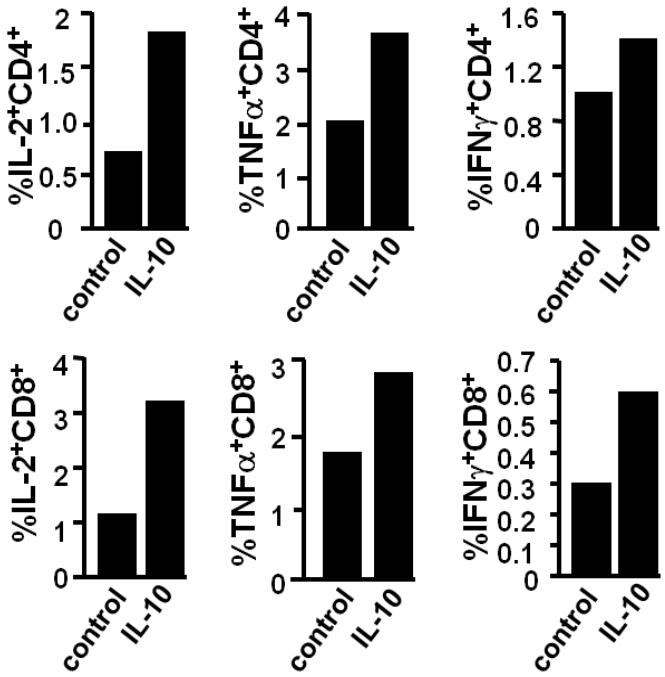


Fig. 2.6B

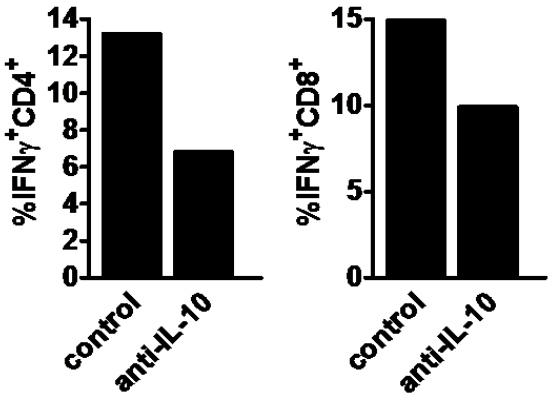


Figure 2.6: IL-10 stimulates effector cytokine expression in $CD4^+$ and $CD8^+$ T cells. A. T cells were isolated from IL-$10^{-/-}$ lymph nodes and cultured with or without 10ng/ml rmIL-10 for 3 days. T cell cytokine expression was determined by intracellular staining. Results are expressed as the percentage of a given cytokine-expressing T cell population in parent T cell populations.
B. Endogenous IL-10 supports effector cytokine production in activated T cells. $CD4^+$ and $CD8^+$ T cells were isolated from IL-$10^{+/+}$ spleens and cultured with anti-CD3, anti-CD28, and 1ug/ml anti-IL-10 for 3 days. One representative experiment of three is shown in A and in B. *, $P<0.05$, as compared to controls.

IL-10 deficiency increases immune suppression in the tumor.

It is possible that the reduced numbers and function of effector T cells in tumor-bearing IL-$10^{-/-}$ mice could be attributed to increased immune suppression. We next investigated the well-defined immunosuppressive immune cell subsets in the tumor microenvironment, including MDSCs and Treg cells. The percentages of Gr-1^{+}CD$11b^{+}$ MDSCs were comparable in tumor (Figure 2.7A), TDLNs (Figure 2.7B) and spleen (Figure 2.7C) in IL-$10^{-/-}$ and IL-$10^{+/+}$ mice. However, the absolute numbers of Gr-1^{+}CD$11b^{+}$CD115^{+} monocytic MDSCs were higher in TDLNs and spleen of tumor-bearing IL-$10^{-/-}$ mice than IL-$10^{+/+}$ mice (Figure 2.8). Interestingly, the percentages (Figure 2.9A) of CD4^{+}Foxp3^{+} Treg cells were increased in the tumor and TDLNs of IL-$10^{-/-}$ mice bearing MC38. Increased populations of Treg cells were also observed in TDLNs (Figure 2.9B) of IL-$10^{-/-}$ mice bearing MCA310. These Treg cells were functionally suppressive (data not shown).

We hypothesized that MDSCs might induce Treg cells in tumor-bearing mice as previously reported [251]. In support of this, we observed that both tumor-derived IL-$10^{-/-}$ and IL-$10^{+/+}$ MDSCs from MC38-bearing mice induced Treg cells. However, Treg cells were more efficiently induced by IL-$10^{-/-}$ MDSCs, as compared to IL-$10^{+/+}$ MDSCs (Figure 2.10A). The absolute numbers of Tregs in cultures with IL-$10^{-/-}$ MDSCs were higher than those cultured with IL-$10^{+/+}$ MDSCs (Figure 2.10B).

Fig. 2.7A

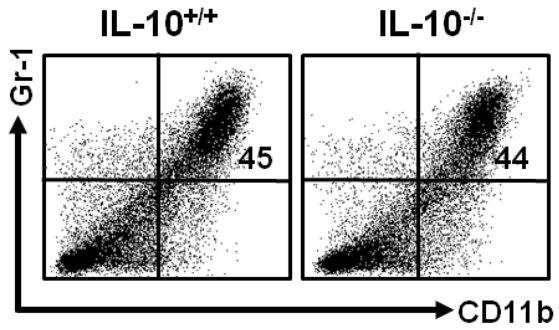


Fig. 2.7B

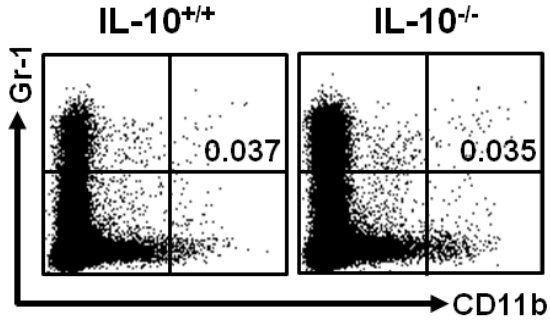


Fig. 2.7C

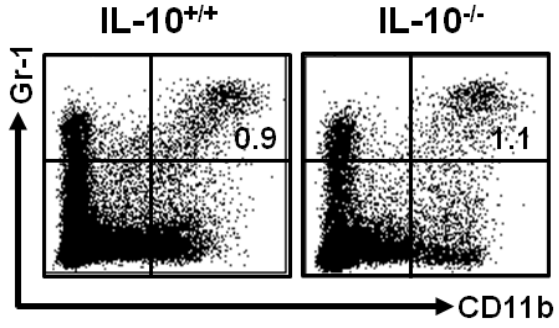


Figure 2.7: MDSC percentages are comparable in IL-10$^{+/+}$ and IL-10$^{-/-}$ mice. Single-cell suspensions were made from A. tumor tissues, B. TDLN, and C. spleen. These cells were stained with anti-Gr-1, CD11b, CD11c, CD19, CD45, CD90, MHC I, and MHC II. Antigen expression was analyzed by FACS. Results are expressed as the mean percent of Gr-1^{+}CD11b^{+}MDSCs in CD45^{+} cells +/- SEM. 4-8 mice per group.

Fig. 2.8A

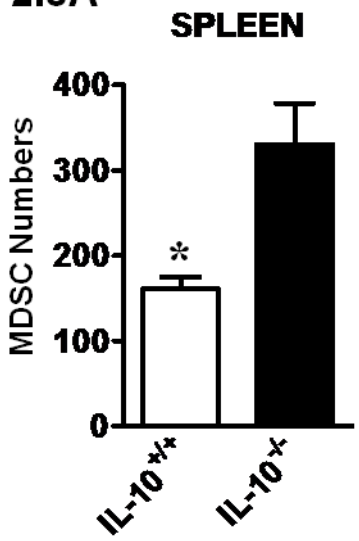


Fig. 2.8B

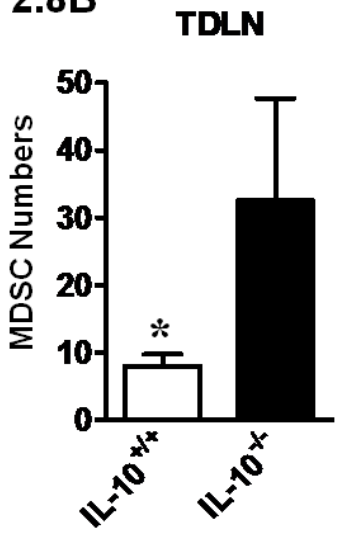


Figure 2.8: IL-10 deficiency increases absolute numbers of MDSC. Single-cell suspensions were made from tumor tissues, spleen, and TDLN. These cells were stained with anti-CD90, CD45, Gr-1, CD11b, and CD115 for membrane antigen expression and analyzed by FACS. 4-8 mice per group. Increased monocytic MDSCs in tumor-bearing IL-$10^{-/-}$ mice. Gr-1^{+}CD11b^{+}CD115^{+} monocytic MDSCs were analyzed in the A. spleen and B. TDLN of tumor-bearing IL-$10^{-/-}$ and IL-$10^{+/+}$ mice. The absolute numbers of Gr-1^{+}CD11b^{+}CD115^{+} monocytic MDSCs were quantified in 2.5 x 10^{5} CD45^{+} cells. Results are expressed as the mean +/- SEM. *P < 0.05, IL-$10^{-/-}$ versus IL-$10^{+/+}$ mice.

Fig. 2.9A

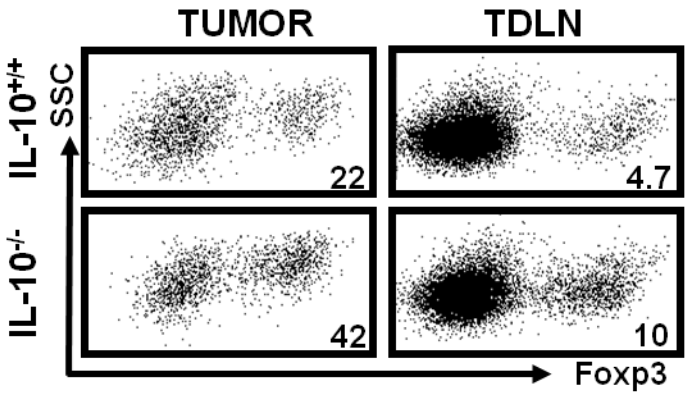


Fig. 2.9B

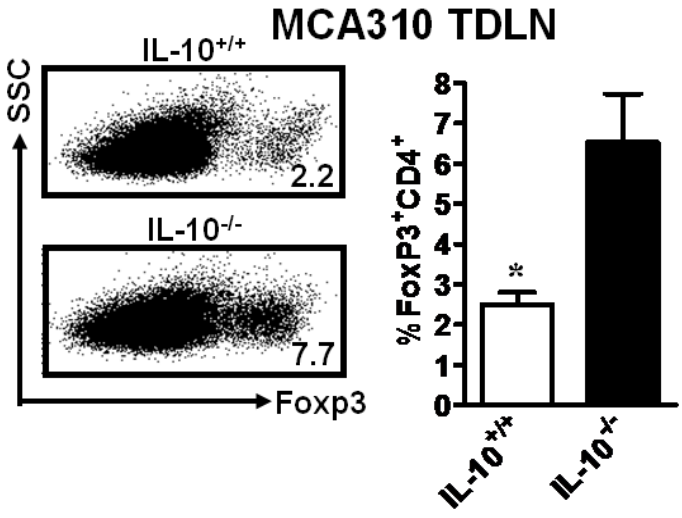


Figure 2.9: IL-10 deficiency increases immune suppression.
Increased Treg cells in tumors and TDLNs in IL-10$^{-/-}$ mice. A. Foxp3^{+}CD4^{+} Treg cells were determined in MC38 and MC38-associated TDLNs in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. B. Foxp3^{+}CD4^{+} Treg cells were determined in MCA310-associated TDLNs in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. Results are expressed as the percentage of CD4^{+}Foxp3^{+} T cells in CD4^{+} T cells or the mean values +/- SEM. *, P < 0.05, IL-10$^{-/-}$ versus IL-10$^{+/+}$ mice.

Fig. 2.10A

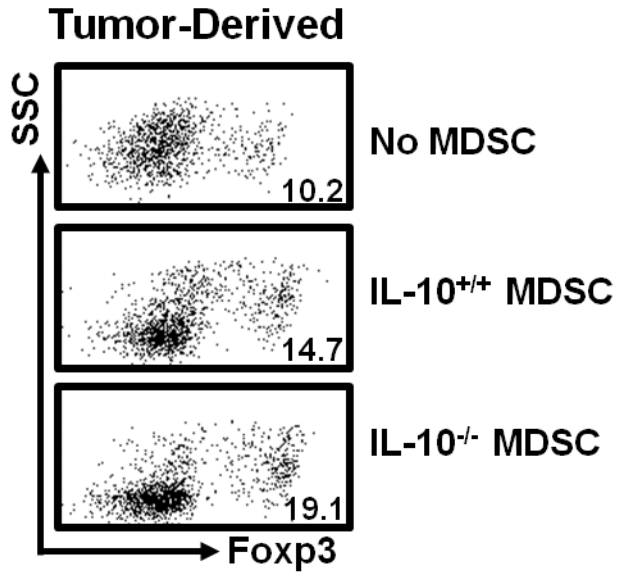


Fig. 2.10B

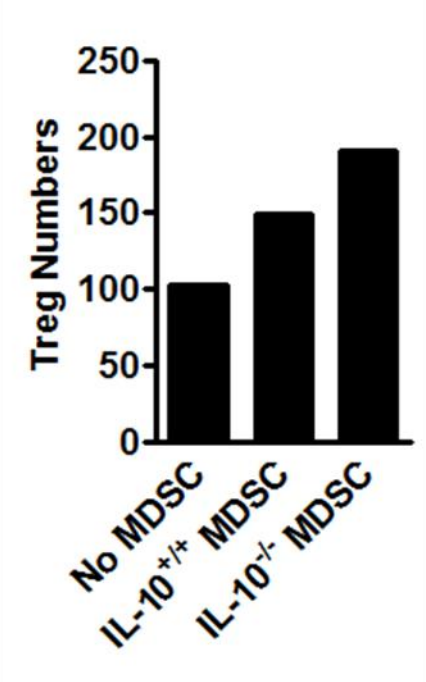


Figure 2.10: Tumor-derived IL-10$^{-/-}$ MDSCs are superior to IL-10$^{+/+}$ MDSCs in Treg induction. MDSCs were isolated from MC38 tumors and co-cultured with IL-10$^{+/+}$ CD4^{+} T cells for 6 days. CD4^{+} FoxP3^{+} cells were analyzed via FACS in cultured T cells. A. Representative FACS plot. B. Absolute numbers of Tregs in the final culture were quantified in 1x10^{3} CD4^{+} T cells. One of three experiments is shown.

We further examined the roles of MDSCs on T cell activation and tumor growth. It is well known that tumor-associated IL-$10^{+/+}$ MDSCs inhibit T cell activation [245]. In an *in vitro* immune suppression assay, similar to IL-$10^{+/+}$ MDSCs, tumor-derived IL-$10^{-/-}$ MDSC suppressed T cell proliferation in a dose-dependent manner (Figure 2.11A). To directly test the effects of MDSCs *in vivo*, we completed immune cell adoptive transfusion experiments. We lethally irradiated IL-$10^{+/+}$ mice and infused them with total mononuclear or MDSC-depleted spleen T cells from IL-$10^{+/+}$ or IL-$10^{-/-}$ mice. Subsequently, we subcutaneously injected MC38 tumor cells into these mice and monitored tumor growth. We observed that MDSC depletion resulted in significantly reduced tumor growth in mice receiving either IL-$10^{-/-}$ or IL-$10^{+/+}$ immune cells. This indicates that IL-$10^{-/-}$ and IL-$10^{+/+}$ MDSCs mediate immune suppression *in vivo*. However, although tumor volumes were bigger in irradiated mice receiving IL-$10^{-/-}$ than in those receiving IL-$10^{+/+}$ immune cells (Figure 2.11B), which is in accordance with our observations of non-irradiated mice (Figure 2.1B,C), we showed that after MDSC depletion, tumor volumes were similar in irradiated mice receiving either IL-$10^{-/-}$ or IL-$10^{+/+}$ immune cells (Figure 2.11B). The data indicate that MDSCs serve as important suppressive components of the IL-$10^{-/-}$ immune system *in vivo*. Altogether, our results suggest that IL-10 deficiency increases immune suppression, both through direct effects on MDSCs and via downstream effects mediated by them.

Fig. 2.11A

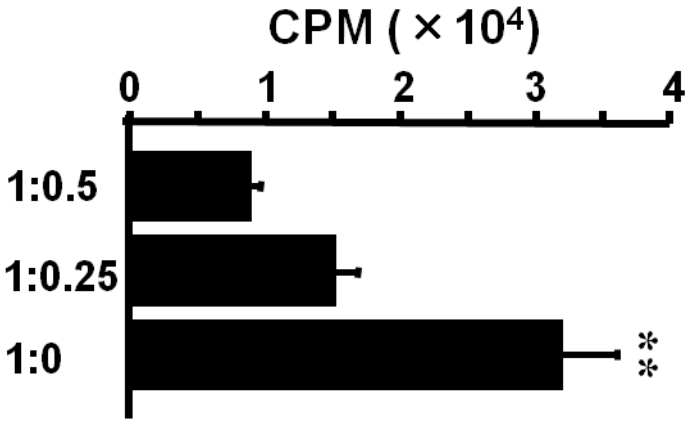


Fig. 2.11B

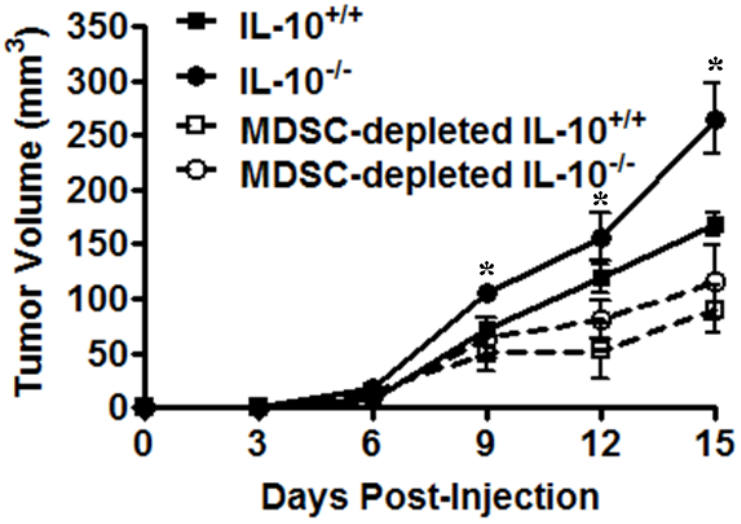


Figure 2.11: IL-10$^{-/-}$ MDSC suppress T cell activation and impede antitumor immunity. A. Tumor-associated IL-10$^{-/-}$ MDSCs mediate immune suppression *in vitro*. MDSCs were sorted from MC38 tumors in IL-10$^{-/-}$ mice and cultured with naïve T cells in different ratios as detailed in Materials and Methods. T cell proliferation was determined by thymidine incorporation. Results are expressed as the mean of counts per minute (CPM) +/- SEM in triplicates. Similar results were observed with IL-10$^{+/+}$ MDSCs. One of three experiments is shown. **P < 0.01 compared to control (ratio 1:0). B. MDSCs suppressed antitumor immunity *in vivo*. IL-10$^{+/+}$ mice were lethally irradiated and given an infusion of IL-10$^{+/+}$ or IL-10$^{-/-}$ whole spleen cells or spleen cells depleted of MDSCs. On the same day, mice were injected subcutaneously with MC38. Tumor growth was measured over two weeks. 3-4 mice per group. *P < 0.05, Day 9-15: IL-10$^{-/-}$ vs. IL-10$^{+/+}$, IL-10$^{-/-}$ vs. MDSC-depleted IL-10$^{-/-}$; and Day 12-15 : IL-10$^{+/+}$ vs. MDSC-depleted IL-10$^{+/+}$.

IL-1 contributes to increased tumor growth in IL-$10^{-/-}$ mice.

After demonstrating the cellular mechanisms which may contribute to increased tumor incidence, growth and foci formation in IL-$10^{-/-}$ mice, we examined the relevant molecular patterns associated with such phenomena. Interestingly, myeloid cells—including MDSCs—expressed higher levels of IL-1α and IL-1β in IL-$10^{-/-}$ than IL-$10^{+/+}$ mice (Figure 2.12A). We then investigated the role of IL-1 in tumor growth in IL-$10^{-/-}$ mice. Anakinra, the recombinant human IL-1 receptor antagonist (IL-1Ra), has demonstrated biological efficacy in mice [268]. We treated tumor-bearing IL-$10^{-/-}$ and IL-$10^{+/+}$ mice with Anakinra or vehicle over a period of three weeks. We observed that IL-$10^{-/-}$ mice treated with Anakinra had reduced tumor burden (Figure 2.12B). Interestingly, tumor growth was comparable in IL-$10^{+/+}$ mice treated with Anakinra and those treated with PBS vehicle (Figure 2.12C). In further support of the relevance of IL-1, we showed that tumor volume was decreased in IL-1 receptor-deficient (IL-$1R^{-/-}$) mice as compared to IL-$1R^{+/+}$ mice (Figure 2.13). It is likely that IL-1 contributes to the increased tumor growth observed in IL-$10^{-/-}$ mice.

IL-1 blockade enhances effector T cell tumor infiltration and reduces tumor angiogenesis in IL-$10^{-/-}$ mice.

We next examined tumor-infiltrating T cells in IL-$10^{-/-}$ mice treated with Anakinra. Immunofluorescent staining revealed that there were more tumor-infiltrating $CD8^+$ T cells in IL-$10^{-/-}$ mice treated with Anakinra as compared to control (Figure 2.14A). The levels of intratumoral IFNγ^+CD8^+ T cells were also increased in mice treated with Anakinra (Figure 2.14B). Although the levels of MDSCs were comparable in the two groups (not shown), the numbers of tumor infiltrating Treg cells were reduced in mice treated with Anakinra (Figure 2.14C). Furthermore, tumor microvessel intensity and size (Figure 2.15) were reduced by Anakinra treatment. Altogether, the data suggest that IL-1 blockade promotes tumor immune surveillance and reduces tumor angiogenesis.

Fig. 2.12A

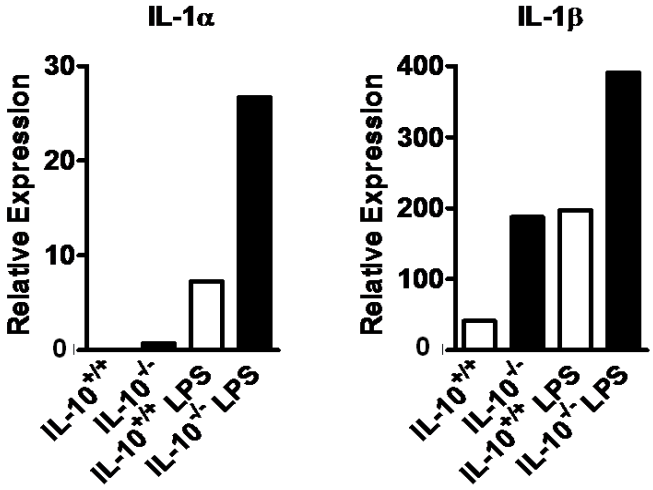


Fig. 2.12B

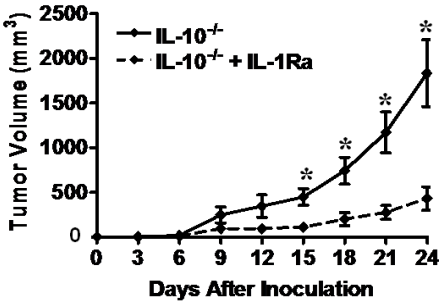


Fig. 2.12C

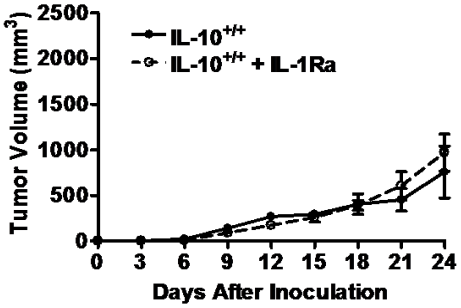


Figure 2.12: IL-1 contributes to increased tumor growth in IL-10$^{-/-}$ mice.
A. IL-10$^{-/-}$ MDSCs expressed high levels of IL-1. MDSCs were isolated from spleen in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice and not stimulated or stimulated with LPS for 8 hours. Real-time PCR was performed to determine the expression of IL-1α and IL-1β. One of 3 experiments is shown. B, C. Anakinra treatment reduced tumor burden in IL-10$^{-/-}$ mice but had no significant effect on tumor burden in IL-10$^{+/+}$ mice. MC38 was injected subcutaneously into IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. The mice were treated with Anakinra or vehicle as described in Materials and Methods. Tumor volume was measured every 3 days. Results are shown as the mean values of tumor volume +/- SEM. n = 4-5 mice per group. *, $P < 0.05$, Anakinra versus control in IL-10$^{-/-}$ mice.

Fig. 2.13

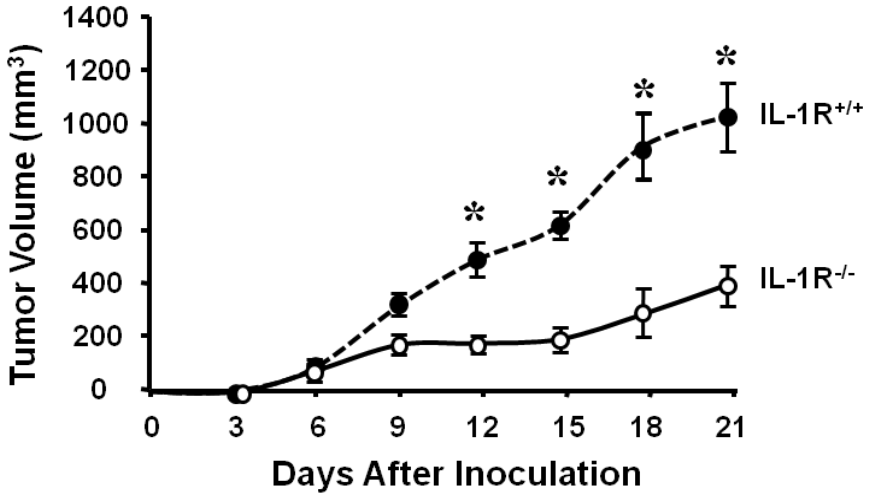


Figure 2.13: Tumor growth is reduced in IL-1R$^{-/-}$ mice. MC38 was injected subcutaneously into IL-1R$^{-/-}$ and IL-1R$^{+/+}$ mice. Tumor volumes were measured every 3 days. Results are shown as the mean values of tumor volume +/- SEM in IL-10$^{-/-}$ mice. n = 3-4 mice per group. *, $P < 0.05$, IL-1R$^{-/-}$ versus IL-1R$^{+/+}$ mice.

Fig. 2.14A

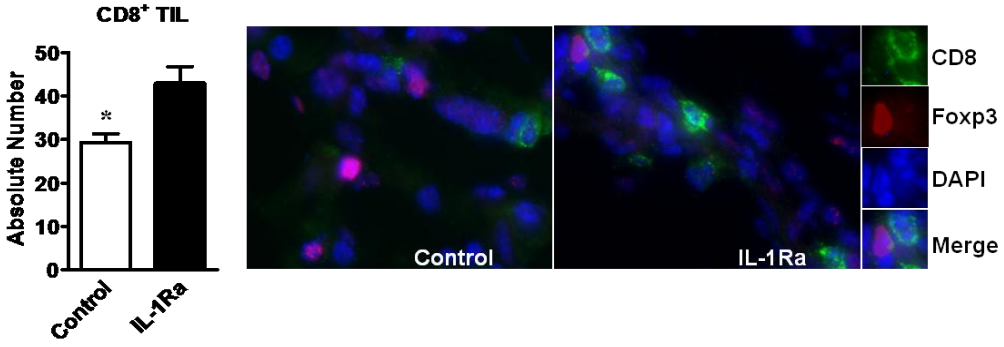


Fig. 2.14B

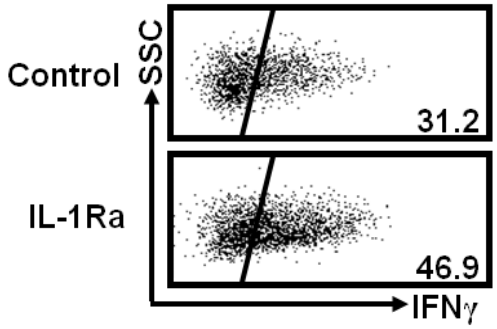


Fig. 2.14C

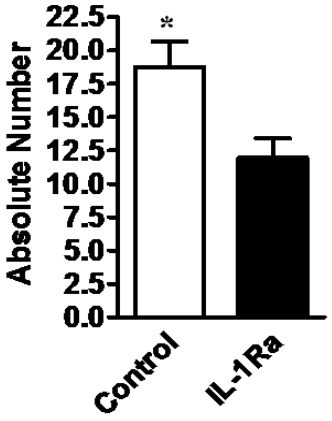


Figure 2.14: IL-1 blockade altered immune phenotype in IL-10$^{-/-}$ mice. A. Increased tumor-infiltrating CD8^{+} T cells in Anakinra-treated IL-10$^{-/-}$ mice. Immunofluorescent staining was performed on tumor tissue sections. The absolute numbers of CD8^{+} T cells were counted. Results are expressed as the mean values +/- SEM per 15 high-powered fields (HPF). B. Increased tumor-infiltrating IFNγ^{+}CD8^{+} T cells in Anakinra-treated IL-10$^{-/-}$ mice. Single-cell suspensions were made from tumor tissues. The cells were stained for CD8 and intracellular IFNγ. Results are expressed as the mean percent of IFNγ^{+}CD8^{+} T cells in CD8^{+} cells +/- SEM. C. Reduced tumor-infiltrating Treg cells in Anakinra-treated IL-10$^{-/-}$ mice. Immunofluorescent staining was performed on tumor tissue sections. The absolute numbers of Foxp3^{+} T cells were counted. Results are expressed as the mean values +/- SEM per 15 HPF. n = 5 mice per group. *, P < 0.05, Anakinra versus control.

Fig. 2.15A

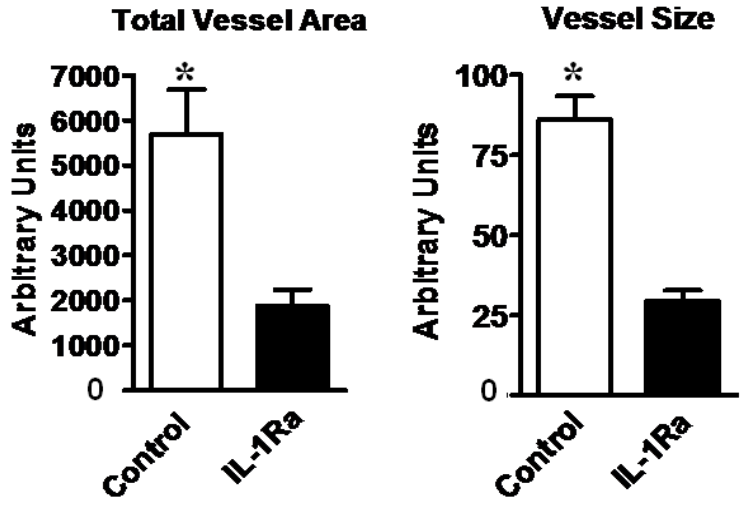


Fig. 2.15B

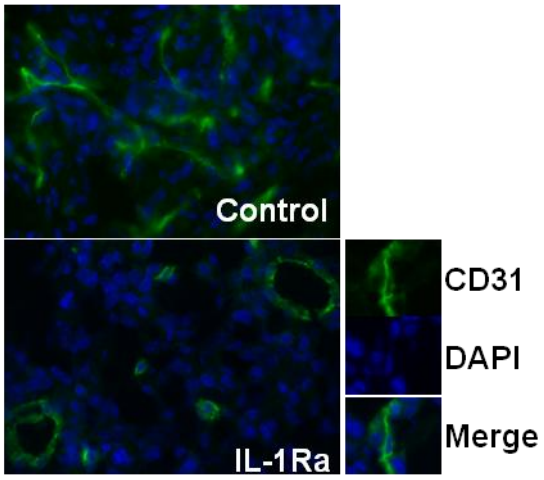


Figure 2.15: Anakinra treatment reduces vascularization in IL-10$^{-/-}$ mice. Immunofluorescent staining was performed to analyze the expression of CD31 in tumor tissue sections. A. The total areas and average size of CD31^{+} vessels were analyzed as detailed in Materials and Methods. Results are expressed as the mean area or size of vessels +/- SEM in 10 HPF. n = 4-5 mice per group. *, $P < 0.05$, Anakinra versus control. B. Representative immunofluorescent sections of untreated IL-10$^{-/-}$ mice (Control) and Anakinra-treated IL-10$^{-/-}$ mice (IL-1Ra).

Discussion.

Here we present a series of experiments demonstrating that endogenous IL-10 plays a stimulatory role in antitumor immunity. Our data challenge commonly-held beliefs about the immune regulatory role of IL-10 and suggest a more complex IL-10 biology, at least in the context of tumor immunology (Figure 2.16).

IL-10 has been thought to be broadly immune suppressive. To our surprise, chemically-induced tumor incidence, transplanted tumor growth, and lung foci formation are increased in IL-$10^{-/-}$ mice. This is associated with a number of immune phenotypic and functional signatures.

We have observed reduced NK cells, $CD8^{+}$ effector T cells and effector T cell cytokine expression in tumor and TDLN of tumor-bearing IL-$10^{-/-}$ mice. This indicates that endogenous IL-10 is crucial for tumor immune surveillance. Consistent with our observations, earlier studies in mice have shown that exogenous IL-10 promoted tumor immunity and led to reduced tumor growth or tumor rejection [43,46,256]. However, the underlying mechanisms by which IL-10 mediates suppression of tumor growth despite its defined immune-inhibitory functions remained elusive. Furthermore, it remained unknown if endogenous IL-10 could mediate protective tumor immunity. Our studies suggest that the biological activities of IL-10 may be highly context-dependent, and vary in the presence of different cellular targets, phases of immune responses and disease model systems.

We show that IL-10 directly targets and stimulates T cell effector cytokine production. The levels of effector cytokines, including TNFα and IFNγ, are reduced in IL-$10^{-/-}$ tumor-bearing mice and may be recovered by addition of IL-10. Additionally, IL-10 blockade reduces effector cytokine production by IL-$10^{+/+}$ T cells. The data indicate that IL-10 may target effector T cells to maintain and enhance their functionalities. In support of this possibility, it has been reported that IL-10 may promote CD8 differentiation and expansion [33-34,269]. In fact, humans treated with IL-10 showed a reduction of IL-1 and increased IFNγ [270-271].

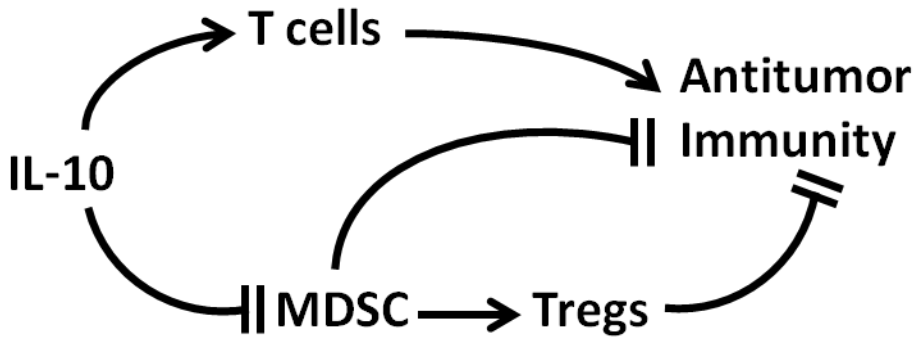


Figure 2.16. Model of IL-10 in antitumor immunity.
1. IL-10 directly supports the development and effector function of T cells.
2. Concurrently, IL-10 minimizes expression of MHC on MDSCs.
3. IL-10 deficiency leads to increased MDSC and Tregs in the tumor environment.
4. Thus, IL-10 directly and indirectly enacts antitumor immunity.

Given that IL-10 can be immune-stimulatory and inhibitory, the balance of these effects may determine whether IL-10 is beneficial or detrimental in a given model system and/or disease stage. The immunoregulatory roles of IL-10 are prominently defined in infectious disease models. In these scenarios, APCs are the immediate and predominant targets of IL-10 [269]. Infectious pathogens rapidly activate Toll-like receptors on APCs, and induce antigen-specific T cell priming. Exogenous or induced endogenous IL-10 inhibits the maturation and function of APCs via reduced MHC expression and IL-12 production, and subsequently suppresses T cell priming and Th1-type responses [272-273]. In a tumor-bearing host, however, when there is no obvious acute phase of immune response [266], tumor-associated antigen (TAA)-specific priming may slowly occur, and IL-10 may be gradually induced. Thus, IL-10 may preferentially target and promote effector T cells during the effector phase of immune responses in tumor-bearing hosts [269]. This may partially explain the discrepancies concerning IL-10 biology in the literature.

It has been reported that IL-1 supports the development of MDSCs in tumor-bearing mice [261-262,274]. In line with this possibility, IL-$10^{-/-}$ myeloid cells express high levels of IL-1, and there are more monocytic MDSCs in IL-$10^{-/-}$ tumor-bearing mice. MDSCs, particularly $CD115^{+}$ monocytic MDSCs, promote Treg cell development [251]. Consistent with this, we have observed that IL-$10^{-/-}$ MDSCs efficiently suppress T cell expansion and are stronger inducers of Treg cells than IL-$10^{+/+}$ MDSCs. It has been suggested that loss of MHC or mutations in MHC expression machinery may result in limited tumor antigen recognition and presentation [275]. Interestingly, IL-$10^{-/-}$ MDSCs express high levels of MHC molecules, and may efficiently present self-antigens to Treg cells, thus activating and expanding Treg cells as we have observed. This may explain why IL-$10^{-/-}$ MDSCs are superior to IL-$10^{+/+}$ MDSCs in Treg cell induction. Perhaps most tellingly, depletion of MDSCs in our immune cell-transfer model profoundly reduces tumor volume, demonstrating that the presence of MDSCs has a negative impact on host immunity *in vivo*. Additionally, tumor burden in mice receiving IL-$10^{-/-}$ cells depleted of MDSCs was reduced by a larger volume than

in those mice receiving IL-10$^{+/+}$ cells depleted of MDSCs. This suggests that MDSCs that develop in the absence of IL-10 may mediate more powerful suppressive activities in tumor-bearing mice. However, although IL-1 promotes the development of MDSCs in tumor-bearing mice [261-262,274], intratumoral and tumor-draining lymph node MDSC number did not change after IL-1Ra administration in IL-10$^{-/-}$ mice. We reason that the effects of IL-1 on MDSCs may be more long-lasting than can be reversed with temporary IL-1 signaling blockade.

IL-10 suppresses the expression of inflammatory cytokines in chronic inflammation models [272-273]. IL-10$^{-/-}$ mice develop chronic colitis in conventional conditions [19]. Although there is no obvious inflammation (including colitis) in IL-10$^{-/-}$ mice when housed in specific pathogen-free facilities, IL-10$^{-/-}$ myeloid cells express high levels of IL-1, which may suggest the presence of microscopic inflammation in these mice. This inflammation may be increased after tumor inoculation or induction. Inflammation is often linked to increased tumor vascularization, and IL-1 has long been known as a pro-angiogenic cytokine [276] and promoter of tumorigenesis [277]. In line with this, IL-1 blockade reduces tumor microvessel density and size in IL-10$^{-/-}$ mice. However, it is also possible that the reduced density of tumor microvessels may be due to the resulting enhanced T cell immunity that includes IFNγ production. IFNγ is a potent anti-angiogeneic factor [278]. Finally, we have demonstrated that IL-1 blockade reduces tumor growth in IL-10$^{-/-}$ mice. Accordingly, the levels of intratumoral Treg cells are reduced, and the numbers of tumor-infiltrating effector T cells and effector cytokines are enhanced. Based upon our observations, IL-10 both directly and indirectly supports T cell immunity by inhibiting IL-1, and reduces IL-1-mediated MDSCs and Treg cells in tumor-bearing hosts.

In summary, in contrast to the established views, endogenous IL-10 is negatively linked to the development of immunosuppressive MDSCs and Treg cells, subverts tumor immune suppression, induces the activation of tumor-infiltrating effector T cells, and in turn contributes to anti-tumor immunity. Our

data indicate that the biological activities of IL-10 can be highly context-dependent.

Chapter 3

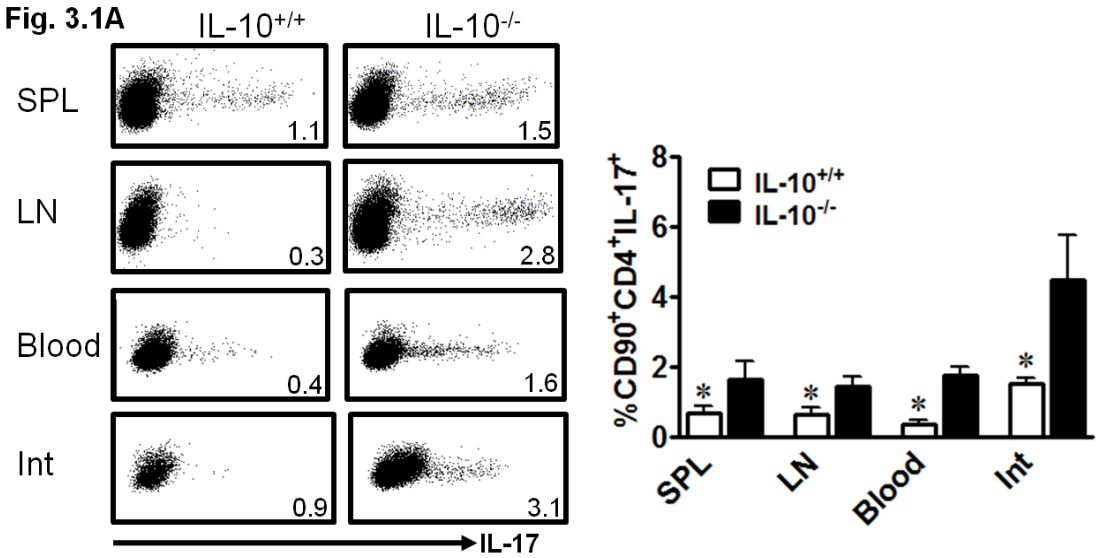
Interleukin-10 and autoimmunity

IL-10$^{-/-}$ mice housed in conventional conditions develop chronic enterocolitis and then colon adenocarcinoma [279]. IL-10$^{-/-}$ colitis shares many architectural signatures with human CD [19], and this disease progression partly mimics IBD-associated cancer in humans. Colitic mice display some histopathological signatures associated with human IBD, including increased numbers of focal ulcerations and transmural lesions, like CD patients [279]. Perhaps not surprisingly, patients with CD or colitis have a highly increased risk of cancer in comparison to healthy individuals [280], and the IL-10$^{-/-}$ mouse now serves as a useful model of spontaneous inflammation-induced cancer. It was also noted early on that a lymphocyte population in colitic mice stimulated by IL-23 produced IL-6 and IL-17 [128]. A subsequent report in 2009 demonstrated an increase in the IL-17 levels of mesenteric lymph node cultures of IL-10$^{-/-}$ mice [281]. We set out to analyze the prevalence and distribution of Th17 cells in IL-10$^{-/-}$ mice, investigate the molecular causes of increased Th17 populations (if this phenomenon is systemic), and examine whether a similar physiological relationship existed in human conditions of disrupted IL-10 production.

IL-10$^{-/-}$ dendritic cells are superior Th17 cell inducers in mice.

First, we examined the cellular and molecular link between IL-10 and Th17 cells in IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. We observed increased levels of Th17 cells in the lymph nodes, spleen, blood, and intestines (also specifically in the colon) of IL-10$^{-/-}$ mice as compared to WT mice (Fig. 3A, B). Interestingly, we also found increased levels of IL-17^{+}CD8^{+} (Tc17) cells in multiple IL-10$^{-/-}$ organs (Fig. 3C). There were no differences in other immune cell subsets, including B cells, macrophages, dendritic cells, granulocytes, and natural killer (NK) cells between IL-10$^{-/-}$ and IL-10$^{+/+}$ mice (not shown). It is of note that there was a significant

decrease in the intestinal regulatory T cell population of IL-10$^{-/-}$ mice, an observation in line with a recent publication documenting the importance of IL-10 in maintaining FoxP3 expression [18]. Thus, Th17 cells are spontaneously and systemically increased in IL-10$^{-/-}$ mice.



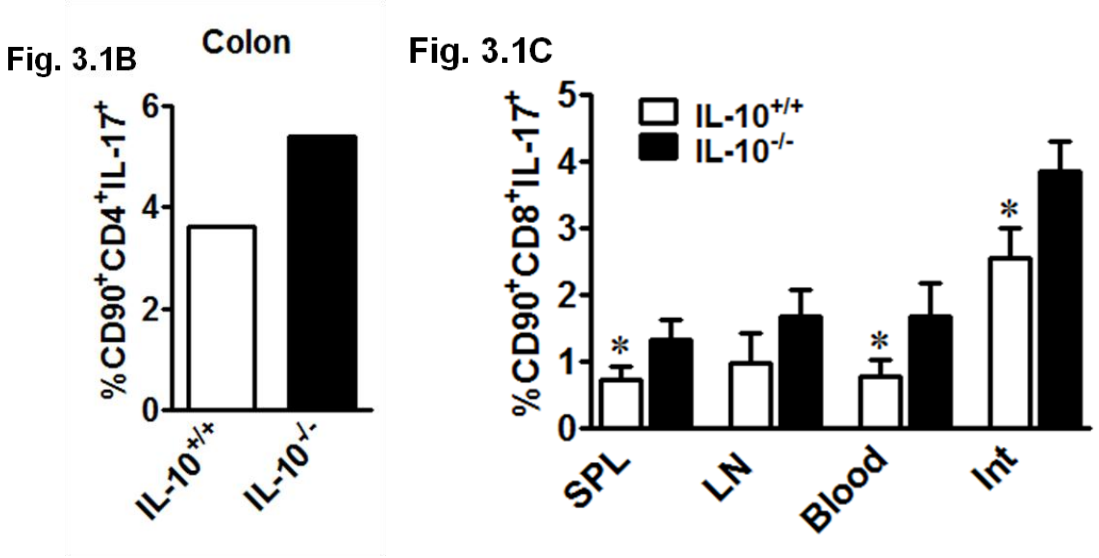


Figure 3.1: IL-17^{+} cells are increased in IL-10$^{-/-}$ mice.
Total mononuclear cells from unchallenged Il-10$^{+/+}$ and IL-10$^{-/-}$ mouse organs were analyzed via flow cytometry. A. Th17 cell percentages in CD4+ T cell parent population. 6 mice per group, *p<0.05. B. Colons from three IL-10$^{+/+}$ and three IL-10$^{-/-}$ mice were pooled, processed and analyzed for Th17 cells via flow cytometry. One experiment of two with three mice per group each. C. IL-17^{+} cell percentages in CD8^{+} T cell parent population. 6 mice per group, *p<0.05.

We next studied the potential underlying mechanisms causing the spontaneous increase of Th17 cells in IL-$10^{-/-}$ mice. To this end, IL-$10^{+/+}$ and IL-$10^{-/-}$ splenocytes were cultured under Th17-polarizing conditions. We observed that there were more Th17 cells in the IL-$10^{-/-}$ spleen cultures and more IL-17 in the IL-$10^{-/-}$ culture supernatant when compared to IL-$10^{+/+}$ cultures (Fig. 3.2A, B). We then investigated the role of IL-$10^{-/-}$ DCs in Th17 cell induction. We co-cultured IL-$10^{+/+}$ T cells with IL-$10^{+/+}$ or IL-$10^{-/-}$ DCs, and examined the resulting cellular phenotypes. We found increased Th17 cells in co-cultures with IL-$10^{-/-}$ DCs as compared to those with IL-$10^{+/+}$ DCs (Fig. 3.2C). Thus, IL-$10^{-/-}$ DCs are superior inducers of Th17 cells.

Fig. 3.2A

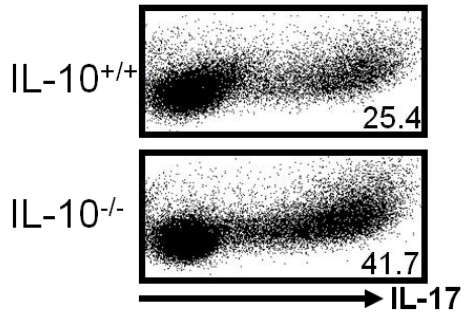


Fig. 3.2B

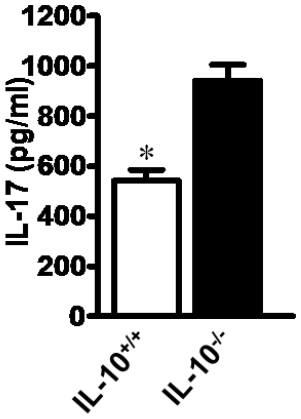


Fig. 3.2C

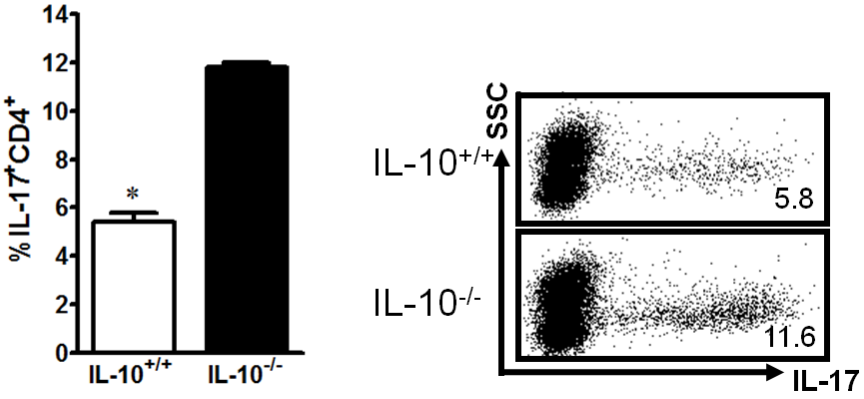


Figure 3.2: IL-10$^{-/-}$ DCs are more powerful inducers of Th17 cells. A. Total mononuclear cells from IL-10$^{+/+}$ and IL-10$^{-/-}$ spleens were plated at 1x10^{6}/ml with Th17-polarizing cytokines. Th17 were analyzed by flow cytometry on day 6. B. Supernatant from Th17 cultures was collected on day 3 and analyzed via ELISA for levels of IL-17. 4 mice per group, *p<0.05. C. Splenic CD11c^{+} DC from IL-10$^{-/-}$ or IL-10$^{+/+}$ mice were cultured in a ratio of 1:5 with IL-10$^{+/+}$ CD4^{+} T cells for 5 days and then analyzed for Th17 via flow cytometry, *P<0.05. Representative of 6 experiments.

Murine IL-10$^{-/-}$ DCs induce Th17 cells via IL-1**.**

We next examined possible mechanisms involved in making IL-10$^{-/-}$ DCs better at Th17 induction. As we mentioned before, multiple cytokine cocktails have been reported to induce Th17 cell polarization. We and others have demonstrated the importance of IL-1 in the development of mouse and human Th17 cells [282-283]. We thus hypothesized that mouse IL-10$^{-/-}$ DCs produce more IL-1 which in turn leads to more potent Th17 induction. We first tested this hypothesis in a co-culture system where IL-10$^{+/+}$DCs were incubated with either IL-10$^{+/+}$ or IL-1 receptor knockout (IL-1R$^{-/-}$) T cells. We demonstrated that the IL-1R$^{-/-}$ T cell population expressing IL-17 was only half the size of that in the IL-10$^{+/+}$ T cell cultures (Fig. 3.3A). This observation confirms the importance of IL-1 signaling in Th17 development. We next investigated the IL-1-producing capacity of IL-10$^{-/-}$ DCs. We observed increased expression of IL-1α, IL-1β, IL-6, and TNFα transcripts in IL-10$^{-/-}$ DCs when compared to IL-10$^{+/+}$ DCs (Fig. 3.3B and not shown). Increased IL-1β protein was also detected in lipopolysaccharide (LPS)-stimulated IL-10$^{-/-}$ DC culture supernatants (Fig. 3.3C). Importantly, the profile of IL-1R expression in IL-10$^{-/-}$ T cells was similar to that of IL-10$^{+/+}$ cells: IL-1R^{+} cells were constrained to the memory (CD62L^{lo}CD45RB^{hi}) population, and IL-1R expression density did not differ between IL-10$^{+/+}$ and IL-10$^{-/-}$ T cell subsets.

Fig. 3.3A

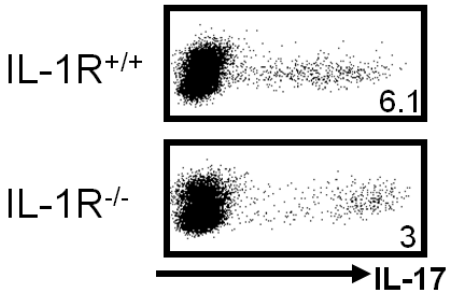


Fig. 3.3B

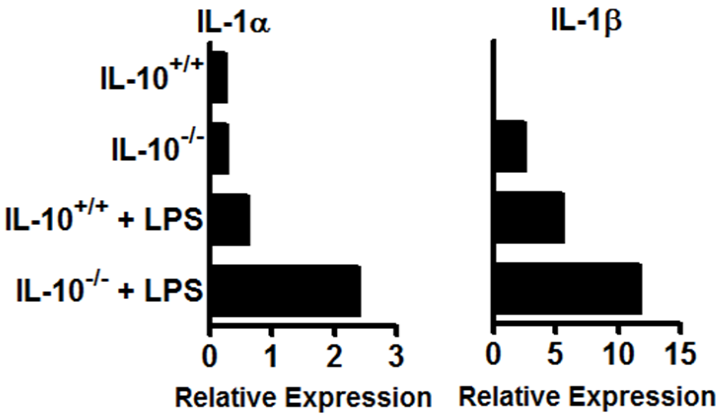


Fig. 3.3C

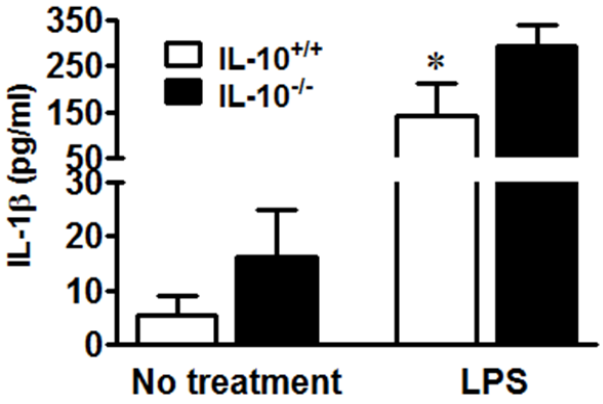


Figure 3.3: IL-10$^{-/-}$ DC produce more IL-1 than IL-10$^{+/+}$ DC. A. Splenic CD11c^{+} DC from IL-1R$^{+/+}$ mice were cultured in a ratio of 1:5 with CD4^{+} T cells from IL-1R$^{+/+}$ or IL-1R$^{-/-}$ mice for 5 days and then analyzed for Th17 via flow cytometry,*p<0.05. Representative of 3 experiments. B. DC from IL-10$^{+/+}$ or IL-10$^{-/-}$ mice were cultured for 8 hours with or without LPS. IL-1α and IL-1β message was quantified via RT-PCR. Representative of 3 experiments. C. DC from IL-10$^{+/+}$ or IL-10$^{-/-}$ mice were cultured for 48 hours with or without LPS. Supernatant was analyzed for IL-1β via ELISA. *p<0.05, average of 3 experiments.

In order to determine whether this increased IL-1 was involved in the stronger Th17 induction documented in our experiments with IL-10$^{-/-}$ DCs, we added anti-IL-1R monoclonal antibody to the co-cultures of T cells with IL-10$^{+/+}$ or IL-10$^{-/-}$ DCs. Blockade of IL-1R, but not IL-6 or TNFα, resulted in significantly decreased Th17 cells and IL-17 levels in both culture supernatants (Fig. 3.4A, B, and not shown). Finally, we investigated whether blockade of IL-1 signaling had any impact on Th17 *in vivo*. We administered the human recombinant IL-1R antagonist (IL-1Ra) Anakinra (Kineret ®), already shown to have efficacy in mice [268], to IL-10$^{-/-}$ mice and analyzed Th17 cells in different organs. As expected, *in vivo* IL-1 blockade decreased Th17 cells (Fig. 3.5 A, B) and Tc17 cells (Fig. 3.5C) in most IL-10$^{-/-}$ organs evaluated. Interestingly, treatment did not affect Th17 population size in the intestine in general or, upon closer examination, in the colon particularly. We speculate that because the gastrointestinal tract is one of the sites of the most rapid cell turnover in the body, and because potential microscopic inflammation in the gastrointestinal tract may induce rapid Th17 recruitment, Th17 cells may be able to repopulate rather quickly in this organ. It is also possible that IL-1Ra cannot penetrate or reach the GI tissue efficiently (recall that IL-1R distribution is widespread, and IL-1Ra may saturate receptors in other organs before reaching the gut). These data indicate that increased IL-1 production by IL-10$^{-/-}$ DCs is at least partly responsible for the stronger Th17 induction in IL-10$^{-/-}$ mice.

Fig. 3.4A

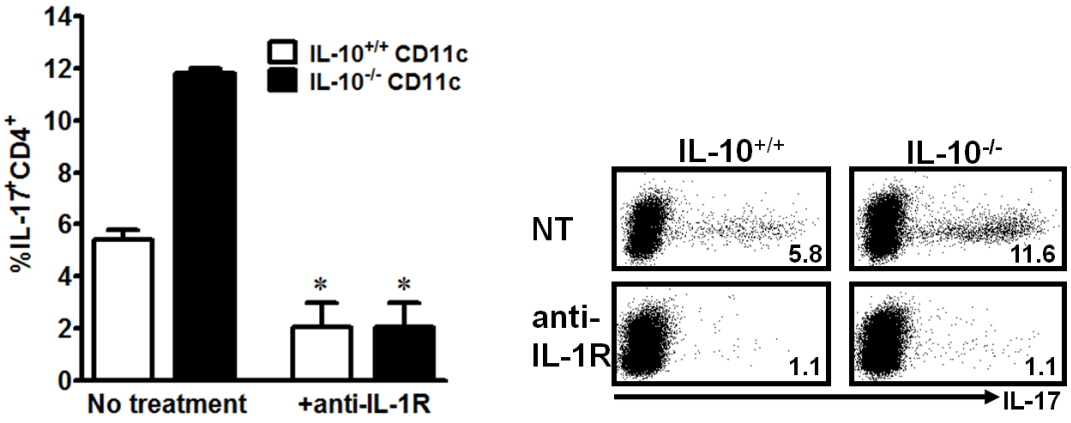


Fig. 3.4B

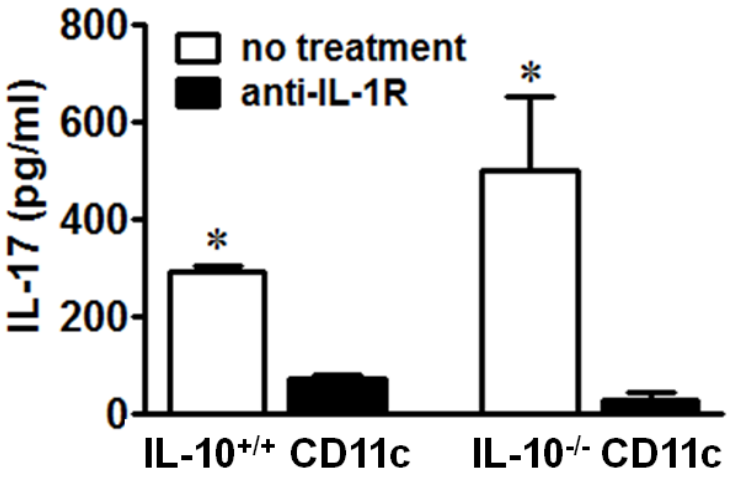


Figure 3.4: Blockade of IL-1R signaling decreases Th17 populations *in vitro*. A. Splenic CD11c^{+} DC from IL-10$^{-/-}$ or IL-10$^{+/+}$ mice were cultured in a ratio of 1:5 with CD4^{+} T cells from IL-10$^{+/+}$ mice for 5 days with or without anti-IL-1R antibody and then analyzed for Th17 via flow cytometry. NT = no treatment. *,P<0.05. Representative of 4 experiments. B. ELISA of supernatants from day 3 of cultures in A. Representative of 4 experiments. *,P<0.05.

Fig. 3.5A

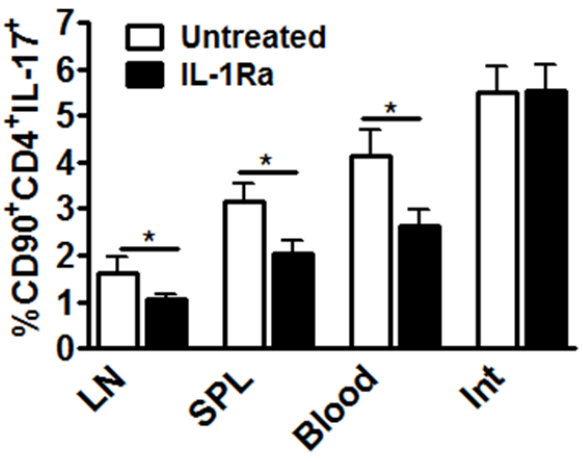


Fig. 3.5B

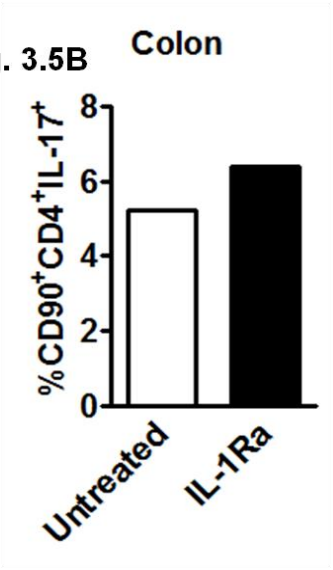


Fig. 3.5C

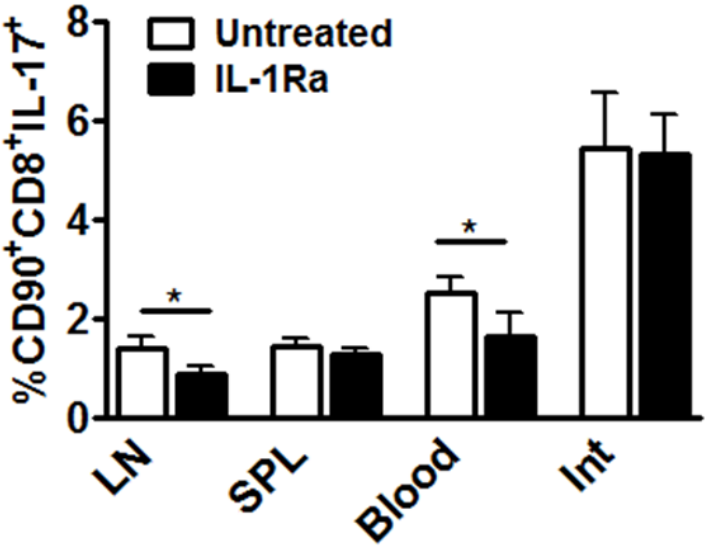


Figure 3.5: *In vivo* IL-1Ra administration reduces endogenous Th17 in IL-10$^{-/-}$ mice. A. Mice were treated with Anakinra or saline solution for 7 days. Single-cell suspensions from organs of interest were analyzed via flow cytometry for Th17 cells. *p<0.05, 5 mice per group. B. Colons from three Anakinra-treated and three saline-treated mice were pooled, processed and analyzed for Th17 cells via flow cytometry. One experiment of two. C. Mice were treated with Anakinra or saline solution for 7 days. Single-cell suspensions from organs of interest were analyzed via flow cytometry for Tc17 cells. *, P<0.05, 5 mice per group.

Increased Th17 cells and IL-17 in the intestine of patients with Crohn's disease.

After determining the causal relationship among IL-10, DCs, IL-1 and Th17 cell development in murine system, we have further extended our studies to patients with autoimmune disease. Recent studies have emphasized the relevance of Th17 cell function in human autoimmune diseases, including multiple sclerosis [123], colitis [140,284] and psoriasis [98,101,285]. It has been reported that a variety of cytokine cocktails including transforming growth factor beta (TGFβ), interleukins (IL)-6, IL-1, and IL-23 promote Th17 cell development, while IL-2 is able to inhibit Th17 cell development [89,286-291].

We have chosen to study patients with CD. To this end, we isolated the lamina propria mononuclear cell fraction from fresh inflammatory colonic tissues in CD patients or from "approximately normal" adjacent colonic tissues in patients with colorectal cancer. Th17 cells were detectable in the CD colon tissues, "normal" colon tissues, and peripheral blood from the non-CD patients (Fig. 3.6A). However, the numbers of Th17 cells were significantly higher in the CD colon tissues than in both the other tissue compartments from non-CD patients (Fig. 3.6A). We also detected higher levels of IL-17 in the briefly cultured lamina propria mononuclear cells from patients with CD. This indicates that IL-17 was spontaneously released in these cultures by Th17 cells from CD patients (Fig. 3.6B). After stimulation with IL-1 and IL-23, the levels of IL-17 were further increased in cultures of lamina propria mononuclear cells from patients with Crohn's disease (Fig. 3.6B). Interestingly, blood levels of IL-17 were significantly increased in patients with Crohn's disease as compared to normal donors (Fig. 3.6C). The data suggest that Th17 cells may be induced in the local pathological environment in patients with CD.

Fig. 3.6A

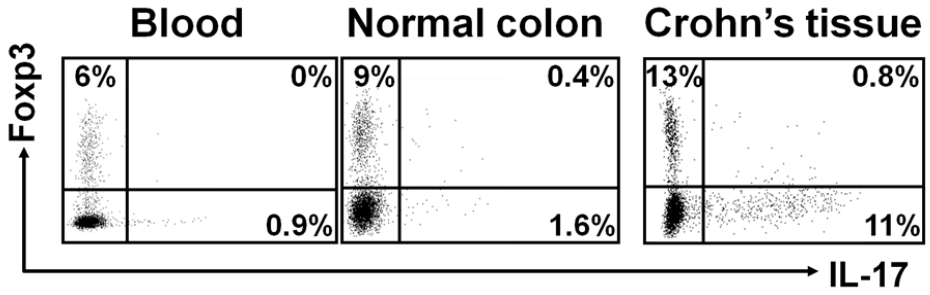


Fig. 3.6B

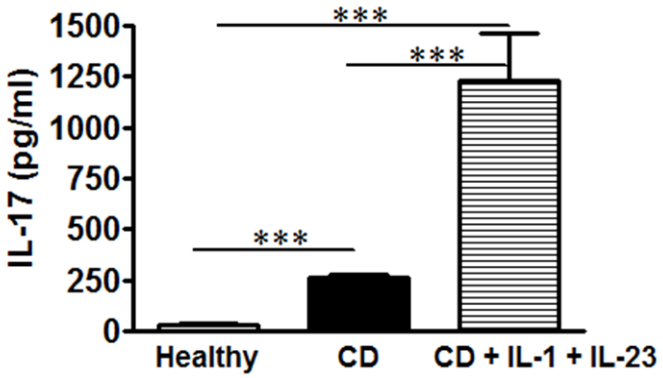


Fig. 3.6C

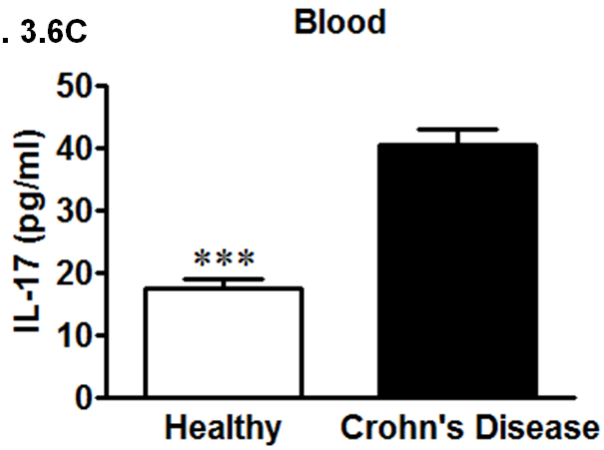


Figure 3.6: Th17 cells and IL-17 are increased in Crohn's disease patient tissue. A. Single-cell suspensions were created from healthy blood or colon tissue or colon tissue from CD patients. Cells were stained with antibodies to CD3, CD4, CD8, IL-17, IFNγ, and FoxP3 and antigen expression was analyzed via flow cytometry. B. Lamina propria mononuclear cells from noncancerous tissues excised from colon biopsies or from CD patient colons were cultured for two days with or without IL-1 and IL-23 treatment. Culture supernatants were subjected to ELISA for analysis of IL-17 protein. C. Blood serum from healthy or CD patients was subjected to ELISA for IL-17 quantification. N=16. ***P<0.0001.

Th17 cells are associated with decreased IL-10 and increased IL-1 in patients with Crohn's disease.

We next examined the potential mechanisms by which Th17 cells were induced in the local pathological environment in patients with Crohn's disease. IL-10 gene polymorphisms that result in defective IL-10 production are observed in patients with CD [292]. We quantified IL-17 and IL-10 in mononuclear lamina propria cells from patients with CD. Interestingly, IL-10 message levels were negatively associated with those of IL-17 in CD patients (Fig. 3.7A). It is possible that decreased IL-10 in Crohn's disease patients may allow increased IL-1 in the intestinal milieu and thus contribute to larger local Th17 populations and greater IL-17 production, as we have demonstrated in the murine system. Accordingly, DC from CD patients produced large amounts of IL-1 upon stimulation with LPS (Fig. 3.7B). Furthermore, IL-10 message levels correlated inversely with IL-1β message levels (Fig. 3.7C) in myeloid APCs from these tissues. Blockade of IL-1R signaling reduced CD-isolated $CD4^+$ T cell-derived IL-17 production (Fig. 3.7D). Thus far, the data suggest that reduced IL-10 expression by intestinal APCs may promote Th17 cell development through increased IL-1 production in patients with CD.

Fig.3.7A

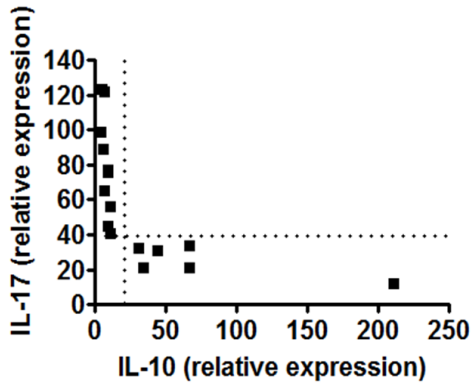


Fig.3.7B

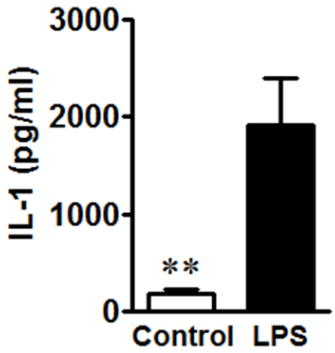


Fig. 3.7C

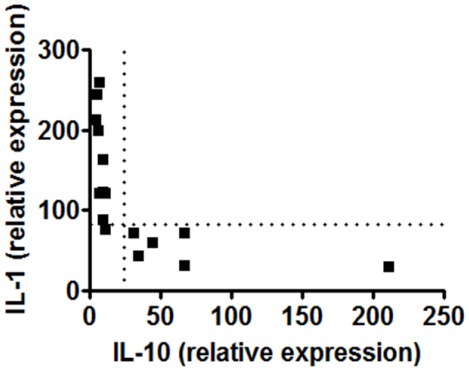


Fig. 3.7D

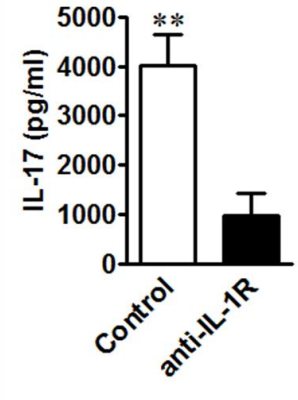


Figure 3.7: IL-17 in Crohn's disease patients is associated with increased IL-1 and decreased IL-10. A. IL-17 and IL-10 message from fresh CD mononuclear lamina propria cells were quantified via RT-PCR. ***, P<0.0001, Chi-squared (χ^2) test. B. IL-1 protein was quantified in supernatant of colon DC isolated from CD patients cultured for two days. N = 6. **, P<0.005. C. IL-1 and IL-10 message from fresh CD mononuclear lamina propria cells were quantified via RT-PCR. ***, P<0.0001, Chi-squared (χ^2) test. D. IL-17 protein in supernatant from two-day co-cultures of CD colon $CD4^+$ T cells and myeloid DC with or without the addition of anti-IL-1R antibody was analyzed via ELISA. N = 6. **, P<0.005.

Th17 cells promote inflammation in patients with Crohn's disease.

To define the inflammatory functionality of intestinal Th17 cells, we isolated and activated intestinal $CD4^+$ T cells from patients with CD. Autologous fresh colon tissue cells were exposed to supernatants from Crohn's T cells for a short time. In the absence of CD T cells, colon tissue cells produced minimal amounts of IL-1β, IL-6, and IL-8 (Fig. 3.8A, B, C). Interestingly, the levels of IL-1β, IL-6 and IL-8 protein were dramatically increased in the presence of CD T cell supernatants (Fig. 3.8A, B, C). Blockade of IL-17 receptor (IL-17R) reduced the production of IL-1β, IL-6, and IL-8 stimulated by CD T cells (Fig. 3.8A, B, C). Crohn's Th17 cells may thus play an active pro-inflammatory role in the local disease environment. Taken together, these data demonstrate a potent cellular and molecular link between IL-10 and Th17 cells in mice and in humans with CD.

Fig. 3.8A

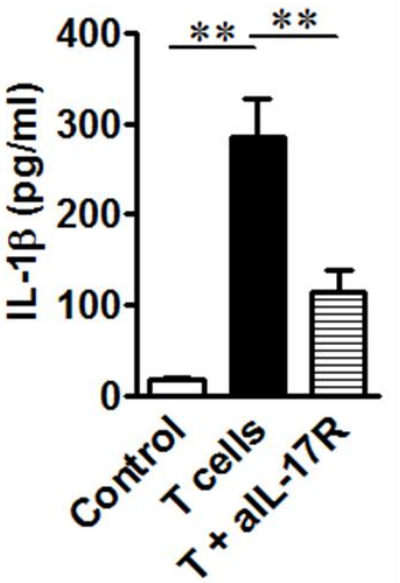


Fig. 3.8B

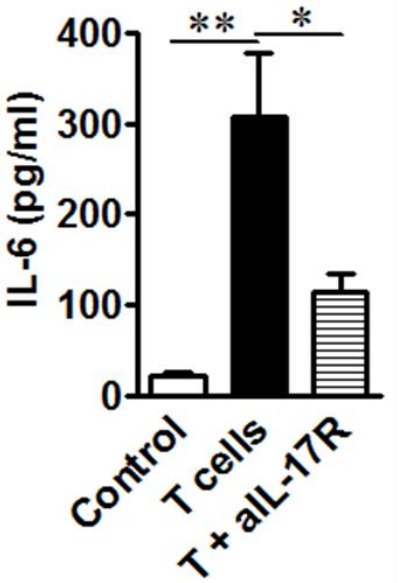


Fig. 3.8C

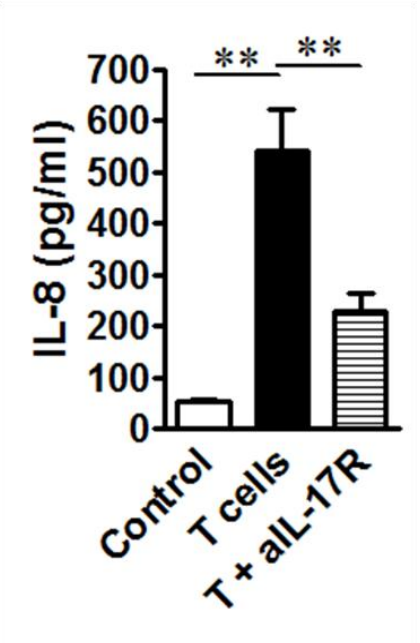


Figure 3.8: T cell-derived IL-17 stimulates production of proinflammatory cytokines in Crohn's disease tissue. Noncancerous colon cells from colon cancer patients were cultured for 40 hours with medium alone or anti-CD3/anti-CD28-activated Crohn's disease T cells with or without with anti-IL-17R antibody. Culture supernatant was subjected to ELISA for quantification of A. IL-1β, B. IL-6, and C. IL-8 protein levels. N = 5. *, $P<0.05$ and **, $P<0.01$.

Gender	Age	Diagnosis
M	31	Crohn's Disease
M	38	Crohn's Disease
M	29	Crohn's Disease
M	29	Crohn's Disease
F	43	Crohn's Disease
F	32	Crohn's Disease
F	25	Crohn's Disease
F	21	Crohn's Disease
F	34	Crohn's Disease
F	32	Crohn's Disease
F	43	Crohn's Disease
F	24	Crohn's Disease
F	25	Crohn's Disease
F	28	Crohn's Disease
F	33	Crohn's Disease
F	43	Crohn's Disease
M	34	Colon Carcinoma
M	42	Colon Carcinoma
F	40	Colon Carcinoma
F	38	Colon Carcinoma
F	22	Colon Carcinoma

Table 3.1: Patient Characteristics.

Discussion.

In the present study, we established a cellular and molecular relationship between IL-10 and Th17 cell development in mice and in humans (Fig. 3.9). This link is likely involved in the immune pathogenesis of chronic inflammatory conditions in humans, such as CD.

Th17 cells play a role in the inflammatory response associated with multiple human autoimmune diseases [125,127,140,293] and cancer [81]. Th17 cells and IL-17 are detected in patients with CD [135-139,294-296]. However, the functional relevance of CD Th17 cells remains poorly understood. We have sorted T cells from fresh CD colon tissues and tested the effects of their cytokine products—particularly IL-17—on the signature inflammatory gene expression described in CD colon tissues. We have found that these T cells induce the production of IL-1, IL-6, and IL-8 by CD patient colon tissue cells. Neutralization of IL-17 results in a significant abrogation of IL-1, IL-6, and IL-8 production in affected colon tissues. Our data indicate that functional IL-17 is derived from Th17 cells in CD colon tissues. In line with this likelihood, elevated inflammatory cytokines are detected in the freshly isolated colon environment in patients with CD. It has been reported that recombinant IL-17 induces IL-6 expression in other systems [66,297-299]. As IL-1, IL-6 and IL-8 play crucial roles in CD [300-304], it is likely that Th17 cells promote the production of inflammatory cytokines and contribute to the immunopathogenesis of human CD.

We have demonstrated a cellular and molecular link between IL-10, IL-1, and Th17 cells in patients with CD and in IL-$10^{-/-}$ mice. In CD patients, intestinal lamina propria cells produce less IL-10, and the levels of intestinal IL-10 are negatively associated with IL-17. Both IL-$10^{-/-}$ mouse DCs and human colitic DCs are superior inducers of Th17 cells via their increased IL-1 production. Blockade of the IL-1 signaling pathway reduces Th17 cell development both *in vitro* and *in vivo*. In agreement with these observations, we and others have shown that IL-1 is crucial for inducing Th17 cells in humans and mice [79,282]. In patients with psoriasis, psoriatic DCs potently induce Th17 cells in an IL-1-dependent manner [98]. Notably, it has been reported that IL-10 suppresses IL-1

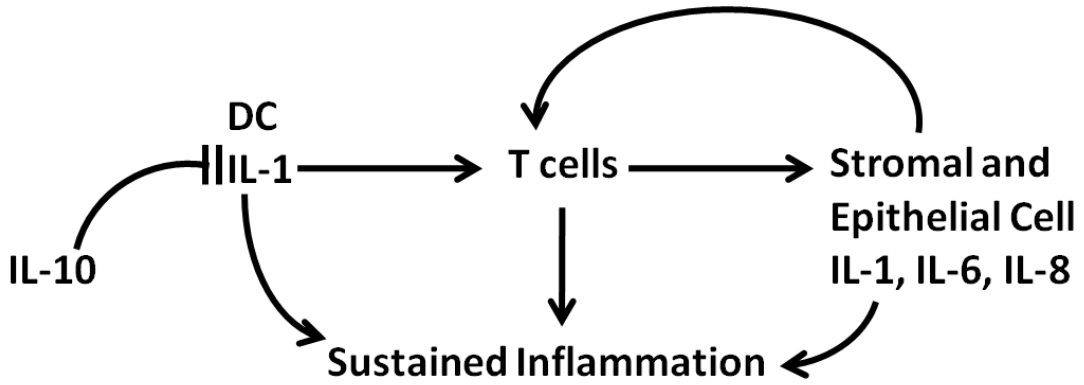


Figure 3.9. Model of IL-10 in autoimmunity.
1. IL-10 downregulates DC expression of IL-1, which directly abrogates Th17 development.
2. This in turn decreases Th17-mediated upregulation of the proinflammatory cytokines IL-1, IL-6, and IL-8.
3. Lower inflammatory mediator levels minimize Th17 self-perpetuation, curtail inflammation, and reduce the potential of developing autoimmunity.

production [305-306] and that IL-1 is involved in controlling Th17 cells in the mouse model of EAE [283]. Exogenous IL-10 can suppress the *in vitro* development of Th17 cells from $CD4^+$ T cells in patients with RA [307]. However, our study is the first to demonstrate a role for IL-10 in Th17 development through the control of IL-1 expression by DCs in both mouse and human systems. Our data indicate that IL-1 plays a key role in Th17 cell development in human autoimmune diseases, and support the notion that IL-1 signaling blockade is a meaningful strategy to treat patients with autoimmune diseases. IL-10, via downregulation of IL-1, is thus able to limit development of Th17 cells in mice and humans, and in doing so executes some of its anti-inflammatory effects. In future experiments, it would be interesting to investigate the cytokines that these mouse and human Th17 cells produce in addition to IL-17. Th17 cells are capable of producing multiple proinflammatory cytokines, and $IL\text{-}17^+IFN\gamma^+$ double-positive cells have been identified in numerous inflammatory settings [81,308-309]. Further characterization of these Th17 populations might yield new insights into their contributions to the development of pathology.

The next logical step is to investigate how IL-10 controls IL-1 production by APCs. It has recently been shown that IL-10 dampens MyD88-dependent signaling in DCs and leads to LPS hyporesponsiveness [310]. Because IL-1 signaling can be mediated by MyD88, this may explain how IL-10 controls endotoxin-induced IL-1 production. It is also possible that IL-10 controls IL-1 expression machinery, such as IL-1 converting enzyme (ICE) and components of the inflammasome [311-312]. However, it remains to be determined if IL-10 suppresses IL-1 production induced by other stimuli, including the necrotic tissue often found in a chronic inflammatory environment.

One key question is why IL-10 production is reduced in patients with Crohn's disease. Recent genetic studies have shed some light on the explanation. It has been reported that a nucleotide-binding oligomerization domain containing 2 (NOD2) mutation commonly observed in patients with Crohn's disease leads to inhibition of IL-10 transcription [292]. However, we have not examined the gene profile of NOD expression in our patient

populations. Since 30% of patients with Crohn's disease have NOD mutations, it is likely that alterations in NOD2 transcription may at least partially contribute to the reduced IL-10 production in our patient population. Further genetic studies are required to confirm this possibility.

In summary, we have demonstrated that IL-10 suppresses Th17 cell development in mice and humans through modulation of IL-1 production by DCs. The data document a cellular and molecular link between IL-10, IL-1, and Th17 cells, and suggest that IL-10 may inhibit inflammation via control of Th17 cell development.

Chapter 4
Conclusions

Perhaps the broadest conclusion that may be drawn from this work is one that is already commonly accepted: IL-10 is a pluripotent cytokine that controls the development of several immune cell populations and the expression of multiple proteins crucial in immune signaling. In fact, both of the experimental series contained in this work explore effects that IL-10 mediates through the classic pro-inflammatory cytokine IL-1. In one series, IL-10 is shown to affect Th17 cell population development and size through the control of IL-1, and the relationship between IL-10 and Th17 cells is inversely correlated. Thus, IL-10 down-regulates IL-1 production in myeloid cells, and in doing so hinders expansion of Th17 populations by decreasing bioavailability of IL-1. The increased Th17 populations in the IL-$10^{-/-}$ mouse, then, result at least in part from an increase in IL-1 expression. This finding is not far from the generally accepted role for IL-10 as an immunosuppressive molecule: IL-10 keeps pro-inflammatory cytokine levels in check, and its absence leads to a stimulatory environment that supports the development or expansion of certain immune cell subsets typically associated with inflammation. This data serves to reinforce the commonly-held view of IL-10 as a regulator and restrainer of immune responses. In this context, our novel observation is that IL-10 suppresses Th17 cells through inhibition of DC-derived IL-1, and in turn negatively controls autoimmunity in both mice and humans.

However, from the second set of experiments in this work, we find fairly convincing evidence that IL-10 displays immune-supporting functions, especially in the context of tumor immunity. Although IL-10 has been shown to serve as a development factor for $CD8^+$ T cells [34,313], we find that it can also support effector cytokine production in both $CD8^+$ and $CD4^+$ T cells. Its absence causes

an increase in MDSC accumulation in tumor-bearing animals (whether this effect is direct or occurs through other mediators remains to be determined). These MDSC are potent suppressors of T cell proliferation and also more powerful inducers of regulatory T cells *in vitro* and likely *in vivo*. Here, it is likely that some phenotypic signatures in the tumor-bearing mice result from IL-10's control of IL-1 expression, especially in the context of MDSC recruitment and function in the tumor environment [261-262,274]. Upon IL-1 signal blockade, effector cytokine production was partially rescued, and Treg numbers within the tumor environment were decreased. Although the role of IL-1 in support of T cell effector cytokine expression must still be explored, its impact on intratumoral Treg cell populations may be easier to elucidate. Because IL-1 contributes to MDSC recruitment to and retention in the tumor microenvironment, and because a functional signature of MDSC is the induction of Treg cells from parent $CD4^+$ T cell populations [251], it is logical to surmise that these interactions occur sequentially and culminate in expanded Treg cell populations within the tumor. Interestingly, blockade of IL-1 signaling did not decrease MDSC populations in tumor-bearing animals. This suggests either that IL-1's effects on MDSC are more durable than can be reversed with temporary abrogation of signaling, or that a factor (or factors) distinct from IL-1 may contribute to MDSC recruitment. Future research will be required to determine which scenario is correct. For this set of data, it is clear that IL-10 supports antitumor immunity both through maintenance of effector T cell populations and their effector cytokine production, and by moderation of pro-tumor immune suppression mediated by MDSC and Tregs.

As with any set of experiments that challenges the commonly-held beliefs about certain cells or mediators, this work raises as many questions as it answers. One of the most important is: how precisely does IL-10 regulate IL-1 expression in myeloid cells? As we mentioned above, it is possible that this occurs through IL-10's regulation of MyD88 [310], but this may not be the only mechanism. The process whereby IL-1 is activated and released from the cell is rather complicated: IL-1 exists as pro-IL-1β before it is cleaved by ICE/ caspase

1, and caspase 1 requires the scaffolding of the NLRP3 inflammasome in order to cleave pro-IL-1β. Caspase 1 itself is produced as a zymogen and must be cleaved in order to become active. Active Caspase 1 interacts with a protein called Apoptosis-associated speck-like protein containing a CARD (PYCARD) [314]. It is possible that IL-10 controls one or more factors involved in IL-1 processing and release from cells. The most straightforward experiment may be the direct analysis of the expression levels and activation of these factors within IL-10-deficient myeloid cells (both stimulated and unstimulated) and wild-type cells. This could be accomplished by examination via RT-PCR and protein analysis by Western Blot. Alternatively, IL-10 may contribute to the regulation of the autophagy pathway. Autophagy is a catabolic process normally active in homeostasis, involving the sequestration and degradation of cytosolic macromolecules, surplus or damaged organelles, and certain intracellular pathogens. Under normal physiological conditions, cellular autophagy can be induced by nutrient deprivation, exposure to inhibitors of mTOR, or stimulation by IFNγ or TNFα. It is inhibited by the Th2 cytokines IL-13 and IL-4. In settings of infection, autophagy can be initiated by the recognition of pattern-associated molecular patterns (PAMPs) by TLRs on myeloid cells. Signaling through TLRs induces the sequestration of pro-IL-1β into autophagosomes, where it is degraded. Inhibition of autophagy leads to an increase in the processing and secretion of IL-1β from antigen-presenting cells; this is dependent on the NLRP3 inflammasome and TRIF [315]. In order to investigate whether IL-10 can, in fact, regulate IL-1β expression via control of autophagy, the process should be examined in IL-10-sufficient and deficient myeloid cells. Stimulation of cells by cytokines or inhibitors of mTOR could be used to induce or abrogate autophagy, and then cellular release of IL-1β could be measured by ELISA. Additionally, to more closely examine the process, pro-IL-1β could be localized and quantified in cellular compartments (via fluorescence microscopy) after cell stimulation.

An overarching question remains for both sets of experiments. Given that the phenotypes we discovered and discussed were observed in IL-$10^{-/-}$ mice, they could be the result of a global lack of IL-10 expression during birth,

development and maturation of these animals. In order to determine whether the molecular signatures we observe result from or are complicated by the complete absence of one protein, it will be essential to examine the same parameters in wild-type, C57Bl/6 mice that have developed normally but undergo IL-10 blockade. This blockade may be genetic (global or tissue-specific) or biological (administration of anti-IL-10 or anti-IL-10R antibodies). Using one or more of these methods to abrogate IL-10 signaling for a few weeks before tumor inoculation, colitis induction, or systemic examination of Th17 populations would be ideal. In this way, one could more concretely identify the cellular and molecular phenotypes as the result of a (temporary) lack of IL-10 in a more physiological setting.

Finally, because IL-10-deficient mice are known for their gastrointestinal phenotypes, and because more and more often, investigators are observing profound effects of intestinal flora on disease development, it is crucial to investigate the role of commensals in dissecting the phenotypes we observe. Two very recent papers have examined the induction of IL-10 production in adaptive immune cells by commensal bacteria. Pils and colleagues documented an increase in T cell IL-10 mRNA levels after DSS exposure of wild-type mice housed in SPF but not germ-free conditions. Intriguingly, germ-free mice developed more severe intestinal disease, and some experiments had to be terminated early because of mouse mortality [316]. This suggests that the bacteria present in mice living in SPF conditions—in these experiments, Charles River altered Schaedler flora (CRASF)—have significant roles in regulating immune responses. In the same year, Round and Mazmanian observed that regulatory T cell development could be influenced by commensal flora. Monocolonization of germ-free mice with *Bacteriodes fragilis* increased both suppressive activity and anti-inflammatory cytokine production by intestinal regulatory T cells [317]. Interestingly, polysaccharide A (PSA) from *B. fragilis* was determined to be the molecule responsible for converting $CD4^+$ T cells to IL-10^+ $FoxP3^+$ regulatory T cells during commensal colonization of the gut. For this induction, signaling through TLR2 was required, once again emphasizing the

complex relationship between innate immune cues and adaptive immune development in the gastrointestinal tract. Interestingly, exposure of mice to PSA could cure TNBS-induced colitis. A thorough investigation of the commensal contribution to the phenotypes we have observed in IL-10$^{-/-}$ mice would take years. At baseline, it would be necessary to establish the differences in gut colonization of wild-type and IL-10$^{-/-}$ mice, using microarrays or high-throughput PCR techniques specific for certain species of bacteria. It would perhaps be easier to establish how the differences in flora contributed to increased Th17 populations in IL-10$^{-/-}$ mice and their higher susceptibility to tumor development than to the individual adaptive immune signatures we observe in tumor-bearing IL-10$^{-/-}$ mice. A comparison of immune cell populations in IL-10$^{-/-}$ and wild-type mice housed in germ-free conditions might also prove informative. Mono- or oligocolonization of IL-10$^{-/-}$ mice kept in germ-free conditions could be undertaken to observe the possible kinetic induction of Th17 populations; again, these experiments are tedious and would need to be carried out with extreme caution to minimize accidental colonization of mice with unknown commensals or pathogens.

From an immunological perspective, the biological effects of given cell populations, cytokines, and other mediators are all context-dependent. In this case, we are exploring the downstream effects of IL-10 in two very different contexts: tumor immunity and autoimmunity. It is well established that Tregs and MDSC constitute major suppressive populations in the tumor microenvironment. Settings of malignancy (and especially advanced malignancy) are generally suppressive. In addition to MDSC and Treg expansion, immune populations responsible for T cell activation and stimulation are dysregulated. Myeloid and plasmacytoid DC, as well as tumor-associated macrophages, lose expression of costimulatory molecules and upregulate inhibitory molecules, including B7-H1 and B7-H4 [149,213,318]. Their cytokine profiles also change dramatically [319]. In tumor microenvironments, IL-10 limits IL-1 production and MHC expression in myeloid cells. Lower MHC expression could effect a decrease in presentation of tumor peptides by myeloid cells to regulatory T cells, and thus a decrease in

tumor tolerance. Lower IL-1 production decreases MDSC exposure to this crucial cytokine, and thus minimizes their development and function in the tumor site. Fewer tumor-associated MDSC results in abrogated induction of Tregs from local $CD4^+$ T cells, and perhaps then prevents or lowers suppression of local antitumor T cell populations. At the same time, we have demonstrated that IL-10 directly supports the development and effector function of T cells. Therefore, in our experimental setting, IL-10 affects both innate and adaptive immunity to the benefit of the host.

Multiple groups have reported decreased Treg numbers and dysregulated Treg function in conditions of autoimmunity [320-321][and others]. As we have already discussed, the myeloid cell populations in the gut of mice and humans affected with IBD are massively dysregulated. Their numbers, locations, and molecular phenotype are changed, making them more susceptible to certain activatory signals [158-159]. Their molecular signatures are different (whether they possess NOD2 mutations or not), and it is likely that their increase in proinflammatory cytokine production and decrease in suppressive cytokine production contributes greatly to the maintenance of the local inflammatory milieu. In this context, the predominant effect of IL-10 may be on APCs, where IL-10 suppresses IL-1 expression. Additionally, because T cells in IBD are more strongly retained in the gut, and some of them have demonstrated higher resistance to apoptosis, mDC-derived IL-1 may more easily reach T cell targets and expand populations of memory Th17 cells. More Th17 cells means higher levels of IL-17, IL-23 and other Th17 signature cytokines in the gut, and signaling via these cytokines leads to more profound induction of other proinflammatory cytokines from stromal and epithelial tissue. In this way, it is straightforward to hypothesize the impact of IL-10 on multiple downstream populations as mediated through its direct effects on myeloid cells. In both tumor immunity and autoimmunity, we see the net effect of IL-10's interactions with its environment and on its target cells, and this can be profoundly different.

Taken together, the data presented herein expand upon previously-held conceptions regarding IL-10. We have presented an additional manner in which

this cytokine moderates pro-inflammatory responses: through constraint of Th17 development. Yet, we have also demonstrated that IL-10 stimulates and supports host immunity in the context of malignancy. It is crucial, then, to carefully evaluate the type of immune response in question before assigning a role (or roles) to the cytokine of interest—in this case, IL-10. The inquisitive investigator will no doubt explore other ways or elucidate further mechanisms whereby this cytokine may support immune responses. In light of the current evidence, however, we must remind ourselves that, especially in the case of IL-10, “context is everything.”

Appendix

Materials and Methods

Mice and Tumor models

6-12 week old female C57BL/6 mice were purchased from Charles River. IL-$10^{-/-}$ mice (strain #2251) and IL-$1R^{-/-}$ mice (strain #3245) were purchased from the Jackson Laboratory (Jackson Laboratory, Maine) and bred in-house. The mice were maintained in specific pathogen-free conditions. This research was approved by the committee on Use and Care of Animals at the University of Michigan. 1×10^6 MC38 mouse colon carcinoma cells were inoculated subcutaneously into the left flank of IL-$10^{-/-}$, IL-$10^{+/+}$, or IL-$1R^{-/-}$ C57/BL6 mice. Tumor size was measured each three days using calipers fitted with a Vernier scale. Tumor volume was calculated based on three perpendicular measurements. For other experiments, $2x10^5$ MCA310 cells were inoculated intravenously into the tail vein of IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. At 2 weeks post-inoculation, mice were sacrificed and their lungs were harvested, perfused with India Ink, and fixed in paraformaldehyde. The numbers of lung foci were quantified with the aid of a magnifier.

Patients

Blood was collected from patients with Crohn's Disease and healthy volunteers. Fresh colon tissues were collected from patients with CD who underwent colonic resections or diagnostic biopsies. Fresh "approximately normal" colon tissues adjacent to colorectal carcinoma were also collected as control tissues. All patients with CD were in remission and were not treated with steroid drugs or antibiological therapy during the 2 months before the study. Patient information is presented in Table 3.1. This research was approved by local Institutional Review Boards.

AOM/DSS treatment

Mice were given 10mg/kg azoxymethane (Sigma) via intraperitoneal injection. Five days later they were allowed free access to water containing 2% dextran sodium sulfate (DSS, MP Biomedicals/ Fisher) for five days, followed by 16 days of regular water. This cycle was repeated twice and mice were sacrificed 2 weeks after the end of the last DSS cycle or at the end of 9 weeks. Colons were harvested, flushed of feces, and slit open longitudinally to count tumors with the aid of a magnifier.

IL-1Ra treatment

The human recombinant IL-1 receptor antagonist Anakinra (Kineret®) was administered at 150mg/kg to mice intraperitoneally for 5 days before tumor inoculation and each day thereafter for three weeks. In experiments not involving tumor inoculation, Anakinra (Kineret®) was administered at 150mg/kg to mice intraperitoneally for 8 days. On the eighth day, mice were sacrificed and their organs were harvested for phenotyping. Mice not receiving Anakinra were injected with PBS vehicle.

Flow cytometry analysis

Single-cell suspensions were made from human colon tissue or blood, mouse spleen, lymph nodes, tumor-draining lymph nodes and/or tumor. Cells were labeled with fluorescence-conjugated antibodies to CD45, CD11c (both Invitrogen), CD4, CD8, Gr-1, CD11b, CD90, CD115, IL-17, and/or FoxP3 (all eBioscience), CD3, IFNγ, TNFα, lL-2, MHC I, NK1.1, CD49b, CD19 (all BD Pharmingen), and/or Foxp3 (eBioscience). For cytokine profiles, the cells were stimulated with 50ng/mL PMA (Sigma) and 1 µM Ionomycin (Sigma-Aldrich, St. Louis, MO) for 4 hours in the presence of GolgiPlug and GolgiStop (BD Biosciences, San Jose, CA). Cells were first stained extracellularly with specific antibodies, then fixed and permeabilized with Fix/Perm solution (eBioscience), and finally stained intracellularly with specific antibodies. Samples were acquired on a special order LSR II flow cytometer (BD Biosciences), and data were

analyzed as previously published with DIVA software (BD Biosciences) [213,257].

Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described [213,257]. Briefly, harvested tissues were frozen in OCT and then fixed with paraformaldehyde. Permeabilized tissues were stained with rat anti-mouse CD8 (1:50, BD Pharmingen), rat anti-mouse CD31 (1:00, BD Pharmingen), and/or rabbit anti-mouse Foxp3 (1:500, Abcam) followed by goat anti-rat and goat anti-rabbit secondaries conjugated to Alexa Fluor 488 and Alexa Fluor 568, respectively (both 1:2000; Molecular Probes/Invitrogen). Nuclei were stained with DAPI (Molecular Probes/ Invitrogen). Fluorescent images were acquired on a fluorescence microscope (Leica) and analyzed by ImagePro Plus software.

MDSC Suppression and Treg Induction

For the immunosuppressive assay, MDSCs were isolated from tumor or spleen in tumor-bearing mice and cultured with irradiated spleen cells from wild-type (IL-$10^{+/+}$) mice. These cells were stimulated for 3 days with anti-CD3 (2.5μg/ml) and anti-CD28 (1.25μg/ml) in the presence of different concentrations of MDSCs. Thymidine was added in the last 16 hours. T cell proliferation was determined by thymidine incorporation. In Treg induction experiments, tumor-associated MDSCs were cultured with IL-$10^{+/+}$ $CD4^+$ T cells for 6 days. On day 6 the cells were removed from culture, permeabilized, and stained with antibodies to FoxP3, CD4, CD90, and CD11b. FoxP3 expression in total $CD4^+$ T cells was analyzed via FACS.

Cytokine expression

T cells were isolated from spleen with Mouse T Cell or $CD4^+$ or $CD8^+$ Enrichment Kit (StemCell Technologies). The enriched T cells were cultured with 10 ng/ml IL-10 (R&D Systems) or 1ug/ml anti-IL-10 for 3 days. IL-2, TNFα, and IFNγ were analyzed on a special order LSR II flow cytometer (BD Biosciences).

Real-time reverse-transcriptase polymerase chain reaction (RT-PCR)

$CD11b^+$ cells were isolated from IL-$10^{+/+}$ or IL-$10^{-/-}$ splenic single-cell suspensions with CD11b positive selection microbeads (Miltenyi), and $CD11c^-$ $CD11b^+$ cells were further sorted with FACSAria. $CD11c^+$ cells were isolated from IL-$10^{+/+}$ or IL-$10^{-/-}$ splenic single-cell suspensions with a $CD11c^+$ positive selection kit (StemCell Technologies) and cultured for 8 hours with or without LPS stimulation. In other experiments, fresh DC from Crohn's Disease patients were isolated and evaluated directly. mRNA was isolated with Trizol (Gibco BRL). Cytokine transcripts were detected by real-time RT-PCR as previously described [81]. Gene-specific primer pairs and Fast SYBR Green Master Mix (Applied Biosystems) were used in a Multiplex instrument (Eppendorf). Data analysis is based on the Ct method with normalization of raw data to a housekeeping gene (HPRT).

ELISA Cytokine Detection

Supernatant was collected from 3-day cultures of mouse T and $CD11c^+$ cells, or after 48-hour LPS stimulation of $CD11c^+$ cells, or after two-day cultures of fresh human colon lamina propria or $CD4^+$ T cells, or co-cultures of colon cells with or without T cells from Crohn's Disease patients. In other experiments, fresh serum from the blood of healthy volunteers or Crohn's Disease patients was directly subjected to ELISA. IL-1β, IL-17, IL-6 and IL-8 were detected using murine or human DuoSet kits (R&D Systems, Minneapolis, MN) with standard curves ranging from 8-1000pg/ml and a detection limit of 8pg/ml.

DC and T cell culture

Mouse spleens were harvested, processed, and subjected to Ficoll density-gradient centrifugation, rinsed, and resuspended as single-cell suspensions. $CD11c^+$ DC and $CD4^+$ T cells were isolated using positive ($CD11c^+$) and negative ($CD4^+$ T) selection kits (Stemcell Technologies). Cells were co-cultured in a ratio of 1:5 for 5-6 days with anti-CD3 and anti-CD28 antibodies. For single cultures, T or $CD11c^+$ cells were plated at $1x10^6$ cells/ml, with anti-CD3 and anti-CD28 antibodies added for T cells and 1ug/ml LPS or no stimulation for $CD11c^+$ DC.

Colon and T cell Cultures

Noncancerous colon cells (2 x 10^6/ ml) from colon cancer patients were cultured for 40 hours with medium alone, anti-CD3/anti-CD28-activated T cells isolated from Crohn's disease patients, or anti-CD3/anti-CD28-activated T cells isolated from Crohn's disease patients plus anti-IL-17R antibody (R&D, clone 133617). The ratio of colon cells to T cells was 1:5.

Statistics

Most experiments were evaluated using the Mann-Whitney test, with $P < 0.05$ considered significant. Some cases were evaluated with Student's T test, also with $P < 0.05$ considered significant. Statistics were performed in the GraphPad Prism program suite (GraphPad Software, Inc., La Jolla, CA) and Statistica program suite (StatSoft, Tulsa, OK).

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