CHARACTERIZATION OF THE SOURCE AND FUNCTION OF INTERLEUKIN-10 DURING LYME ARTHRITIS DEVELOPMENT

by

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ABSTRACT

IL-10 is a non-redundant inflammatory modulator that suppresses the development of arthritis in *Borrelia burgdorferi* infected mice. Previous microarray studies of infected B6 and C3H joint tissue found IL-10 to be induced only in B6 mice, leading to the hypothesis that the inability to produce IL-10 was a factor in arthritis development in C3H mice. Expression of IL-10 was robust in the skin from around the ankle joint in both B6 and C3H mice, suggesting that arthritis in C3H mice develops through pathways not regulated by IL-10.

B6 IL-$10^{-/-}$ mice were previously found to have a robust and sustained interferon-inducible response in joint tissues, and the current study identified high concentrations of IFN-γ in sera. Infection of IL-10 reporter mice demonstrated that macrophages and $CD4^+$ T cells were the primary sources of IL-10 in the infected joint and skin tissues, which suggested that early local production of IL-10 dampened the pro-arthritic interferon response. In fact, treatment of IL-$10^{-/-}$ mice with anti-IFN-γ reduced arthritis severity and suppressed interferon-inducible transcripts to wild type levels, thereby linking dysregulation of IFN-γ to disease in the IL-$10^{-/-}$ mouse. Arthritis in IL-10 deficient mice was associated with elevated numbers of NK cell, NKT cell, α/β T cell, and macrophage infiltration of the infected joint. FACS lineage sorting revealed NK cells and $CD4^+$ T cells as sources of IFN-γ in the joint tissue of IL-$10^{-/-}$ mice. These findings suggest the presence of a positive feedback loop in the joint tissue of infected IL-$10^{-/-}$ mice, where

production of inflammatory chemokines, infiltration of IFN-γ producing cells, and additional production of inflammatory cytokines result in arthritis. This mechanism of arthritis is in contrast to that seen in C3H mice, where arthritis development is linked to transient production of Type I interferon, and develops independently of IFN-γ. Due to the sustained interferon response driven by NK cells and T cells, we propose the IL-$10^{-/-}$ mouse as a potential model to study the prolonged symptoms observed in some human Lyme disease patients.

To Brandi, Josie, and Jamison

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CHAPTER 1

INTRODUCTION

Lyme Disease

Lyme borreliosis (also termed Lyme disease) is the manifestation of one or more lesions that result following infection with the tick-borne spirochetes *Borrelia burgdorferi, Borrelia afzelii, and Borrelia garinii* (1-3). These three species have been documented in Europe and Asia, and are collectively termed *Borrelia* sensu lato. *Borrelia burgdorferi* is the only species found in North America, and therefore the term 'sensu strico' is added to the end of the species name. Whether in Europe or North America, these bacteria are transmitted through the bite of hard-bodied ticks of the *Ixodes* genus (2, 3). In the U.S., there are up to 30,000 new cases of Lyme disease reported each year, making it the most common vector-transmitted infection in America.

Lyme disease was initially misdiagnosed as 'juvenile rheumatoid arthritis' in children, and 'early onset rheumatoid arthritis' in adults, which was sometimes preceded by a characteristic rash (4). The high prevalence of these conditions in Lyme, Connecticut suggested environmental factors were the cause of these syndromes. It was not until 1982, when Burgdorfer *et al* discovered a new species of *Borrelia* present in Long Island *Ixodes* tick populations that was recognized by patients' sera that a connection could be made (3). Shortly after this discovery, two independent groups isolated these newly identified spirochetes from Lyme disease patients, establishing these aptly named (*Borrelia* ***burgdorferi***) spirochetes as the causative agent of Lyme disease (2, 5).

Several days after tick inoculation, most patients develop a hallmark 'bulls eye' rash, termed 'erythema migrans', at the site of the tick bite. This rash is thought to result from the localized innate immune response to disseminating spirochetal migration into

the dermal microvasculature (6, 7). In the absence of appropriate antibiotic therapy, the bacteria will disseminate from the site of inoculation hematogenously, and case a systemic infection (6). At this temporal point of infection, patients experience what are termed 'early' symptoms, which include fever, fatigue, body aches, and general malaise (8, 9). Disseminating spirochetes infect multiple tissues, but preferentially invade secondary skin sites, nervous tissue, cardiac tissue, and connective tissue of the joint, and cause disease several months after the initial infection (2, 10-12).

Lyme Arthritis

Arthritis is the most common late stage symptom associated with *B. burgdorferi* infection, with up to 60% of untreated individuals developing Lyme arthritis (13, 14). In addition, up to 30% of individuals who receive treatment will go on to develop arthritis. This self-limiting, infection-associated arthritis is intermittent if untreated, and typically affects only one large joint, though multiple joints may be affected over the course of infection (14-16). This Lyme arthritis is characterized by swelling, edema, hyperproliferation of the synovium, and a moderate inflammatory infiltrate that consists primarily of granulocytes (15, 17, 18). Excluding patients who develop no adverse effects from *B. burgdorferi* infection, the severity of arthritis varies greatly among untreated individuals, ranging from subjective joint pain, to intermittent attacks of arthritis, to chronic erosive disease (14). The range in arthritis severity observed in humans may be due to the genetic propensity for arthritis development in the host, the arthritic potential of the infecting *B. burgdorferi* strain, or a combination of both.

Antibiotic-Refractory Lyme Arthritis

Most human patients with Lyme arthritis and other late stage symptoms respond to antibiotic therapy, after which arthritis eventually resolves (19). However, in some cases arthritis persists for many months after treatment designed to eradicate infection, suggesting that there is a subset of individuals that maintain a long-term inflammatory response in the absence of active infection (19, 20). This condition has been termed "antibiotic-refractory Lyme arthritis" and more recently recognized as "Post-treatment Lyme disease" (21, 22). Genetic population studies identified several MHC class II alleles that were associated with antibiotic-refractory Lyme arthritis patients, which led to a hypothesis that this chronic condition was the result of T cell cross-reactivity with *B. burgdorferi* antigens and self antigens found in the host joint tissue (19, 23, 24). The alleles identified, HLA-DRB1*0401, HLA-DRB1*0101, and HLA-DRB1*0404 have also been associated with rheumatoid arthritis susceptibility (24-27). The identification of a peptide from the *B. burgdorferi* lipoprotein, outer surface protein A (OspA), had sequence homology to the human protein LFA-1, further supported this idea (25). The LFA-1 peptide bound to HLA-DRB1*0401, and some patients had T cells that reacted to the OspA peptide presented by this allele. Thus the link to autoimmunity was proposed to result from patient T cell cross reactivity between two similar peptides derived from two interspecies proteins (25). Subsequent studies examining T cell receptor promiscuity found that cross reactivity was common, and that epitope mimicry was not robust evidence for autoimmune disease (28). In addition to autoimmunity, other less studied hypotheses exist to explain the development of antibiotic-refractory Lyme arthritis. One of these hypotheses is that the local immune response in the joint to the invading

spirochetes is poorly regulated and results in sustained inflammation in the synovium (19). Interestingly elevated levels of IFN-γ, and extremely high levels of the T cell, NK cell, and monocyte recruiting chemokines CXCL-9 and CXCL-10, are found in patients with antibiotic-refractory Lyme arthritis when compared to patients with antibiotic-responsive Lyme arthritis (20). Additionally, recent data have shown that certain *B. burgdorferi* isolates are capable of inducing more robust immune responses than other conventional strains (29). Thus, it is unclear why Post-Treatment Lyme Disease patients are genetically susceptible.

Borrelia burgdorferi

Borrelia burgdorferi are classified as spirochetes, due to their characteristic, corkscrew-like shape. These bacteria are 0.2-0.5μM in diameter, and 10-30μM in length and contain periplasmic flagellae, which make these bacteria extremely motile (30). *B. burgdorferi* are morphologically similar to classic gram-negative organisms; however, they lack the biosynthetic pathways required for synthesis of lipopolysacharide (LPS), a feature that was accepted based on early biochemical and functional studies prior to publication of the *B. burgdorferi* genome sequence (31-34). Rather than LPS, these spirochetes have abundant tripalmitoyl-S-glyceryl-cystein (Pam3Cys) lipoproteins on their surface. Genes encoding more than 150 putative lipoproteins possessing the signal peptidase II sequence are found in the *B. burgdorferi* chromosomal genome, and which render them particularly immunogenic (34-37). Two strains of *B. burgdorferi*, N40 and B31, are routinely used in the laboratory, and were both isolated from ticks in Long Island (3, 38). Both strains contain multiple linear and circular plasmids, some of which

are essential for bacterial survival, and some of which contain virulence factors (39-41). The complex 'organization' of the *B. burgdorferi* genome has made it difficult to identify bacterial mechanisms of virulence (39).

The life cycle of *B. burgdorferi* is dependent on its ability to infect its mammalian host, and persist within both the tick vector and the mammalian host. Larval *Ixodes scapularis* ticks hatch from eggs in the spring, and acquire the infection from a previously infected rodent, rendering the tick infectious for life. The following spring, the ticks, now in the nymphal stage, acquire their second blood meal, likely from a rodent. It is this cycle of larval acquisition, and nymphal transmission that propagates the bacteria. After nymphal ticks acquire their blood meal, they molt and mature to adult ticks. The adult ticks acquire a final blood meal from white-tailed deer, mate, lay non-infectious eggs, and die (42). Thus, due to its complex life cycle, *B. burgdorferi* must persist within its mammalian host for an entire year. One such mechanism by which *B. burgdorferi* persist is to alter the expression of dominant surface lipoproteins to avoid the host adaptive immune response (43). The gene for one such immuno-dominant *B. burgdorferi* lipoprotein, *vlsE*, is found on a plasmid associated with 15 silent "vamp-like sequences" or cassettes, portions of which re-combine with a central, upstream *VlsE* expression cassette to generate an altered surface lipoprotein through gene conversion (44, 45). Taking into account the duration required for a host antibody response, and the millions of possible combinations of *VlsE*, *B. burgdorferi* spirochetes are capable of persisting in their hosts for the lifetime of the host organism.

Mouse Models of Lyme Arthritis

Animal models of Lyme disease, particularly mouse models, are a powerful tool in elucidating the pathogenesis of *B. burgdorferi*. In nature, mice and other rodents are the primary reservoir of these bacteria, and are therefore a logical choice for disease modeling. Currently, no murine model of neuroborreliosis or Post-Treatment Lyme Disease exists that accurately models these conditions. The acute, infection-associated, Lyme arthritis, however, has accurately been modeled using the C3H/He (C3H), BALB/c and C57BL/6 inbred mouse strains. Unlike human rheumatoid arthritis, murine Lyme arthritis is an inflammatory arthritis that is induced by a known, and natural trigger. The process of arthritogenesis can therefore be monitored from beginning, and the identified mechanisms will likely provide insight into general features of many arthritic diseases. Many of the symptoms associated with *B. burgdorferi* infection in human are similar in these mice, and the Lyme arthritis phenotypes found among these strains represent a range in arthritis phenotypes, modeling the spectrum in disease severity seen in humans (46). In infection models that utilize intradermal needle inoculation, spirochetes reach the joint by 1 week of infection, and achieve maximal density at two weeks after infection, while arthritis develops 3-4 weeks after infection (47-49). Although arthritis develops in multiple joints, it is most prominently seen in the tibiotarsal (ankle) joints (46). In C3H mice, arthritis is severe, with robust infiltration of neutrophils along with the accumulation of edema in the tibiotarsal joint, joint space, and contiguous supportive tissues, as well as proliferation of synoviocytes of the tibiotarsal tendon sheath (46, 47). These lesions have also been observed in B6 mice, but the severity of disease is greatly decreased in comparison (46, 48). Interestingly, arthritis development in BALB/c mice is

dependent on the inoculum dose, where mice infected with a low number of spirochetes develop mild arthritis, but severe arthritis is observed at higher inoculum dose (46). The range in arthritis severity between B6 and C3H mice is not due to a failure of one strain to adequately manage the infection, as both strains harbor similar numbers of joint spirochetes, suggesting that the type of host immune response, the magnitude of this response, and/or the regulation of this response is genetically regulated (48).

The Host Response to *B. burgdorferi*

Cells of both the innate and adaptive immune system play critical roles in the control of *B. burgdorferi* infection, although it should be noted that the host immune system alone is incapable of sterile clearance of these spirochetes (50-52). During the early stages of *B. burgdorferi* infection, multiple cells of the innate immune system are activated, including macrophages, neutrophils, and NK cells (53-56). The early and robust immune response to *B. burgdorferi* is thought to be due, in part, to the abundant lipoproteins found on the surface of these spirochetes, which are recognized by Toll like receptor 2 (TLR2) (57-59). The *B. burgdorferi* genome encodes approximately 150 putative lipoproteins (34). Recognition of these lipoproteins by cells of the innate immune system ultimately results in the activation of NF-κB, and the production of both pro- and anti-inflammatory cytokines thought to modulate the development of Lyme arthritis (53, 57, 60). Mice lacking TLR2 harbor 10-fold more bacteria in their joints than do WT mice, and mice lacking the common TLR adapter molecule MyD88 harbor 100-fold more bacteria than their WT littermates (61, 62). This indicates that there are MyD88 dependent pathways that participate in immune clearance of *B. burgdorferi* that

are independent of TLR2. Interestingly, arthritis severity in TLR2$^{-/-}$ mice, on both the C3H and B6 backgrounds is greater than in WT mice of the same strain (61, 63, 64). In addition, because arthritis development was not halted in the absence of TLR-2, additional pathways must exist that may be involved in bacterial recognition. This will be further discussed below.

It is still largely unknown how innate immune cells kill *B. burgdorferi in vivo*, however, multiple cell types, particularly those of myeloid lineage, including microglia, have been reported to kill *B. burgdorferi* in vitro (65-67). Macrophages but not neutrophils are capable of phagocytosing *B. burgdorferi* in the absence of opsonizing antibody *in vitro* (56, 68). This may emphasize an important early role played by macrophages in host defense. Interestingly, killing of *B. burgdorferi* by human PMN *in vitro* is more efficient when the neutrophils are lysed (69). Calprotectin, a protein found abundantly within these lysates was found to account for a portion of this activity. Interestingly, both historic and contemporary finding have demonstrated the anti-microbial properties of host histone molecules as well as neutrophil extracellular traps (NET), which may account for the remainder of this killing activity (70-72). It is widely held that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are bactericidal. However, the viability of *B. burgdorferi* is not altered when subjected to hydrogen peroxide and other oxidative stresses (73). Additionally, *B. burgdorferi*–infected mice lacking either iNOS, or essential components of the NADPH oxidase pathway are not impaired in their ability to control infection (74-76). This is partially explained by the fact that *B. burgdorferi* does not use free iron or iron-bound enzymes, which are sensitive to reactive oxygen species (77).

The adaptive immune response, particularly the B cell response is critical in the management of *B. burgdorferi* infection. The fact that arthritis develops in the absence of T and B lymphocytes in C3H mice has often overshadowed the observation that arthritis fails to resolve without these cells (78-80). Mice that lack B and T cells, or are deficient in antibody production have elevated levels of *B. burgdorferi* in their tissues, and this defect can be ameliorated by passive transfer of human immune sera and B cells (81-83). Interestingly, *B. burgdorferi* and their lipoproteins act as potent B cell mitogens, that stimulate polyclonal activation of these cells through TLR2-dependent and independent pathways (60, 84). A consequence of this polyclonal proliferation of B cells in infected individuals is the development of lymphoadenopathy (swollen lymph nodes). Only a fraction of the antibody produced by these proliferating B cells is antigen specific, and this may be a mechanism used by the bacteria to confound its host (84, 85).

T cell Polarity in Lyme Arthritis

Early histopathological studies from human Lyme disease patients proposed that T cells were directly involved in tissue damage that results in arthritis (6). These findings prompted the examination of T cell polarity, particularly the T_H1 polarized T cell subset, as the culprits that lead to Lyme arthritis development (86). Indeed, a study examining arthritis development in the susceptible C3H strain injected with neutralizing antibody to IFN-γ (a pro-inflammatory T_H1-associated cytokine) showed that ankle swelling was reduced following this treatment (87). Another set of studies, seeking to show that arthritis could be exacerbated in the absence of IL-4 (a cytokine that restricts development of T_H1 T cells), demonstrated that arthritis was exacerbated in the normally

arthritis-resistant BALB/c strain, and also showed that addition of exogenous IL-4 resulted in reduced arthritis severity in C3H mice (88, 89). However, subsequent studies using gene-targeted knockout mice for IFN-γ (on the C3H background), IL-4, and IL-4/IL-13 receptor on the DBA (arthritis resistant) B6, or BALB/c background did not confirm these studies (90, 91). One explanation for the discrepancy in the study utilizing recombinant IL-4 in C3H mice is that the authors examined resolution, rather than development of Lyme arthritis. A second, and perhaps more telling, reason for the discrepancy between all of these experiments is that the former experiments, utilizing neutralizing antibody and recombinant proteins failed to examine the histopathological evidence of arthritis. The edema associated with Lyme arthritis is responsible for increased ankle swelling, but contains a sparse inflammatory infiltrate, and may or may not be an indicator of joint inflammation (92, 93). A final study utilizing C3H *scid* mice (these mice have no B or T cells) authoritatively demonstrated that these mice develop severe arthritis, despite the lack of T cells (78). Because arthritis develops in the absence of T_H1 polarity, the role of these polarized $CD4^+$ T cells, as well as the cytokine, IFN-γ remains elusive in mice and little can be extrapolated to human disease. The apparent lack of T cell involvement in the C3H model of Lyme arthritis appears contradictory to what has been reported in human patients, and reflects another possible weakness of the murine Lyme arthritis model. This issue will further be discussed in Chapter 2.

Development of Inflammatory Lyme Arthritis

The development of Lyme arthritis is preceded by spirochetal invasion of the joint, and this event is thought to be required for arthritis development (33, 94). In mice,

invasion begins at 7 days of infection, and at 14 days of infection, bacterial numbers in the joint reach their highest levels (47). In the arthritis susceptible C3H mice, but not in the arthritis-resistant B6 mice, the PMN and monocyte recruiting chemokines, CXCL-1 and CCL-2 were found to be at their highest levels 4 days prior to, and at the peak of arthritis severity (95). By histopathological examination of infected joint tissue, the 2-week time point is also when a myeloid infiltrate, consisting of primarily of granulocytes, but also includes monocytes and macrophages, is detected, and is more prominent in C3H mice than in B6 mice (46, 47). C3H mice lacking the chemokine receptor, CXCR2 (receptor for KC), but not mice deficient for CCR2 (receptor for MCP-1) developed reduced arthritis severity (95, 96). This suggests that prior to arthritis development, *B. burgdorferi* induce expression of these chemokines in local joint cells, which ultimately results in arthritis development in C3H mice. The fact that both the arthritis susceptible (C3H) and arthritis resistant (B6) strains harbor similar numbers of spirochetes in their joint tissue, and therefore similar stimulation potential for the production of the above chemokines, suggests arthritogenic mechanisms are genetically controlled (48). Indeed genetic linkage analysis of B6 and C3H intercross populations revealed the presence of multiple quantitative trait loci (QTL), two of which reciprocally transfer the severe arthritis phenotype, located on mouse chromosomes 5 and 11 (75, 93, 97).

In an effort to understand the mechanism(s) underlying the development of Lyme arthritis, microarray analyses were performed on infected joint tissue from C3H and B6 mice at 1, 2, and 4 weeks of infection with *B. burgdorferi* (98). A striking feature of this experiment was that, at the earliest time point after infection in the arthritis susceptible C3H mouse, there was evidence of a robust interferon-inducible response that was absent

in the arthritis-resistant B6 mice (98). Because this interferon-induced response was correlated to severe arthritis development in C3H mice, and others had reported that C3H IFN-$\gamma^{-/-}$ mice retain the severe arthritis phenotype, it was hypothesized that type I interferon may have a role in promoting Lyme arthritis development in C3H mice (90, 98). Indeed, C3H mice injected with antibody that prevents signaling through the Type I interferon receptor had reduced ankle swelling and overall lesion development compared to C3H mice receiving control treatments (99). Interestingly, a dissection of the interferon-induced response in the joints of infected C3H mice demonstrated that Type I interferon was only partially responsible for the induction of interferon-responsive genes. In fact, the most robustly induced transcripts appeared to be due to NK cell-derived IFN-γ (99). To circumvent this complicating issue, bone marrow derived macrophages (BMDM), which do not produce IFN-γ, from multiple gene knockout mouse strains were utilized to identify the pathways that induce Type I interferon production. It was found that induction of Type I interferon was independent of TLR2, TLR4, TLR9, and MyD88, but was dependent on feedback through the Type I interferon receptor (99). Similar findings in human peripheral blood mononuclear cells (PBMC's) suggested that *B. burgdorferi* have the capacity to enact this pathway in human Lyme disease (100). Later findings in BMDM revealed this feed-forward loop to be dependent on IRF-3, and additionally identified *B. burgdorferi* RNA as a ligand that is capable of inducing a Type I interferon response (101). Finally, two independent groups demonstrated that production of both IFN-α and IFN-β in human mononuclear cells was reduced when cells were pretreated with inhibitors of TLR-7, TLR-8, and TLR-9, receptors that recognize microbial RNA and CpG DNA (102-104). Petzke *et al*. also demonstrated that

plasmacytoid dendritic cells (pDC) produced Type I interferon in response to *B. burgdorferi* stimulation, an observation not observed in purified murine pDC's (99, 102). Interestingly, in the heterogeneous PBMC population, expression of Type I interferon was relatively modest when compared to the robust induction of IFN-γ (100, 102). This observation indicates that the induction of IFN-γ may overshadow the contribution of Type I interferon in the human interferon response.

In addition to the robust interferon response observed by microarray at 1 week of infection, both strains of mice displayed hallmark transcripts of the immune response at 2 weeks of infection, characteristic of immune cell infiltration, as well as the including chemokines in both the arthritis-susceptible and arthritis-resistant strain (98). At both 2 and 4 weeks of infection, during the peak of arthritis severity in C3H mice, hallmark transcripts previously associated with the murine collagen induced arthritis model were apparent (98, 105). Of these genes, matrix-metalloproteinases (MMP) were of particular interest, as they are thought to be the cause cartilage and bone destruction. MMP-3 was induced in both strains of mice, while MMP-9 was seen only in C3H mice at 2 weeks of infection. C3H mice deficient in MMP-9 display reduced arthritis severity, but unchanged bacterial numbers (106). Interestingly, MMP-3, but not MMP-9 was predominant in the synovial fluids of patients with acute Lyme arthritis, while MMP-9 was more prominent in synovial fluids of antibiotic-refractory Lyme arthritis patients (107).

Expression and Function of Interleukin-10

Interleukin-10 (IL-10) is a nonredundant, pleiotropic, anti-inflammatory cytokine, whose primary effects are on macrophages and monocytes (108-110). Although IL-10 was initially shown to act on T cells and NK cells, the majority of these mechanisms have shown to be indirect (108). IL-10 enacts its suppressive activity by inducing the release of soluble receptors for some pro-inflammatory cytokines, reducing expression of MHC class II and co-stimulatory molecules, inducing instability of pro-inflammatory cytokine and chemokine mRNA, and induces expression of TIMP (Tissue inhibitor of matrix metalloproteinase) (108-114). Multiple cell types, including $CD4^+$ and $CD8^+$ T cell subsets, dendritic cells, macrophages, B cells, and keratinocytes have been reported as sources of this cytokine (115). IL-10 was originally found to be a TH2-associated cytokine, but it is now understood that all known $CD4^+$ T cell subsets are capable of producing this cytokine (116). Production of IL-10 by T_H1, T_H2, and T_H17 subsets require the identical signals needed for differentiation and the activation of ERK, which is thought to come from IL-21 and/or IL-27. Additionally, T_H1, T_H2, and T_H17 cells require activation of STAT4, STAT6, and STAT3, respectively, for optimal IL-10 expression (116). The signals that induce IL-10 expression in regulatory T cells are largely unknown. In myeloid lineage cells, induction of IL-10 utilizes TLR signaling through MyD88 and/or TRIF, which activate signaling molecules such as ERK and p38. This ultimately allows transcription factors such as CREB, SP1, SP3, C/EBP and NF-κB to associate with enhancer sequences in the IL-10 promoter (117-122). Interestingly, T cells and myeloid-lineage cells each have a unique DNase I hypersensitivity site (HSS) within the IL-10 locus. For T cells, this site is several KB downstream of the start site,

while the myeloid HSS is several KB upstream of the promoter region (116). Thus, the mechanisms required for IL-10 expression differs greatly between cell types, and may reflect differences in both the duration and magnitude of the IL-10 response.

Interleukin 10 in Lyme Arthritis

An initial, and intriguing observation made about IL-10 was that BMDM derived from arthritis resistant B6 mice stimulated with *B. burgdorferi* lipoproteins produced much higher levels of IL-10 than macrophages derived from arthritis-susceptible C3H mice (61, 123). Inversely, C3H macrophages produced higher concentrations of the pro-inflammatory cytokines IL-6 and TNF-α. Further, B6 IL-$10^{-/-}$ mice infected with *B. burgdorferi* harbored 5-10 fold fewer spirochetes in their joint tissue, but developed more severe arthritis than WT mice (123). Microarray analyses of infected joint tissue from B6 IL-$10^{-/-}$ mice revealed a delayed (compared to C3H), but sustained induction of interferon-inducible transcripts that was correlated to an increase in arthritis severity (98). Although the initial comparison of B6 IL-$10^{-/-}$ mice and C3H mice was performed in the context of genes that were highly induced in C3H mice, further scrutiny revealed unique and/or robust induction of cytokines and chemokines (98). Finally, the observation that IL-10 overexpression failed to reduce arthritis severity in C3H mice led to the hypothesis that arthritis in the IL-$10^{-/-}$ mouse developed by a distinct mechanism from that observed for C3H mice (124).

Preview of Thesis Research

This dissertation is based heavily on two observations made by microarray analyses of *B. burgdorferi*-infected joint tissue. The first observation is that, in the absence of IL-10, there is a robust, and sustained induction of interferon-inducible genes, which is correlated to an increase in arthritis severity in B6 IL-$10^{-/-}$ mice. The second observation is that IFN-γ is robustly expressed at both 2 and 4 weeks of infection. The experiments described herein were designed with goal to 1: characterized the source of the anti-arthritic IL-10, and 2: test the hypothesis that sustained IFN-γ expression was the driving force for arthritis development in IL-$10^{-/-}$ mice.

Prior to this study, the function of IFN-γ in Lyme arthritis development was largely unknown, as mouse models failed to see phenotypes related to the absence of this cytokine. Over the course of these studies, a group with access to patients with Post-Treatment Lyme Disease published an important paper that reported elevated levels of IFN-γ and extremely high levels of the chemokines, CXCL-9 and CXCL-10 in the synovial fluid of patients with antibiotic-refractory, but not antibiotic-responsive, patients (20). Of the known mouse models of Lyme arthritis, only the IL-$10^{-/-}$ mouse has a similar phenotype. Thus, the IL-$10^{-/-}$ mouse model may provide an opportunity to elucidate the role of IFN-γ in Lyme arthritis in humans. A second set of publications from groups not studying Lyme disease identified loss of function alleles in the *il10r1* gene (125, 126). Although this is not thought to be the cause of severe disease seen in some Lyme arthritis patients, the IL-$10^{-/-}$ mouse phenotype is relevant to human disease, and may also provide insight into the balance between insufficient and overwhelming inflammatory responses.

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CHAPTER 2

LOCALIZED PRODUCTION OF IL-10 SUPPRESSES EARLY INFLAMMATORY CELL INFILTRATION AND SUBSEQUENT DEVELOPMENT OF IFN-γ-MEDIATED LYME ARTHRITIS

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Abstract

IL-10 is a nonredundant inflammatory modulator that suppresses the development of arthritis in *Borrelia burgdorferi* infected mice. Infected IL-$10^{-/-}$ mice were previously found to have a robust and sustained interferon-inducible response in joint tissue, and elevated IFN-γ in sera. Infection of IL-10 reporter mice demonstrated that macrophages and $CD4^{+}$ T cells were the primary sources of IL-10 in the infected joint tissue, which suggested that early local production of IL-10 dampened the pro-arthritic interferon response. In fact, treatment of IL-$10^{-/-}$ mice with anti-IFN-γ reduced the increase in arthritis severity and suppressed interferon-inducible transcripts to wild type levels, thereby linking dysregulation of IFN-γ to disease in the IL-$10^{-/-}$ mouse. Arthritis in IL-10 deficient mice was associated with elevated numbers of NK cell, NKT cell, α/β T cell, and macrophage infiltration of the infected joint. FACS lineage sorting revealed NK cells and $CD4^{+}$ T cells as sources of IFN-γ in the joint tissue of IL-$10^{-/-}$ mice. These findings suggest the presence of a positive feedback loop in the joint tissue of infected IL-$10^{-/-}$ mice, where production of inflammatory chemokines, infiltration of IFN-γ producing cells, and additional production of inflammatory cytokines result in arthritis. This mechanism of arthritis is in contrast to that seen in C3H mice, where arthritis development is linked to transient production of Type I interferon, and develops independently of IFN-γ. Due to the sustained interferon response driven by NK cells and T cells, we propose the IL-$10^{-/-}$ mouse as a potential model to study the prolonged symptoms observed in some human Lyme disease patients.

Introduction

Lyme disease is the manifestation of one or more lesions that result following infection with the spirochete *Borrelia burgdorferi* (1), which is transmitted to humans and animals through the bite of infected *Ixodes* ticks (2-4). These bacteria establish infections of skin, cardiac, nervous, and/or connective tissue of the joint (5), and can cause disease at these sites (4). Lyme arthritis occurs in up to 60% of infected humans not treated early with antibiotics, and may develop months after the initial infection (6). This arthritis is characterized by swelling, edema and a moderate inflammatory infiltrate that consists primarily of granulocytes (7). These symptoms and lesions can be recurrent if untreated (6-8). Most human patients with Lyme arthritis respond to antibiotic therapy, after which the arthritis eventually resolves (5). However, in some cases arthritis persists after treatment designed to eradicate infection suggesting that there is a subset of individuals that maintain a long-term inflammatory response in the absence of active infection (5, 9).

The acute, infection-associated arthritis seen in humans has been modeled using inbred mice, in particular, the C3H/HeN (C3H) and C57BL/6 (B6) strains. In mice infected intradermally, *B. burgdorferi* reach maximal numbers in the joint tissue at 2 weeks post infection, and arthritis severity peaks at 4 weeks after infection (10-12). Genetic susceptibility to arthritis development is clearly illustrated in inbred mice, and is independent of spirochete numbers in the joint (11, 13, 14). In C3H mice, arthritis is severe, with robust infiltration of neutrophils along with the accumulation of edema in the tibiotarsal joint, joint space, and contiguous supportive tissues, as well as proliferation of synoviocytes of the tibiotarsal tendon sheath (10, 13). These lesions have also been

observed in B6 mice, but the severity of disease is greatly decreased in comparison (11, 13). Severe arthritis development in C3H mice has not consistently been linked to skewing of T cell responses, such as IFN-γ-producing T_H1 cells (15-17), or a lack of T_H2 responses (18, 19), and in fact, arthritis in C3H *scid* mice is equivalent in severity to wildtype (WT)[3] C3H mice (20). Rather than a T cell driven disease, arthritis in C3H mice has been linked to the production of Type I interferon (21), MMP-9 (22), and chemokines that signal through *CXCR2* (23), and is also regulated through the CD14 pathway (24).

Much insight has been gained from the C3H mouse models of Lyme arthritis, as these mice have a robust phenotype that accurately mimics many symptoms associated with Lyme borreliosis in humans. However, the C3H mouse does not completely model the spectrum of pathology observed in human Lyme arthritis (6). The apparent lack of T cell involvement in this mouse model appears contradictory to what is reported in some human patients (25), where both T_H1 (26) and γ/δ (27, 28) T cells have been implicated. Indeed, this holds true for a select group of Lyme arthritis patients who experience persistent arthritis after appropriate antibiotic therapy. It has been hypothesized that these long-lasting symptoms are due to one or more of the following: 1) autoimmunity, 2) persistent undetectable levels of infection, 3) persistence of bacterial antigens, or 4) dysregulated inflammatory responses (1). Recent findings from Shin *et al*. demonstrated a possible dysregulated inflammatory response in patients with antibiotic-refractory arthritis, as synovial fluid from these individuals contained elevated concentrations of the cytokines IL-1β, IL-6, IFN-γ, and others, as well as extremely high concentrations of the chemokines *CXCL-9* and *CXCL-10* (9).

The C57BL/6 (B6) mouse model is a useful tool in the study of Lyme arthritis, as it is resistant to the development of severe disease. Multiple gene-targeted knockouts are available on this background, and thus, the B6 model provides an opportunity to explore specific deficiencies of acute and chronic inflammatory responses and mediators that may exacerbate Lyme arthritis (29-35). IL-10 deficiency in B6 mice is one such knockout model, and this deficiency results in increased arthritis severity, even with 5-10 fold fewer spirochetes in the joint tissues of infected mice (36). Infection of IL-10-/- mice with a related spirochete, *Borrelia turicatae* also leads to increased disease severity but enhanced control of the pathogen (37). Production of pro-inflammatory cytokines by murine immune cells stimulated with *B. burgdorferi* is regulated by IL-10 (38), and in fact, previous microarray analyses of infected joint tissue from B6 IL-$10^{-/-}$ mice revealed a sustained induction of the cytokines IL-1β, IL-6, and IFN-γ and chemokines *CXCL-9*, and *CXCL-10* (39). All of these pro-inflammatory transcripts showed no signs of resolution, as they all had increased expression at 4 weeks of infection compared to earlier measurements. In fact, the most highly induced gene in IL-$10^{-/-}$ mice, *CXCL-9*, displayed >500 and >800 fold induction at 2 and 4 weeks of infection, respectively. These findings are strikingly similar to the cytokine and chemokine profiles observed by Shin *et al*., in the synovial fluid of patients with treatment-refractory Lyme arthritis (9).

In the current study, we have extended our analysis of the B6 IL-$10^{-/-}$ mouse as a Lyme arthritis model resulting from a dysregulated inflammatory response to low levels of joint spirochetes. We identify the pro-inflammatory cytokine IFN-γ as a primary target of IL-10 in the joint tissue of the normally arthritis resistant B6 mice, and determine that macrophages and $CD4^+$ T cells in infected joint tissue are the primary

sources of this regulatory cytokine. Furthermore, we identify both NK cells and $CD4^+$ T cells as the primary sources of IFN-γ in the infected joints of IL-$10^{-/-}$ mice. We propose that disruptions of the delicate balance between pro and anti-inflammatory signaling in both innate and adaptive immunity result in unique patterns of lesions during *B. burgdorferi* infection of joint tissue. Furthermore, the IL-$10^{-/-}$ mouse provides an opportunity to dissect inflammatory events that are normally highly regulated, and result in pathological development when this balance is disrupted.

Materials and Methods

Mice, infections, and assessment of arthritis severity

C57BL/6 (B6), B6.129P2-*IL-10tm1Cgn*/J (IL-$10^{-/-}$), (B6.129S6-*Il10*^{tm1Flv}/J (*tiger),* mice were purchased from The Jackson Laboratories. B6-LY5.2/Cr ($Ly5.1^+$) congenic mice were purchased from NCI. All mice were housed in the University of Utah Animal Research Center (Salt Lake City, UT) and strict adherence to institutional guidelines were followed. To avoid colitis development, IL-$10^{-/-}$ mice were kept on antibiotic water (trimethoprim and sulfamethoxazole) until one day prior to infection. Mice were infected with 2×10^4 bacteria of the clonal *Borrelia burgdorferi* strain N40 as described (39). Ankle measurements were obtained using a metric caliper before and at 4 weeks of infection. Joint tissue was prepared for assessment of histopathology as described (21). Ankle measurements and histopathology were assessed in a blinded fashion. Infection was confirmed in mice sacrificed prior to 14 days of infection by culturing bladder tissue in BSK II media containing 6% rabbit serum, phosphamycin, and rifampicin. ELISA quantification of *B. burgdorferi*-specific IgM and IgG concentrations were used to

confirm infection in mice euthanized at and after 14 days of infection as described (11, 34).

Preparation of single cell suspensions from mouse tissue

Single cell suspensions were prepared from rear ankle joint tissue as described previously (21), with a few modifications. After excision, joints were placed into Petri dishes containing 3 ml digestion buffer. For flow cytometry experiments, the buffer consisted of 1mg/ml collagenase A (Roche), 10mM NaN_3, and 5% NCS in HBSS. For experiments where joint cells were sorted for transcriptional analysis, the buffer consisted of endotoxin-free Liberase TM (Roche) at 0.2 mg/ml in RPMI 1640. Collagenase digestions were incubated for 2 hours at 37° C, while Liberase digestions were incubated for 1 hour at 37° C. After incubation, digestion reactions were filtered through a 100 μm cell strainer, centrifuged, and the red blood cells lysed. Single cell suspensions of lymph nodes were prepared by smashing lymph nodes through a 100μm cell strainer into Petri dishes containing flow cytometry buffer. Blood was obtained by cheek puncture, and collected in eppendorf tubes containing acid citrate dextrose (ACD). Blood leukocytes were isolated as described (40).

Flow cytometry

All flow cytometry data were analyzed using BD CellQuest Pro software. Sorting experiments were performed using a BD FACSAria II. All other FACS data were collected on a BD FACS Canto II flow cytometer. 7'AAD (eBioscience) was used in all experiments (excluding samples stained for FoxP3), and dead cells were excluded from

analyses, as were doublet cells. At least 10^5 viable, nondoublet events were acquired for each sample. All antibodies used for flow cytometry were purchased from either BioLegend or eBioscience. Unconjugated F_c blocking antibody (clone 93 eBioscience) was included in all antibody-labeling experiments. Fluorochrome-conjugated antibodies used in this study were as follows: FITC conjugated α-CD4 (RM4-4), α-CD8α (53-6.7), α-B220 (RA3-6B2), α-F4/80 (BM8), α-TCR-β (H57-597); PE conjugated α-CD3ε (145-2C11), α-NK1.1 (PK136), α-NKp46 (29A1.4), α-Ly6C (HK1.4), α-F4/80 (BM8), α-FoxP3 (FJK-16s); PE-Cy-7 conjugated α-CD4 (GK1.5), α-CD11b (M1/70), α-TCR-β (H57-597); APC conjugated α-CD3ε (145-2c11), α-CD11c (N418), αCD45.1/Ly5.1 (A20), α-F4/80 (BM8), α-TCR-β (H57-597); Alexa Fluor 700 conjugated α-CD8α (53-6.7), α-Ly6G/Ly6C (RB6-8C5); Pacific Blue conjugated α-CD45.2 (104), α-B220 (RA3-6B2), α-TCR-β (H57-597).

Injection of monoclonal antibodies

Antibodies used in neutralization or depletion studies were purchased from Bio X Cell, and were aggregate and endotoxin free, and sterile. Antibodies used were as follows: α-IFN-γ (XMG1.2), α-CD4 (GK1.5), and α-NK1.1 (PK-136). Isotype control antibodies used were: Rat IgG1 (HPRN), Rat IgG2b (LTF-2) termed isotype 2, and Mouse IgG2a (C1.18) termed isotype 1. All antibodies were delivered by intra peritoneal injection. For neutralization of IFN-γ, mice were initially injected with 1mg α-IFN-γ or isotype control antibody one day prior to infection. Additional doses of 0.5mg α-IFN-γ or isotype control were administered every 4-5 days. For depletion of $CD4^+$ T cells, mice were injected with 200μg α-CD4, or isotype control antibody, one day prior to, and the

day of infection. Depletion was maintained with additional 200μg injections every 5 days. Depletion efficiency was determined to be >95% at time of sacrifice in both the spleen and blood. For depletion of NK cells, mice were injected with 250μg α-NK1.1, or isotype control antibody one day prior to, and the day of infection. Depletion efficiency was 80-90% in the spleen and blood.

Isolation of RNA and quantitative RT-PCR

For all experiments examining gene expression in joint tissue, mice were killed, and the skin was removed from the tibiotarsal joints. Ankle joints were excised, placed in eppendorf tubes, and frozen immediately in dry ice/ethanol, and stored at -80°C. Isolation of total RNA from joint tissue was performed by acid guanidine extraction as described (41). 5μg joint RNA was reverse-transcribed as described (21). Primers sequences used in this study were: *CXCL-10* Forward (5'-GAA ATC ATC CCT GCG AGC CTA TCC-3'), Reverse (5'-GCA ATT AGG ACT AGC CAT CCA CTG GG-3', *F4/80* Forward (5'-TGG GAA AGA CTG GAT TCT GGG-3'), Reverse (5'-GGA GCC ATT CAA GAC AAA GCC-3'); *CD4* Forward (5'-CAA GAA GCA GAG TGA AGG AAG GAC-3'), Reverse (5'-CAG CAG CAG CAG CAA GCG-3'); *CD49b* Forward (5'-ATA AGG AGT GTG GCA GCG ATG G-3'), Reverse (5'-CCC CTC TGT TTT TCA GGA TGA CTG-3'); *IL-10CD* (does not recognize sterile transcript produced by IL-$10^{-/-}$ mouse) Forward (5'-GCT CTT ACT GAC TGG CAT GA-3'), Reverse (5'-TTC CGA TAA GGC TTG GCA AC-3'. Primer sequences for *β–actin* (39), *CXCL-9* (21), *IGTP* (39), *IIGP* (39), and *Vα14-Jα18* iTCR (42) used in this study can be found with their respective citations. All RT-PCR experiments were conducted on a Roche LC-480.

Isolation of DNA and quantification of joint spirochetes

For quantification of joint spirochetes at 4 weeks post infection, total DNA was isolated as described (12). Quantitative PCR was performed on a Roche LC-480. Spirochete quantification was performed by amplification of the *B. burgdorferi recA* gene, followed by normalization to the mouse *nidogen* gene.

Generation of radiation chimeras

IL-$10^{-/-}$ mice 5 weeks of age were lethally irradiated with 2 doses of 525 cGy 3 hours apart using a GE Isovolt Titan. Twenty-four hours post irradiation; splenocytes were harvested from donor mice (either IL-$10^{-/-}$ $Ly5.2^{+}$, or WT congenic B6 mice that were $Ly5.1^{+}$). Irradiated mice were then injected *i.v.* with 2×10^{7} splenocytes in a 200µl volume. Chimerism was determined at 2, 4, 6, and 14 weeks after irradiation by flow cytometry analysis of blood leukocytes. After full reconstitution, B cells and myeloid lineage cells were found to be >90% donor-derived, while T cells were approximately 60% donor-derived. For a summary of reconstitution efficiency see (Appendix A).

ELISA analysis of serum IFN-γ

Blood was obtained from mice by submandibular puncture at the time of euthanasia. Blood was allowed to clot, centrifuged, and serum was collected, and stored at -20°C prior to analysis. IFN-γ concentration in serum samples were detected by sandwich ELISA using clone R46A2 as the capture antibody, and biotinylated antibody (XMG1.2) for detection.

Data and statistical analyses

All graphical data represent the mean ± SEM. Statistical analysis was performed using Prism 5.0c software. Unless otherwise indicated, data were analyzed by one-way ANOVA with the Bonferoni post-hoc test for pair-wise comparisons. Categorical data for histopathology was assessed by the Mann Whitney U test. Statistical significance is indicated by (*), (**), or (***) with p values of < 0.05, < 0.01, and < 0.001 respectively.

Results

Localized production of arthritis-modulating IL-10 in joint tissue is dependent on infiltrating leukocytes

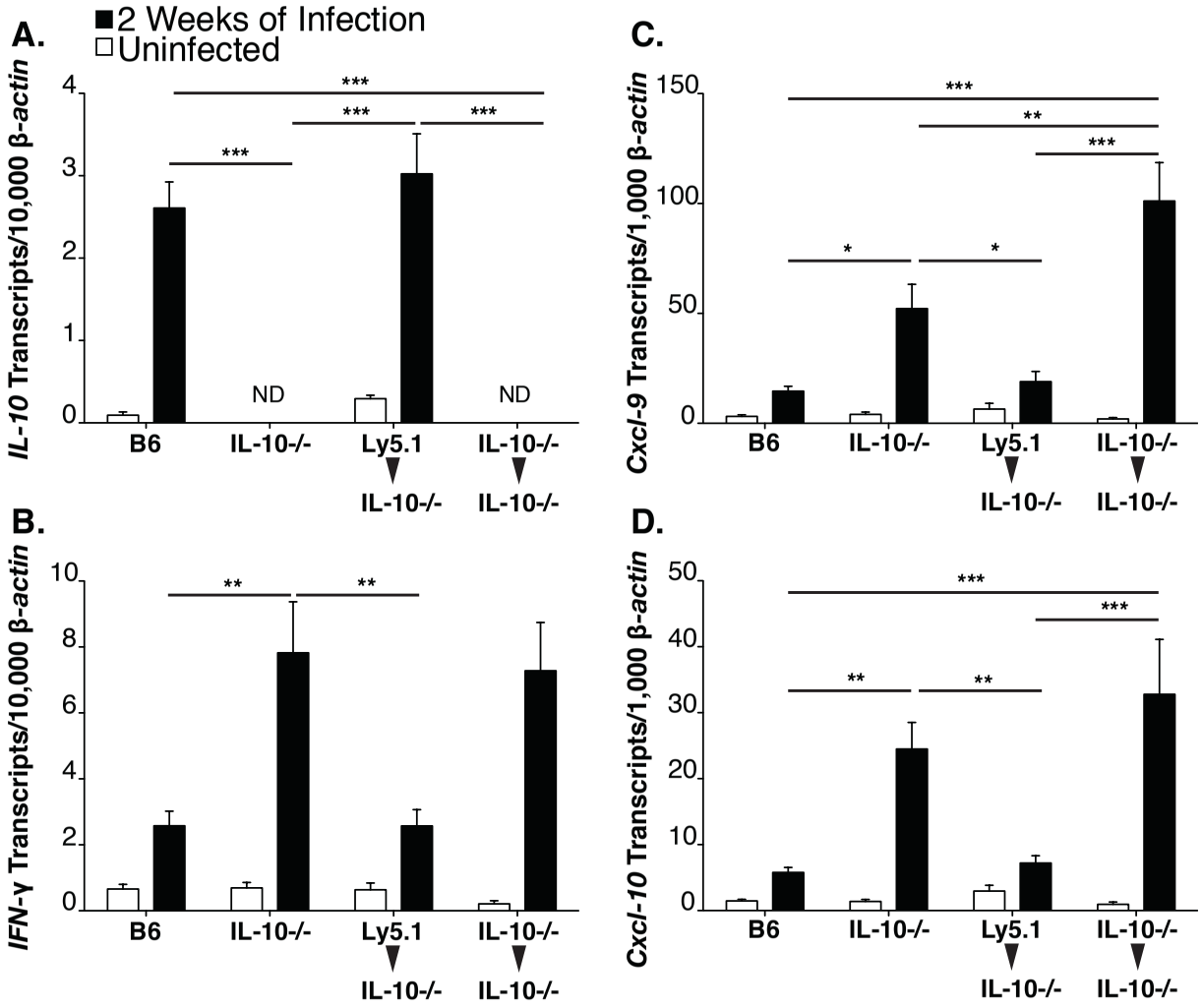
Previous publications with IL-$10^{-/-}$ mice have established a unique role for IL-10 in modulating the inflammatory response to *B. burgdorferi*, restricting the severity of arthritis while permitting bacterial survival within joint tissue (36, 43). Microarray analyses of infected joint tissue further revealed a sustained induction of transcripts for IFN-γ and interferon-inducible genes in B6 IL-$10^{-/-}$ mice, but not WT B6 mice, beginning 2 weeks following infection (39). These findings suggested that suppression of arthritis in B6 mice was dependent on a tissue-localized source of IL-10, possibly acting to suppress local production of IFNγ. As arthritis in B6 mice is more severe in the absence of IL-10, we sought to determine the cellular source(s) of this anti-inflammatory cytokine. For the initial characterization, IL-$10^{-/-}$ mice were lethally irradiated, and served as recipients for Ly5.1^{+} WT B6 splenocytes, or autologous Ly5.2^{+} IL-$10^{-/-}$ splenocytes in a protocol designed for complete reconstitution of both hematopoietic lineage cells and hematopoietic stem cells. Mice were infected 12 weeks after

irradiation/transplantation and sacrificed 2 weeks later, whereupon IL-10, IFN-γ, and interferon-inducible gene expression were assessed by RT-PCR. Importantly, expression of IL-10 in the joint tissue of Ly5.1→IL-$10^{-/-}$ chimeric mice was equivalent to IL-10 expression in joint tissue of un-irradiated, infected, WT B6 mice (Figure 2.1A), whereas IL-10 transcripts were not detectable in IL-$10^{-/-}$ mice reconstituted with splenocytes from IL-$10^{-/-}$ mice. Evidence that active IL-10 protein was produced in the joint tissue of Ly5.1→IL-$10^{-/-}$ chimeras is provided by the resulting suppressed expression of *IFN-γ* and the interferon responsive transcripts *CXCL-9* and *CXCL*-10 to levels found in the joint tissue of infected wild type B6 mice (Figure 2.1B, C, D). Thus, leukocyte derived IL-10 is sufficient to suppress the interferon-inducible response in the joint tissue of *Borrelia burgdorferi* infected B6 mice.

Both $CD4^+$ T cells and macrophages are sources of IL-10 in the B. burgdorferi-infected joint

To identify the IL-10-producing cell type(s) infiltrating joint tissues during *B. burgdorferi* infection, an IL-10 reporter mouse in which an IRES-GFP cassette was knocked into the 3' UTR of the IL-10 gene was utilized. The availability of this mouse, designated "*tiger*", on the B6 background allowed direct assessment of the cell types responsible for IL-10 production in response to *B. burgdorferi* by flow cytometry of cells recovered from localized tissue (44). Joint tissue and inguinal lymph nodes were collected from uninfected control mice or from mice at day 14 of infection, the time previously associated with up-regulated levels of IL-10 in WT B6 mice (39). Single cell suspensions were prepared from inguinal lymph nodes and from joint tissue disrupted by

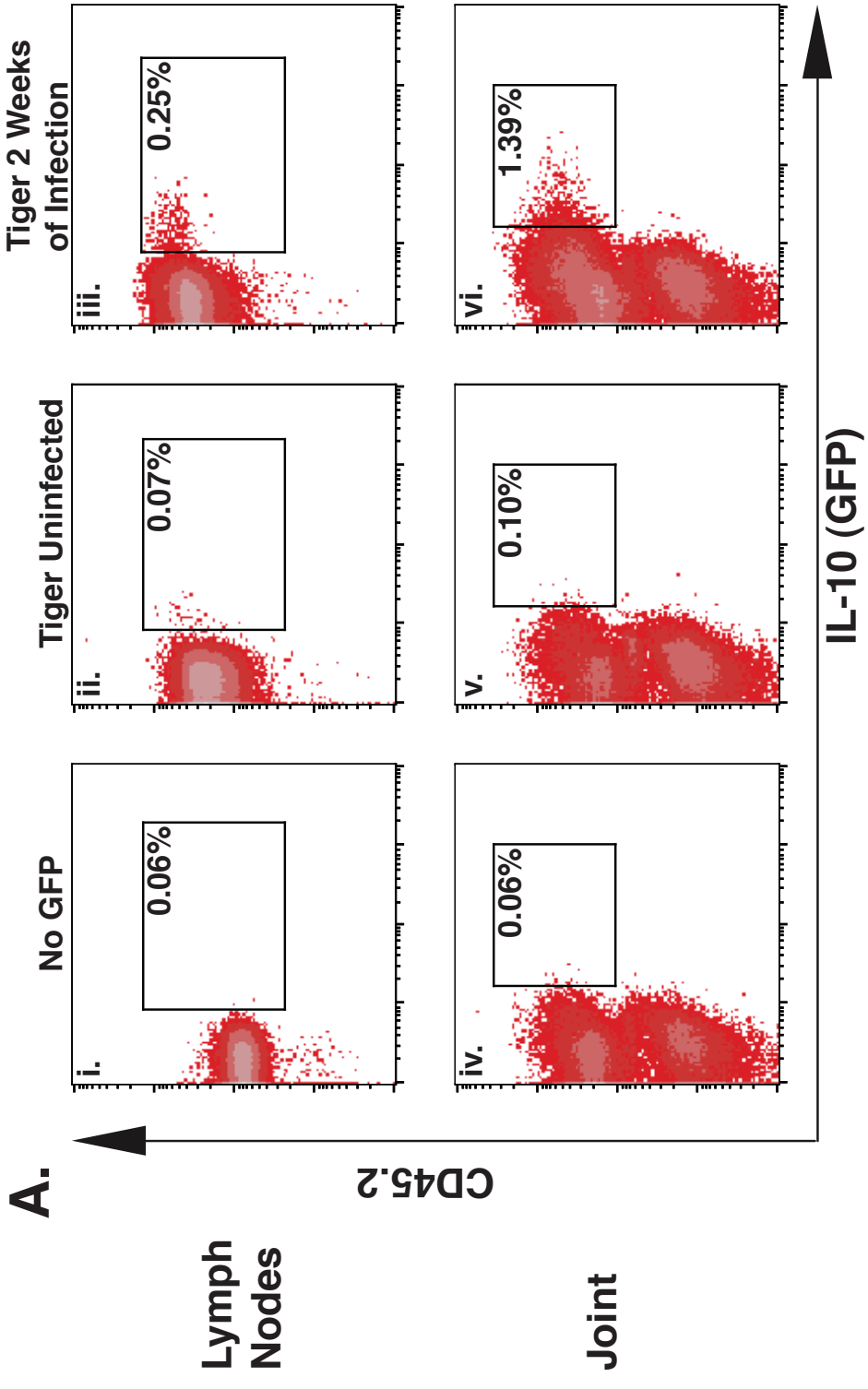
Figure 2.1. *A hematopoietic source of IL-10 regulates expression of interferon-inducible transcripts*. RT-PCR analysis of joint tissue at 2 weeks after infection with *B. burgdorferi* from WT B6 (N=8), B6 IL-10-/- (N=8), WT LY5.1→IL-$10^{-/-}$ chimeras (N=8), and IL-$10^{-/-}$→IL-$10^{-/-}$ chimeras (N=6). Expression of *IL-10* (A), *IFN-γ* (B), *CXCL-9* (C), and *CXCL-10* (D), was normalized to *β-actin*. The term 'ND' indicates that IL-10 transcripts were not detected (A). Statistical analysis was performed as described in Materials and Methods.

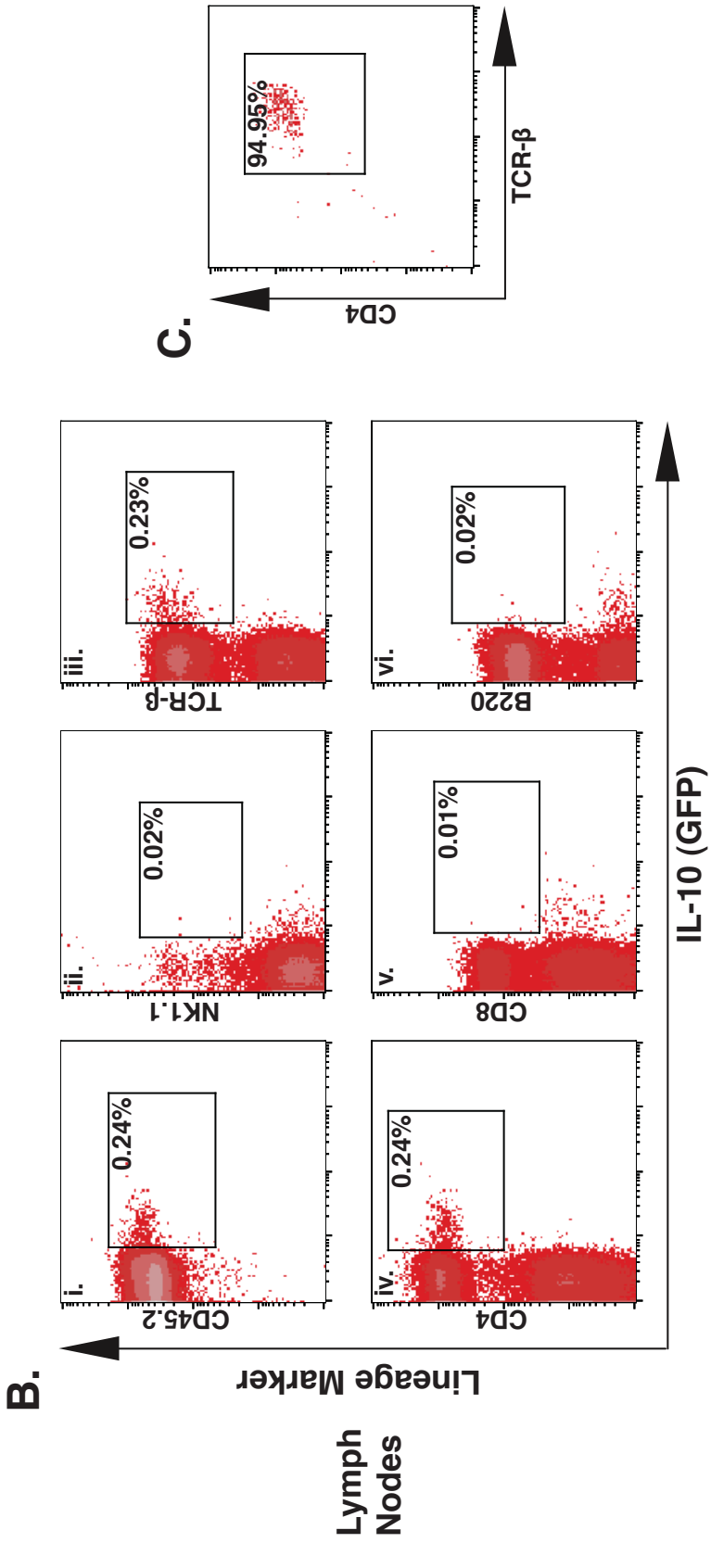

A.
2 Weeks of Infection
Uninfected
IL-10 Transcripts/10,000 β-actin
ND
ND
B6
IL-10-/-
Ly5.1
IL-10-/-
IL-10-/-
IL-10-/-
B.
IFN-γ Transcripts/10,000 β-actin
C.
Cxcl-9 Transcripts/1,000 β-actin
D.
Cxcl-10 Transcripts/1,000 β-actin

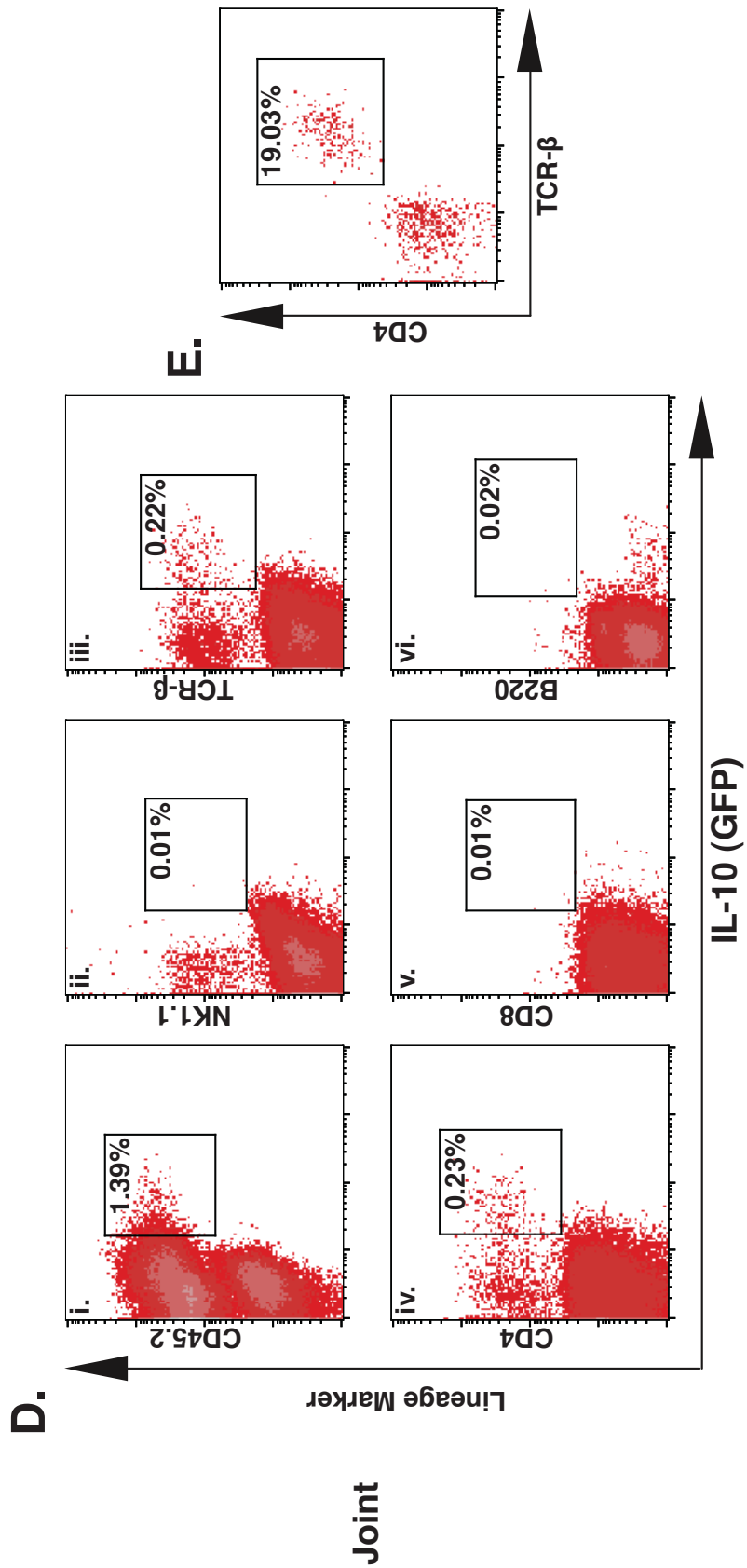
enzymatic digestion, as described in Materials and Methods, and stained with lineage markers. Lymph node and joint cells from uninfected *tiger* mice or control mice showed little to no GFP^+ cells in either the $CD45^+$ or $CD45^-$ fraction (Figure 2.2A, i-ii, iv-v), consistent with the observation that IL-10 expression is extremely low in uninfected mice (Figure 2.1A). However, initial analysis of both tissues from infected *tiger* mice revealed a small population of IL-10 $(GFP)^+$ cells, all of which also expressed the common leukocyte antigen, CD45.2, (Figure 2.2A, iii, vi). The observation that all IL-10 $(GFP)^+$ cells were also $CD45.2^+$, is consistent with data obtained with radiation chimeras, which concluded that anti-inflammatory IL-10 is leukocyte-derived (Figure 2.1A). Further characterization of the IL-10 $(GFP)^+$ cells in the draining lymph nodes of infected *tiger* mice revealed them to also stain as TCR-β^+ and $CD4^+$ (Figure 2.2B, iii, iv) with very few GFP^+ cells staining positive for NK1.1, CD8α, or B220 (Figure 2.2B, ii, v, vi). When total IL-10 $(GFP)^+$ lymph node cells were gated, >90% of these cells were double positive for CD4 and TCR-β (Figure 2.2C), demonstrating that $CD4^+$ T cells are the primary source of IL-10 in the inguinal lymph nodes of *B. burgdorferi* infected mice.

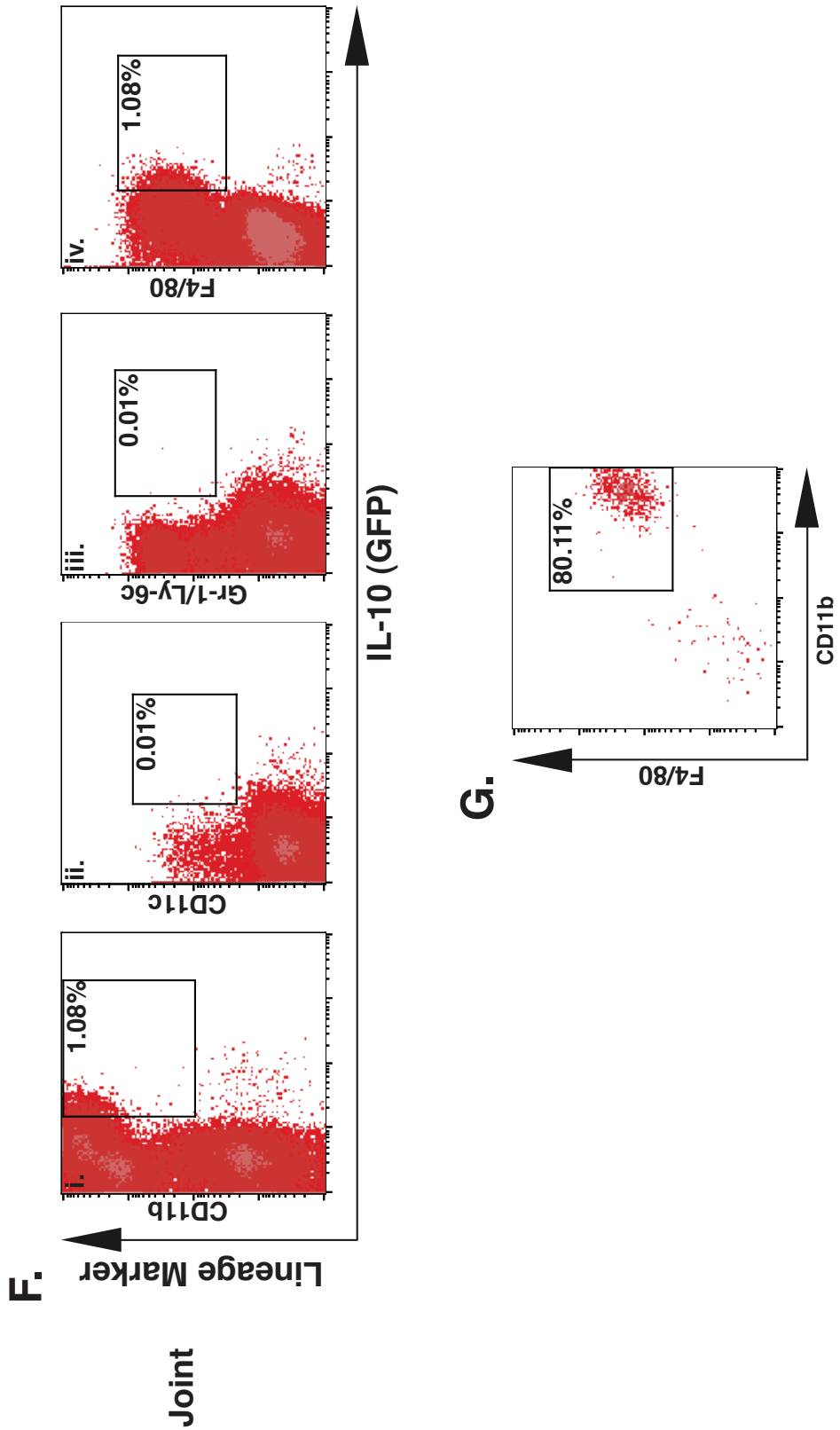
To identify the specific lineages of the $CD45^+$, IL-10 expressing cells in the joint tissue at 2 weeks of infection, single cell suspensions were prepared from infected joint tissue, and stained with the same lineage markers used to analyze lymph node cells. As previously indicated, 1.39% of joint cells were IL-10 $(GFP)^+$ (Figure 2.1A, vi). Further analysis of joint cell lineages identified populations of GFP^+ cells that also stained positive for TCR-β and CD4, at a frequency of 0.22% and 0.23% respectively (Figure 2.2D, iii, iv). As was seen in the infected lymph nodes, IL-10 $(GFP)^+$ cells had little to no

Figure 2.2. *IL-10 expression in vivo is found in both CD4$^+$ T cells and macrophages*. Flow cytometry data in A-E utilized primarily lymphoid markers to designate populations, while date from F-G represent a separate myeloid analysis. Gate boundaries for GFP and lineage markers were established by isotype control staining in cells derived from non-*tiger* mice. Data from A-iii, vi, and B-G were acquired from a single infected *tiger* mouse at 2 weeks after infection. Analysis of three infected *tiger* mice yielded similar results. IL-10 (GFP) expression in CD45$^+$ cells from infected tiger mice, in both the lymph nodes (A-iii) and joint tissue (A-vi). IL-10 (GFP) expression in the tissues of non-tiger or uninfected tiger mice (Ai, ii, iv, v). Analysis of lineage-specific surface markers that were present on IL-10 (GFP)$^+$ cells in infected lymph nodes (B). Frequency of CD4$^+$, TCR-β$^+$ T cells in the total IL-10 (GFP)$^+$ cells in lymph node tissue (C). Analysis of lymphocyte lineage-specific surface markers that were present on IL-10 (GFP)$^+$ cells in infected joint tissue (D). Frequency of CD4$^+$, TCR-β$^+$ T cells in the total IL-10 (GFP)$^+$ cells in infected joint tissue (E). Analysis of myeloid lineage-specific surface markers that were present on IL-10 (GFP)$^+$ cells in infected joint tissue (F). Frequency of CD11b$^+$, F4/80$^+$ macrophages in the total IL-10 (GFP)$^+$ cells in infected joint tissue (G).


A.
No GFP
Tiger Uninfected
Tiger 2 Weeks of Infection
Lymph Nodes
Joint
CD45.2
IL-10 (GFP)
i.
ii.
iii.
iv.
v.
vi.
0.06%
0.07%
0.25%
0.06%
0.10%
1.39%


B.
Lymph Nodes
Lineage Marker
IL-10 (GFP)
i.
CD45.2
0.24%
ii.
NK1.1
0.02%
iii.
TCR-β
0.23%
iv.
CD4
0.24%
v.
CD8
0.01%
vi.
B220
0.02%
C.
CD4
TCR-β
94.95%


D.
Joint
Lineage Marker
IL-10 (GFP)
i.
CD45.2
1.39%
ii.
NK1.1
0.01%
iii.
TCR-β
0.22%
iv.
CD4
0.23%
v.
CD8
0.01%
vi.
B220
0.02%
Ei
CD4
TCR-β
19.03%

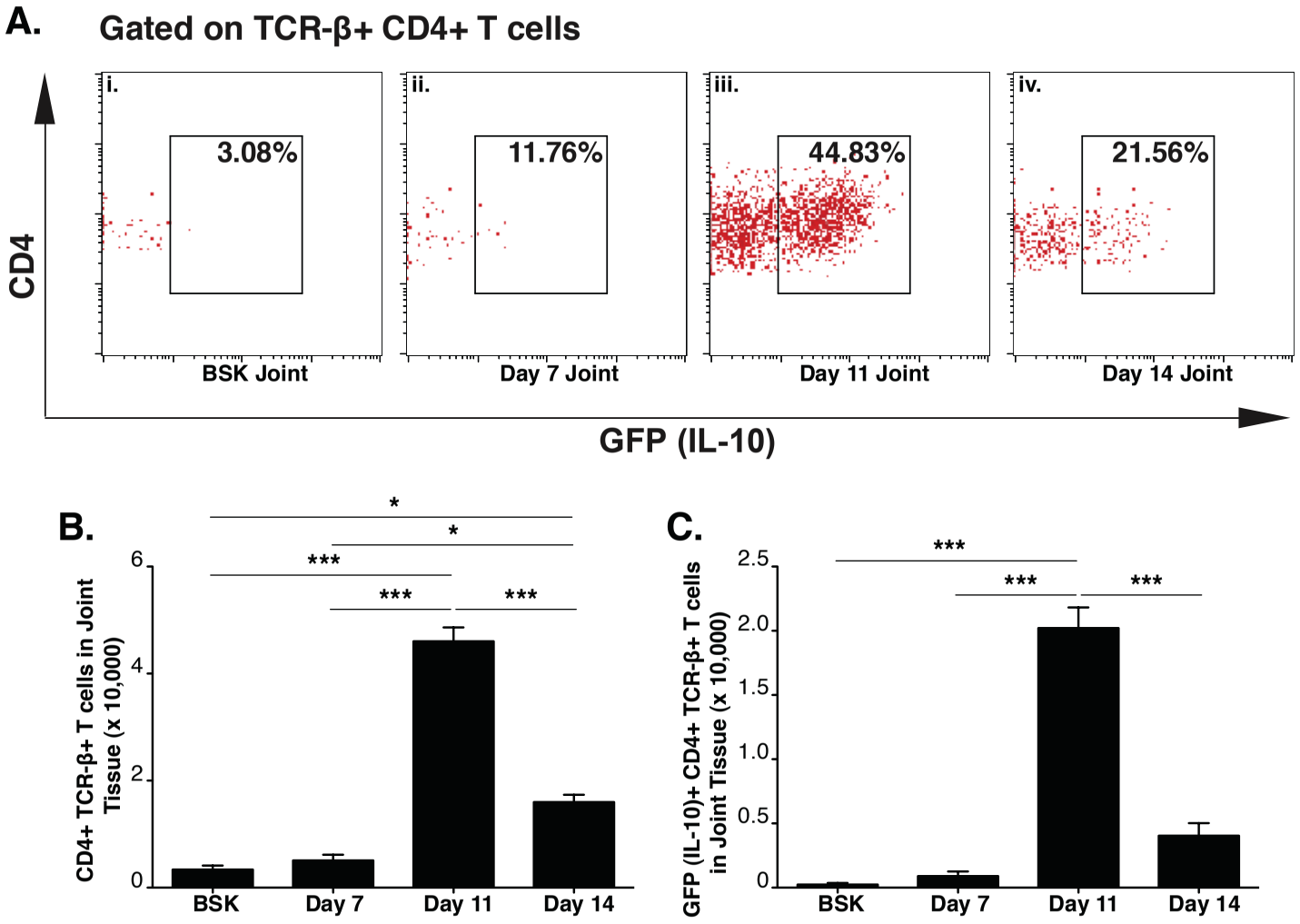

F.
Joint
Lineage Marker
CD11b
i.
1.08%
CD11c
ii.
0.01%
Gr-1/Ly-6c
iii.
0.01%
F4/80
iv.
1.08%
IL-10 (GFP)
G.
F4/80
80.11%
CD11b

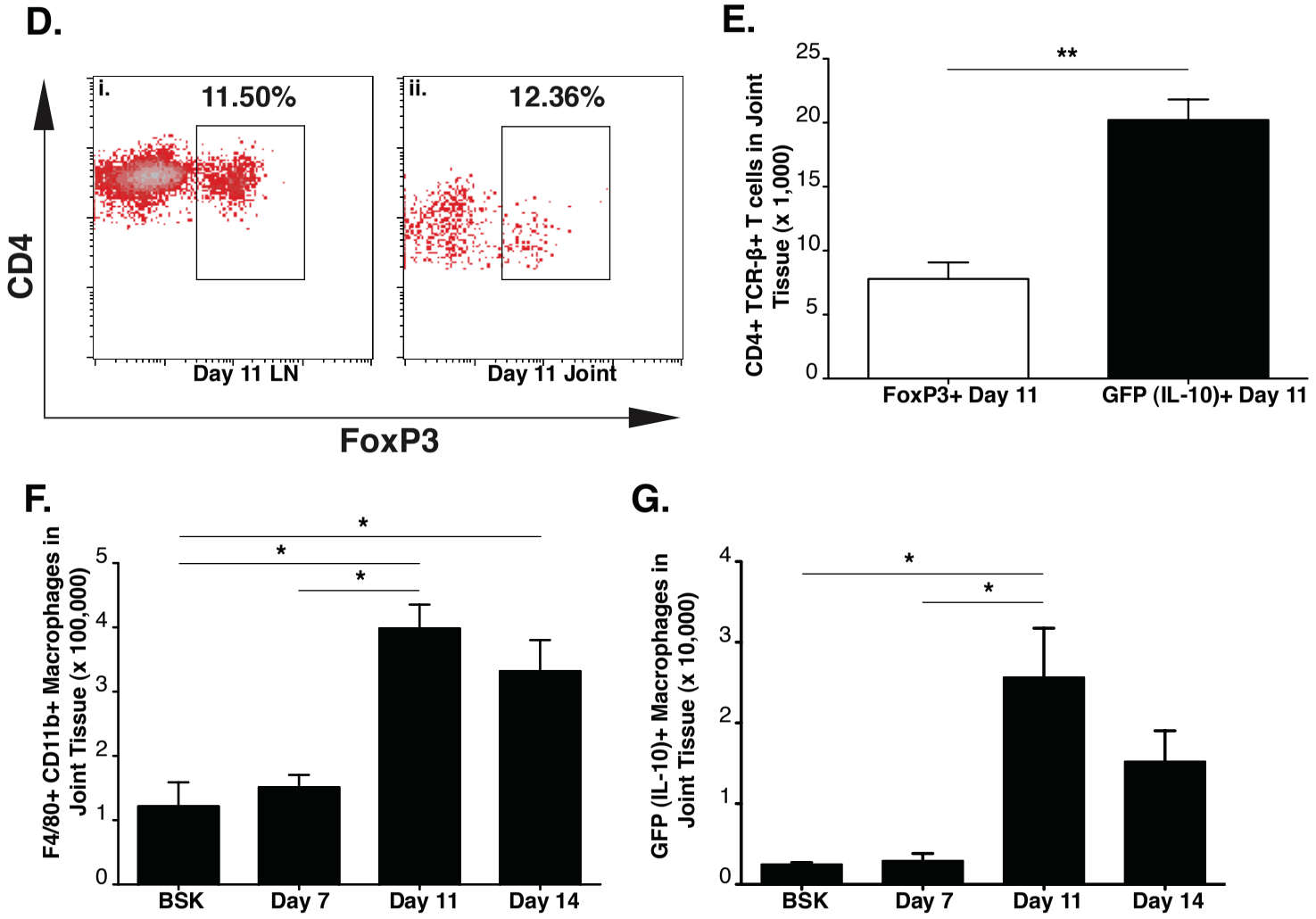
staining for NK1.1, CD8α, or B220 (Figure 2D, ii, v, vi). When total IL-10 (GFP)$^+$ joint cells were gated, 19% stained double positive for TCR-β and CD4 (Figure 2E), demonstrating that CD4$^+$ T cells are significant source of IL-10 in the infected joint. Because CD4$^+$ T cells accounted for one fifth of the total IL-10 expressing cells, a second non-T cell, non-lymphocyte source of IL-10 in the infected joint was suspected. Additional analysis of joint cells from infected *tiger* mice revealed that IL-10 (GFP$^+$) cells stained positive for CD11b and for the macrophage marker F4/80, both at a frequency of 1.08% (Figure 2.2F, i, iv), indicating a myeloid source of IL-10. Very few of the GFP$^+$ cells were positive for CD11c or Gr-1/Ly6c (Figure 2.2F, ii, iii), thus neither dendritic cells nor granulocytes are major sources of IL-10 in the joint. When total IL-10 (GFP)$^+$ cells were gated, 80% stained double positive for CD11b and F4/80, identifying these cells as macrophages (Figure 2.2G), and accounting for the non-T cell source of IL-10 in the infected joint. Together these data indicate that CD4$^+$ T cells are the primary source of IL-10 in the draining lymph nodes of infected B6 mice, while both tissue macrophages and CD4$^+$ T cells produce IL-10 in the joint tissue of *B. burgdorferi* infected mice.

Kinetic analysis of IL-10 expression in joint tissue

The duration and magnitude of IL-10 expression in response to *B. burgdorferi* infection in the joint tissue was further assessed in *tiger* mice by flow cytometry, at 7, 11, and 14 days of expression. CD4$^+$ T cells were quite rare in joint tissue of uninfected animals and at day 7 after infection (Figure 2.3A-i, ii, and 3B). A robust infiltrate of CD4$^+$ T cells was observed, that peaked at day 11 of infection, with 45% of the CD4$^+$ T

Figure 2.3. *Kinetic analysis of IL-10 expressing cells in tiger mice*. Control and infected tiger mice (N=3 per group) were analyzed for IL-10 (GFP) producing $CD4^+$ T cells and macrophages in joint tissue. Flow cytometry analysis of IL-10 expression in joint $CD4^+$ TCR-β^+ T cells from uninfected mice (A-i), or mice at day 7 after infection (A-ii), day 11 after infection (A-iii), and day 14 post infection (A-iv). Total joint $CD4^+$ TCR-β^+ T cells (B) and total IL-10 $(GFP)^+$ $CD4^+$ TCR-β^+ T cells (C). Frequency of $CD4^+$ TCR-β^+ $FoxP3^+$ T_{reg} cells in lymph nodes and joint tissue at day 11 after infection (D). Comparison of total joint IL-10 $(GFP)^+$ $CD4^+$ TCR-β^+ T cells and total joint $CD4^+$ TCR-β^+ $FoxP3^+$ T_{reg} cells in joint tissue at day 11 after infection (E). Total $CD11b^+$, $F4/80^+$ macrophages (F), and IL-10 $(GFP)^+$ $CD11b^+$, $F4/80^+$ macrophages (G) in joint tissue. Statistical analyses were performed as reported in Materials and Methods. Statistical significance in (E) was determined using student's T test.


A.
Gated on TCR-β+ CD4+ T cells
i.
ii.
iii.
iv.
3.08%
11.76%
44.83%
21.56%
CD4
BSK Joint
Day 7 Joint
Day 11 Joint
Day 14 Joint
GFP (IL-10)
B.
CD4+ TCR-β+ T cells in Joint Tissue (x 10,000)
0
2
4
6
*
*
***
***
***
BSK
Day 7
Day 11
Day 14
C.
GFP (IL-10)+ CD4+ TCR-β+ T cells in Joint Tissue (x 10,000)
0
0.5
1.0
1.5
2.0
2.5
***
***
***
BSK
Day 7
Day 11
Day 14


D.
i.
11.50%
ii.
12.36%
CD4
Day 11 LN
Day 11 Joint
FoxP3
E.
CD4+ TCR-β+ T cells in Joint Tissue (x 1,000)
25
20
15
10
5
0
**
FoxP3+ Day 11
GFP (IL-10)+ Day 11
F.
F4/80+ CD11b+ Macrophages in Joint Tissue (x 100,000)
5
4
3
2
1
0
*
*
*
BSK
Day 7
Day 11
Day 14
G.
GFP (IL-10)+ Macrophages in Joint Tissue (x 10,000)
4
3
2
1
0
*
*
BSK
Day 7
Day 11
Day 14

cells expressing IL-10 (GFP), (Figure 2.3A iii) and 3B). By day 14 after infection the percentage and total number of infiltrating $CD4^+$ T cells expressing IL-10 (GFP) had dropped to 21% (Figure 2.3A, iv, 3C). To determine whether regulatory T cells (T_{reg}) were responsible for this IL-10 response, joint cells from day 11 *tiger* mice were also analyzed for the T_{reg} definitive transcription factor, FoxP3. Because, concurrent analysis of IL-10 (GFP) and FoxP3 was not possible due to loss of cytoplasmic GFP from cells treated to allow intranuclear FoxP3 staining, the distributions of GFP^+ and $FoxP3^+$ T cells were assessed. In the day 11 lymph nodes, 12% of the $CD4^+$ T cells were $FoxP3^+$, consistent with other reports that T_{reg} cells account for ~10% of $CD4^+$ T cell population. Only 1-2% of the $CD4^+$ T cells in lymph nodes were IL-10 $(GFP)^+$ (data not shown). In joint tissue at day 11 after infection, only 12% of the $CD4^+$ T cells displayed intracellular staining for FoxP3 (Figure 2.3D-ii), while 45% of the total infiltrating $CD4^+$T cells expressed IL-10 (GFP) (Figure 2.3A iii). Thus, the number of IL-10 $(GFP)^+$ $CD4^+$ T cells was significantly greater than the number of $FoxP3^+$ CD4 T cells in the joint tissue (Figure 2.3E). This observation suggests that the IL-10 producing $CD4^+$ T cells in the joint consist of both T_{reg} and non-T_{reg} cells. As in previous experiments, $CD11b^+$ $F4/80^+$ macrophages constituted a major portion of cells recovered from the joint tissue after collagenase digestion. The maximal infiltration of total and IL-10 (GFP) expressing macrophages into the joint tissue was at day 11 of infection, and levels remained elevated at day 14 (Figure 2.3F-G). Although both the GFP mean fluorescence intensity (MFI) and frequency of IL-10 (GFP) expression in the total $CD4^+$ T cell population was significantly greater than in the macrophage population at 11 and 14 days of infection (Table 2.1), IL-10 (GFP^+) macrophages in the joint were more plentiful than $CD4^+$ T cells

Table 2.1 Comparison of IL-10 (GFP) contributions between macrophages and CD4⁺ T cells in the joint tissue of *B. burgdorferi* infected '*tiger*' mice.

Cell Type	Day of Infection	GFP⁺ (% of population)[a]	Total GFP⁺ Cells (x 1,000)	GFP MFI[b]
CD4⁺ T cells	11	**44.0±3.1**[**c]	20.2±1.6	**57.3±4.9**[*]
Macrophages	11	6.21±1.1	25.7±6.1	23.9±0.4
CD4⁺ T cells	14	**24.6±4.0**[*]	4.0±1.0	**37.2±2.5**[*]
Macrophages	14	4.66±1.2	**15.2±3.8**[*]	20.95±0.5

[a] Values represent the mean ± S.E.M

[b] Mean Fluorescence Intensity

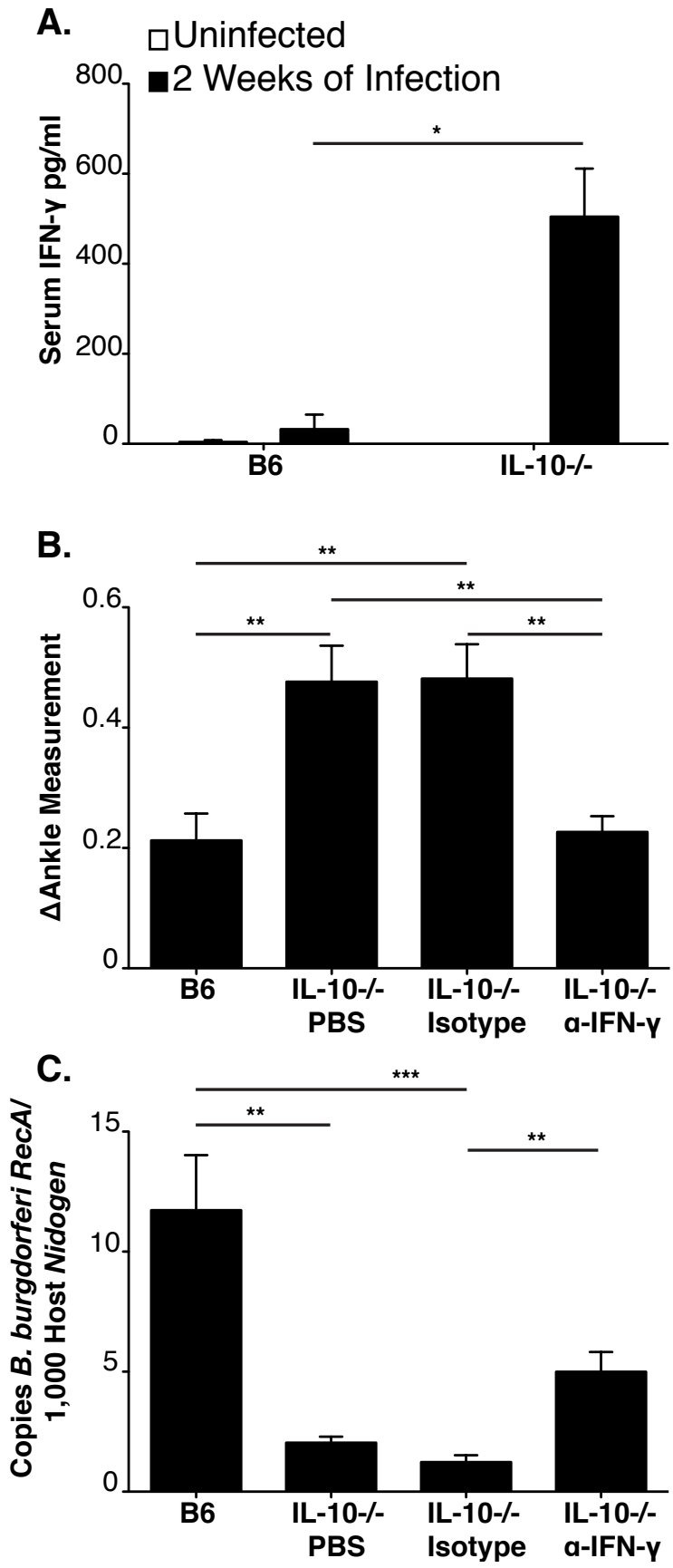
[c] Statistical significance (*) or (**) indicate p values of <0.05 and <0.01 respecively, by student's t-test.

at day 14 after infection (Table I). Thus, on a single cell basis, $CD4^+$ T cells produce more IL-10 than their macrophage counterparts, but as distinct populations, macrophages and CD4+ T cells are comparable in their ability to produce IL-10.

Both systemic and localized production of IFN-γ is associated with increased arthritis severity in IL-$10^{-/-}$ mice

Previous microarray analysis revealed a sustained up-regulation of IFN-γ and interferon-inducible genes in the joint tissue of infected IL-$10^{-/-}$ mice at 2 and 4 weeks after infection (39). Further analysis of serum samples from infected mice revealed a dramatic increase of IFN-γ in infected IL-$10^{-/-}$ mice, relative to modest induction in WT B6 mice, confirming a systemic dysregulated inflammatory response in *B. burgdorferi* infected IL-$10^{-/-}$ mice (Figure 2.4A). Localized and systemic production of IFN-γ was thus hypothesized to be responsible for the increased arthritis severity in IL-$10^{-/-}$ mice infected with *B. burgdorferi*. To directly assess involvement of IFN-γ, *B. burgdorferi* infected B6 IL-$10^{-/-}$ mice were treated with an IFN-γ-neutralizing antibody, an isotype control antibody, or PBS alone, at 5-day intervals throughout the four-week *B. burgdorferi* infection. Arthritis severity at 4 weeks after infection in B6 IL-$10^{-/-}$ mice receiving PBS or control antibody injections was similar, and more severe than WT B6 control mice (Figure 2.4B). However, neutralization of IFN-γ in infected IL-$10^{-/-}$ mice significantly reduced ankle swelling (Figure 2.4B) and histopathology scores (Table II). Interestingly, treatment of infected B6 IL-$10^{-/-}$ mice with the IFN-γ-neutralizing antibody had a modest effect on spirochete numbers in joint tissue relative to control IL-$10^{-/-}$ mice

Figure 2.4. *IFN-γ is responsible for the increased arthritis severity in B6 IL-10$^{-/-}$ mice*. ELISA of serum IFN-γ at 2 weeks of infection in B6 and IL-10$^{-/-}$ mice (N=5 mice per group (A). Arthritis was assessed in WT B6 and IL-10$^{-/-}$ mice at 4 weeks of infection (N≥8 mice per group). B6 IL-10$^{-/-}$ mice were injected with PBS, control antibody, or α-IFN-γ over the course of infection. Ankle swelling was used to assess arthritis severity (B). Quantification of joint spirochetes at 4 weeks of infection, using qPCR (C). Statistical analyses were performed as reported in Materials and Methods.


A.
Uninfected
2 Weeks of Infection
Serum IFN-γ pg/ml
800
600
400
200
0
*
B6
IL-10-/-
B.
ΔAnkle Measurement
0.6
0.4
0.2
0
**
**
**
**
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
C.
Copies B. burgdorferi RecA/ 1,000 Host Nidogen
15
10
5
0
***
**
**
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ

(Figure 2.4C). Thus, the enhanced control of bacterial number in IL-$10^{-/-}$ mice appears to be partially dependent on IFN-γ. We hypothesize that the other functions of IL-10, independent of suppressive effects on IFN-γ, may assist in host defense, as demonstrated by significant effect on mononuclear cell infiltration (addressed in the following sections and Table 2.2). Taken together, these data suggest that IL-10 acts to suppress both arthritis and host defense via its regulatory effects on IFN-γ.

IFN-γ is required for early induction of interferon-responsive transcripts and for chemokines known to perpetuate recruitment of inflammatory cells

The ability of IFN-γ neutralization to suppress the severity of Lyme arthritis in IL-$10^{-/-}$ mice, (Figure 2.4B, Table II), suggested it functioned locally through the induction of inflammatory response and chemokine production in the joint tissue. To characterize the involvement of IFN-γ early in the development of arthritis, prior to the end stage of disease, we assessed the presence of interferon-inducible transcripts at 2 weeks of infection in B6 IL-$10^{-/-}$ mice treated with PBS, isotype control antibody, or IFN-γ-neutralizing antibody. Consistent with previous results, interferon-inducible transcripts, including those for the chemokines *cxcl9* and *cxcl10*, were expressed at low levels in infected WT B6 mice and were highly upregulated in the joint tissue of infected B6 IL-$10^{-/-}$ mice injected with either PBS or control antibody (Figure 2.5A-D). In contrast, IL-$10^{-/-}$ mice treated with IFN-γ-neutralizing antibody displayed a dramatic reduction in expression of these transcripts, similar to that seen in WT B6 mice (Figure 2.5A-D). These results, coupled with those of Figure 2.4B and Table II, implicate IFN-γ in both the up regulation of pro-inflammatory transcripts in the joint tissue beginning at 2 weeks of

Table 2.2 Assessment of Arthritis by Histopathology in B. burgdorferi-infected Joints.[a]

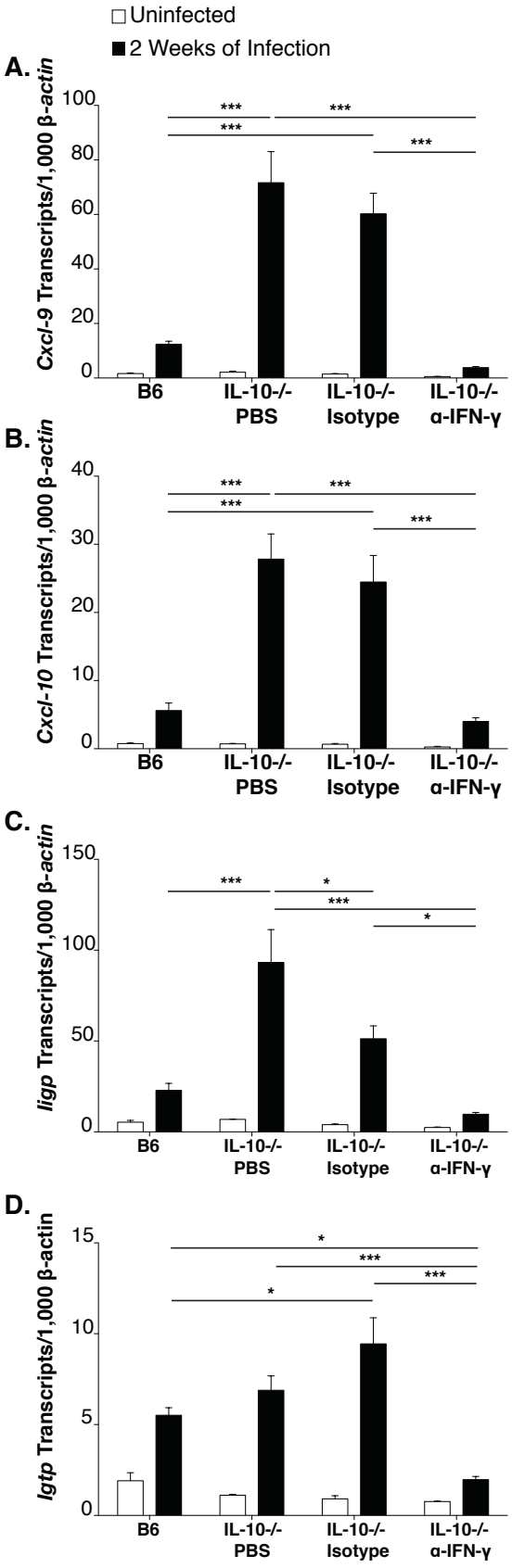
Infected Mouse Strain	Antibody Treatment	Overall Lesion[b]	Neutrophil Infiltration[b]	Mononuclear Infiltration[b]	Sheath Thickening[b]
B6 IL-10$^{-/-}$	PBS (none)	2.88±1.0	2.38±0.46	1.00±0.19	2.38±0.32
B6 IL-10$^{-/-}$	Isotype	2.55±0.77	1.91±0.34	1.27±0.30	2.27±0.24
B6 IL-10$^{-/-}$	α-IFN-γ	**1.50±0.43****	1.17±0.27	**0.33±0.19****	**1.08±0.23****

[a]Assessed at 4 weeks of infection, as described in Materials and Methods

[b]Values represent mean ± S.E.M.

Statistical significance between α-IFN-γ and Isotype is indicated by (**). $p<0.01$ by Mann Whitney U test

Figure 2.5. *IFN-γ is responsible for the robust expression of interferon-inducible transcripts in B6 IL-10$^{-/-}$ mice*. RT-PCR analysis of interferon-inducible transcripts in joint tissue at 14 days of infection in WT B6 and B6 IL-10$^{-/-}$ mice injected with PBS, isotype control antibody, or α-IFN-γ. N≥6 per group. Expression was normalized to *β-actin* and values represent means ± SEM. Expression of interferon-inducible *CXCL-9* (A), *CXCL-10* (B) *Iigp* (C) and *Igtp* (D) transcripts are shown. Statistical analyses were performed as reported in Materials and Methods.

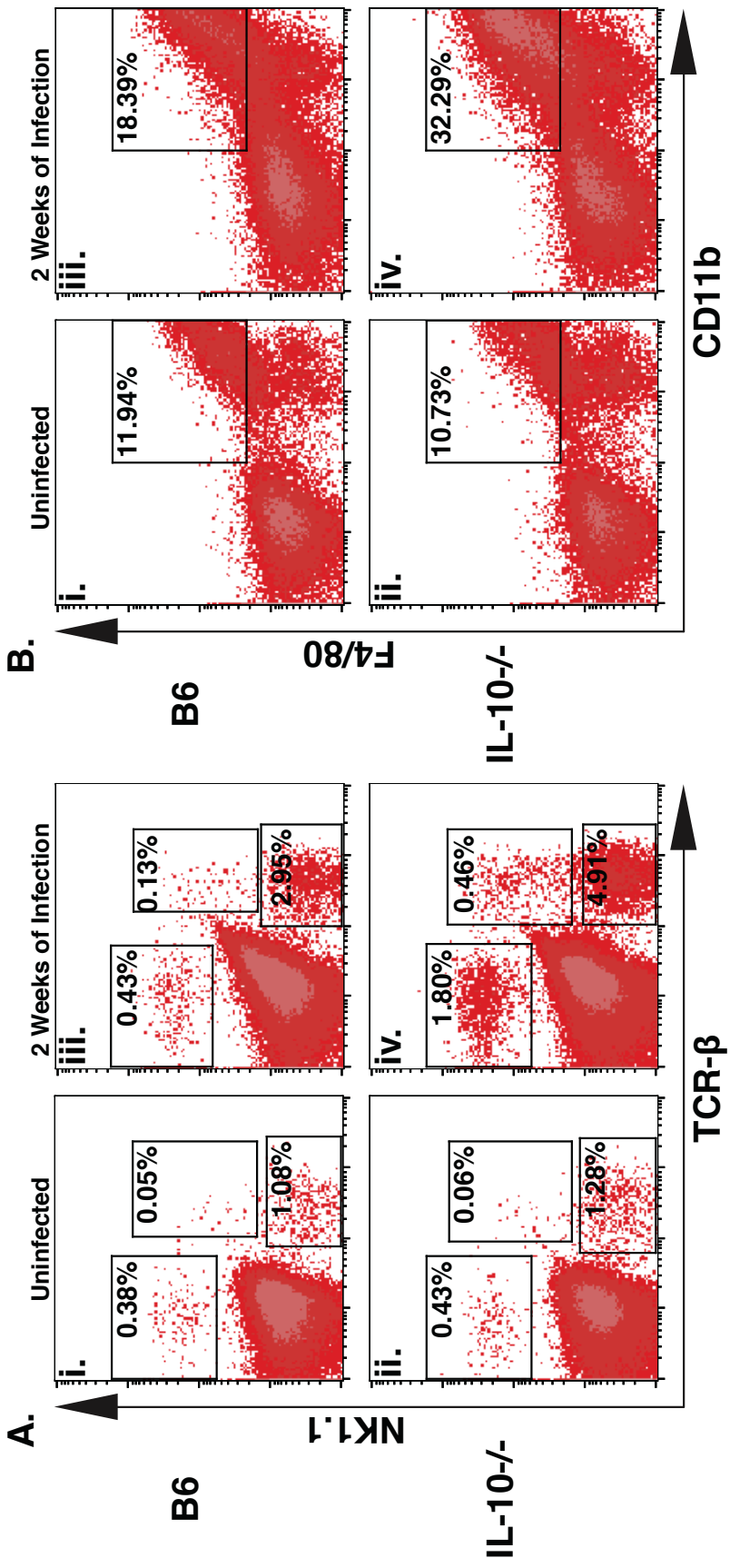

□Uninfected
■2 Weeks of Infection
A.
Cxcl-9 Transcripts/1,000 β-actin
100
80
60
40
20
0
***
***
***
***
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
B.
Cxcl-10 Transcripts/1,000 β-actin
40
30
20
10
0
***
***
***
***
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
C.
Igp Transcripts/1,000 β-actin
150
100
50
0
***
*
***
*
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
D.
Igtp Transcripts/1,000 β-actin
15
10
5
0
*
***
***
*
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ

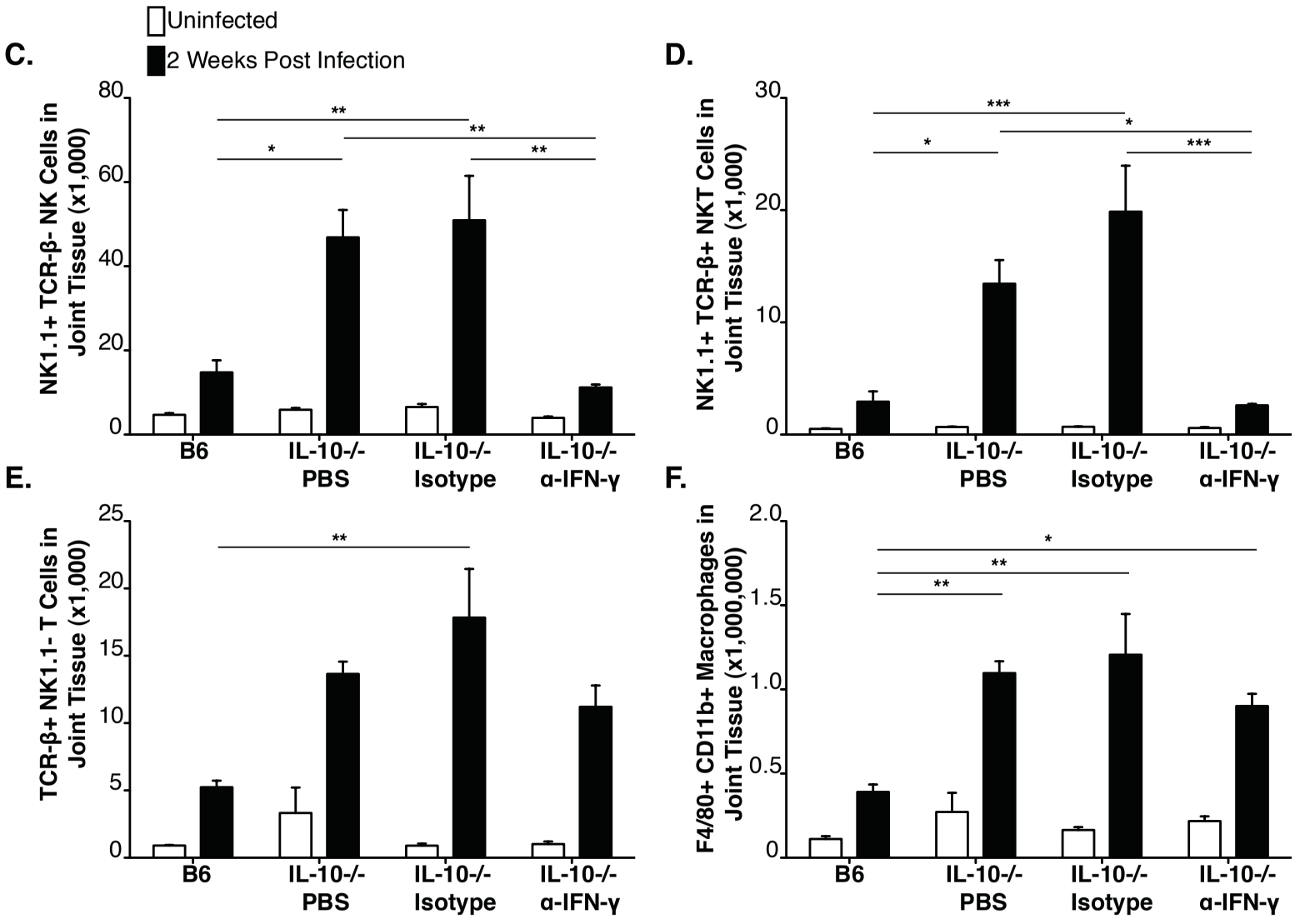
infection and in the increased severity of arthritis seen at 4 weeks of *B. burgdorferi* infection of B6 IL-$10^{-/-}$ mice.

Immune cell infiltration is regulated by IL-10 in infected B6 mice, and is partially dependent on IFN-γ

Histopathology of joint tissue from infected IL-$10^{-/-}$ mice highlighted an IFN-γ-dependent increase in the mononuclear cell infiltrate (Table II). We reasoned that perhaps an early cellular infiltrate into the *B. burgdorferi*-infected joint tissue of IL-$10^{-/-}$ mice should contain potential cellular sources of IFN-γ as well as interferon responsive transcripts. To characterize this cellular infiltrate into the infected joint, single cell suspensions were prepared from joint tissue from WT B6 and IL-$10^{-/-}$ mice at 2 weeks after infection. Increased frequencies of T cells, NK cells, NKT cells, and macrophages were observed into the joint tissue of both WT B6 and B6 IL-$10^{-/-}$ mice, relative to uninfected controls (Figure 2.6A-B) at 2 weeks of infection. Levels of T cells, NK T cells, and macrophages were 2-3 fold greater in joint tissue of infected IL-$10^{-/-}$ mice than in WT B6 mice (Figure 2.6A, iv and 6B, iv). Many of these cell types have been reported to produce IFN-γ during *B. burgdorferi* infection, as well as in other infection models. Because neutralization of IFN-γ in IL-$10^{-/-}$ mice resulted in a decreased mononuclear infiltrate into the joint tissue of IL-$10^{-/-}$ mice (Table II), and this cytokine was also required for the expression of chemokines (*CXCL-9* and *CXCL-10*) known to play a role in the recruitment of NK cells, T cells, and macrophages, it was hypothesized that IFN-γ would be required for the infiltration of one or more of these cells types. To test this hypothesis, B6 IL-$10^{-/-}$ mice were treated with the IFN-γ-neutralizing antibody during a 2-

Figure 2.6. *Ankle infiltration by multiple immune cells is regulated by IL-10 in infected B6 mice, and is partially dependent on IFN-γ.* Flow cytometry analysis of joint single cell suspensions in WT B6 and B6 IL-$10^{-/-}$ mice. Data in A and B were acquired from six pooled joints from 3 B6 and 3 IL-$10^{-/-}$ mice. Frequency of NK cells, NKT cells, α/β T cells in uninfected WT B6 (A-i), uninfected B6 IL-$10^{-/-}$ (A-ii), 2 week infected WT B6 (A-iii) and 2 week infected B6 IL-$10^{-/-}$ (A-iv) ankle joints. Frequency of macrophages in uninfected WT B6 (B-i), uninfected B6 IL-$10^{-/-}$ (B-ii), 2 week infected WT B6 (B-iii), 2 week infected B6 IL-$10^{-/-}$ (B-iv) ankle joints. Infiltration of NK cells (C), NKT cells (D), α/β T cells (E) and macrophages (F) in joint tissue at 14 days of infection in WT B6 and B6 IL-$10^{-/-}$ mice injected with PBS, isotype control antibody, or α-IFN-γ. N≥3 for each group. Statistical analysis was performed as described in Materials and Methods.


A.
B6
IL-10-/-
NK1.1
TCR-β
Uninfected
2 Weeks of Infection
i.
ii.
iii.
iv.
0.38%
0.05%
1.08%
0.43%
0.06%
1.28%
0.43%
0.13%
2.95%
1.80%
0.46%
4.91%
B.
B6
IL-10-/-
F4/80
CD11b
Uninfected
2 Weeks of Infection
i.
ii.
iii.
iv.
11.94%
10.73%
18.39%
32.29%


Uninfected
2 Weeks Post Infection
C.
NK1.1+ TCR-β- NK Cells in Joint Tissue (x1,000)
80
60
40
20
0
**
**
*
**
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
D.
NK1.1+ TCR-β+ NKT Cells in Joint Tissue (x1,000)
30
20
10
0
***
*
*
***
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
E.
TCR-β+ NK1.1- T Cells in Joint Tissue (x1,000)
25
20
15
10
5
0
**
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ
F.
F4/80+ CD11b+ Macrophages in Joint Tissue (x1,000,000)
2.0
1.5
1.0
0.5
0
*
**
**
B6
IL-10-/- PBS
IL-10-/- Isotype
IL-10-/- α-IFN-γ

week *B. burgdorferi* infection. Low levels of infiltrating T cells, NK cells, NKT cells, and macrophages were observed in the infected joint tissue of WT B6 mice at 2 weeks of infection, but high levels of these cells were observed in the infected joint tissue of B6 IL-$10^{-/-}$ mice at the same interval (Figure 2.6C-F). In contrast, B6 IL-$10^{-/-}$ mice treated with the IFN-γ-neutralizing antibody had significantly fewer numbers of infiltrating NK and NKT cells (Figure 2.6C-D), indicating strong dependence on localized effects of IFN-γ. The finding that the number of infiltrating macrophages and T cells in the joint were not reduced by IFN-γ neutralization suggests that recruitment of these cells is not solely dependent on IFN-γ (Figure 2.6E-F), but that their infiltration is regulated by IL-10. This may explain the modest effect of IFN-γ neutralization on enhanced bacterial control in the joint tissue of IL-$10^{-/-}$ mice, which was less dependent on IFN-γ than was arthritis development (Figure 2.4B).

Multiple cell types recruited to the infected joint tissue of IL-$10^{-/-}$ mice produce IFN-γ

Canonical sources of IFN-γ include T_H1 polarized CD4 T cells, NK cells and NKT cells, all of which are found in the joint tissue of *B. burgdorferi* infected IL-$10^{-/-}$ mice and have been previously associated with IFN-γ production during *B. burgdorferi* infection (17, 21, 42, 45-47). Although B cells from the infected lymph node have also been linked to IFN-γ production (48), $B220^+$ cells were not detected in the infected joint tissue of WT B6 or B6 IL-$10^{-/-}$ mice (Figure 2.2D iv, and data not shown). To identify the cell types responsible for IFN-γ production, as well as interferon-inducible gene expression in the joint tissue, IL-$10^{-/-}$ mice were injected with GK1.5, a CD4 T cell-

depleting antibody, or PK136, an NK/NKT cell-depleting antibody, followed by periodic booster injections of depleting antibody during the course of infection with *B. burgdorferi*. Only depletion of $CD4^+$ T cells reduced the presence of IFN-γ in the serum of infected IL-$10^{-/-}$ mice, suggesting these cells are the most important source driving systemic production of IFN-γ (Figure 2.7A). Depletion of $CD4^+$ T cells, but not NK cells, resulted in a significant reduction in *CXCL-9* transcripts, but only a modest reduction of *CXCL-10* transcripts in the joint tissue at 2 weeks of infection in IL-$10^{-/-}$ mice, (Fig 7B-C). This suggests either that depletion of suspected cell types was insufficient in the joint, or that multiple cell types produce IFN-γ in response to *B. burgdorferi* spirochetes in the joint.

To identify the joint infiltrating cells responsible for the production of IFN-γ, single cell suspensions of joint tissue were prepared from B6 IL-$10^{-/-}$ mice 2 weeks after infection, labeled for lineage markers, and FACS was utilized to sort specific populations. Total RNA was then extracted from the sorted cells, and IFN-γ, and lineage specific markers were quantified by RT-PCR in each fraction. Cell lineages were defined in the following manner: NK cells as $NK1.1^+$ TCR-β^-; NKT cells as $NK1.1^+$, TCR-β^+; $CD4^+$ T cells as $NK1.1^-$, TCR-β^+, and $CD4^+$, and Macrophages $F4/80^+$. IFN-γ transcripts were highly enriched in sorted fractions containing NK cells and $CD4^+$ T cells recovered from the infected joint tissue of IL-$10^{-/-}$ mice, but no enrichment of IFN-γ was observed in sorted NKT cells, macrophages, unfractionated joint cells (Figure 2.8A), or whole joint (Figure 2.1B). Thus, as was previously postulated (Figure 2.7), two cell types, NK cells and CD^+ T cells, but not NKT cells, are responsible for localized production of IFN-γ in *B. burgdorferi* infected IL-$10^{-/-}$ mice.

Figure 2.7. *Depletion of CD4^{+} T cells but not NK/ NKT cells reduces expression of interferon inducible transcripts*. Analysis of serum IFN-γ and interferon-inducible transcripts in joint tissue at 14 days of infection (N≥7). B6 IL-10$^{-/-}$ mice were injected with PBS, Mouse IgG2a (Isotype 1), α-NK1.1, Rat IgG2b (Isotype 2), or αCD4 during infection. ELISA analysis of serum IFN-γ (A). Expression of *CXCL-9* (B) and *CXCL-10* (C) in joint tissue was assessed by RT-PCR. Expression was normalized to *β-actin*. Statistical analysis was performed as described in Materials and Methods.

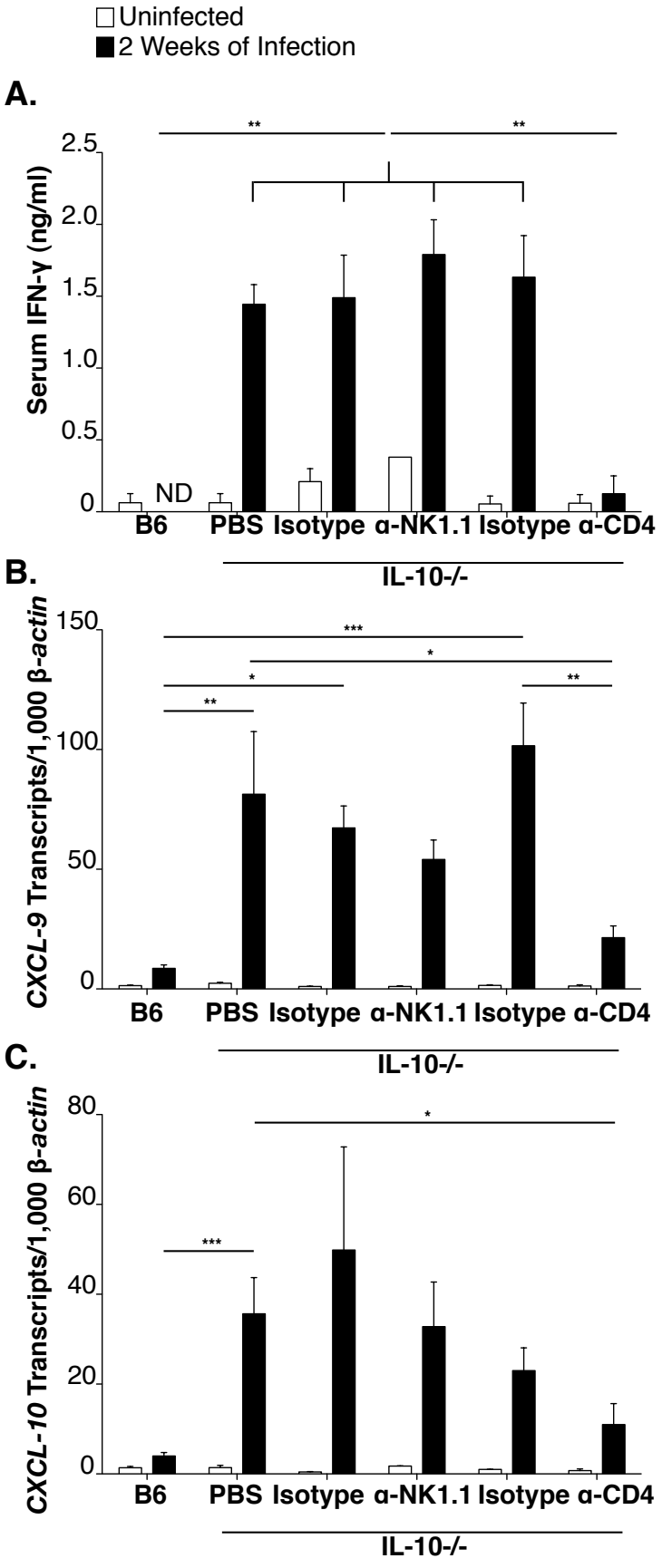
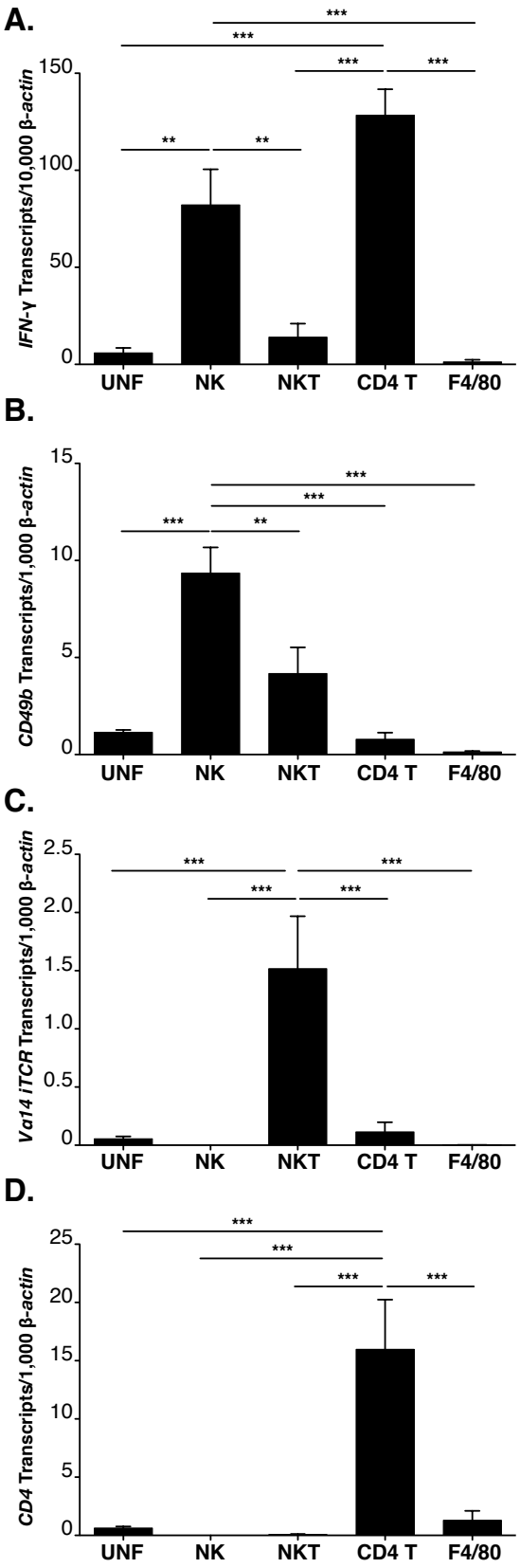

Uninfected
2 Weeks of Infection
A.
Serum IFN-γ (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
ND
**
**
B6
PBS
Isotype
α-NK1.1
Isotype
α-CD4
IL-10-/-
B.
CXCL-9 Transcripts/1,000 β-actin
150
100
50
0
***
*
*
**
**
B6
PBS
Isotype
α-NK1.1
Isotype
α-CD4
IL-10-/-
C.
CXCL-10 Transcripts/1,000 β-actin
80
60
40
20
0
***
*
B6
PBS
Isotype
α-NK1.1
Isotype
α-CD4
IL-10-/-

Figure 2.8. *Joint CD4+ T cells and NK cells express high levels of IFN-γ during B. burgdorferi infection*. RT-PCR analysis of sorted lineages from infected B6 IL-$10^{-/-}$ joint tissue (N=4). NK cells ($NK1.1^+$ TCR-β^-, NKT cells ($NK1.1^+$ TCR-β^+), $CD4^+$ T cells ($NK1.1^-$ TCR-β^+ $CD4^+$ $CD8^-$) and macrophages ($F4/80^+$) were sorted simultaneously. Expression of IFN-γ transcripts (A) and the transcripts of the definitive lineage markers CD49b (B), Vα14 iTCR (C), CD4 (D), and F4/80 (E) by RT-PCR was used to confirm pure sorted populations. Expression was normalized to *β-actin*. Statistical analysis was performed as described in Materials and Methods.


A.
IFN-γ Transcripts/10,000 β-actin
150
100
50
0
***
***
***
***
**
**
UNF
NK
NKT
CD4 T
F4/80
B.
CD49b Transcripts/1,000 β-actin
15
10
5
0
***
***
***
***
**
UNF
NK
NKT
CD4 T
F4/80
C.
Va14 iTCR Transcripts/1,000 β-actin
2.5
2.0
1.5
1.0
0.5
0
***
***
***
***
UNF
NK
NKT
CD4 T
F4/80
D.
CD4 Transcripts/1,000 β-actin
25
20
15
10
5
0
***
***
***
***
UNF
NK
NKT
CD4 T
F4/80

To confirm that the failure to detect IFN-γ transcripts in the NKT cell or other fractions did not reflect a poor recovery of cells, lineage specific transcripts from each of the fractions were assessed by RT-PCR. CD49b (α2 integrin expressed by NK and NKT cells) transcripts were enriched in both the NK cell and NKT cell fractions (Figure 2.8B) as expected, while expression of the NKT cell specific, Vα14-Jα18 iTCR, was found to be enriched only in the sorted NKT cell fraction (Figure 2.8C). CD4 transcripts were enriched in the CD4 T cell fraction (Figure 2.8D).

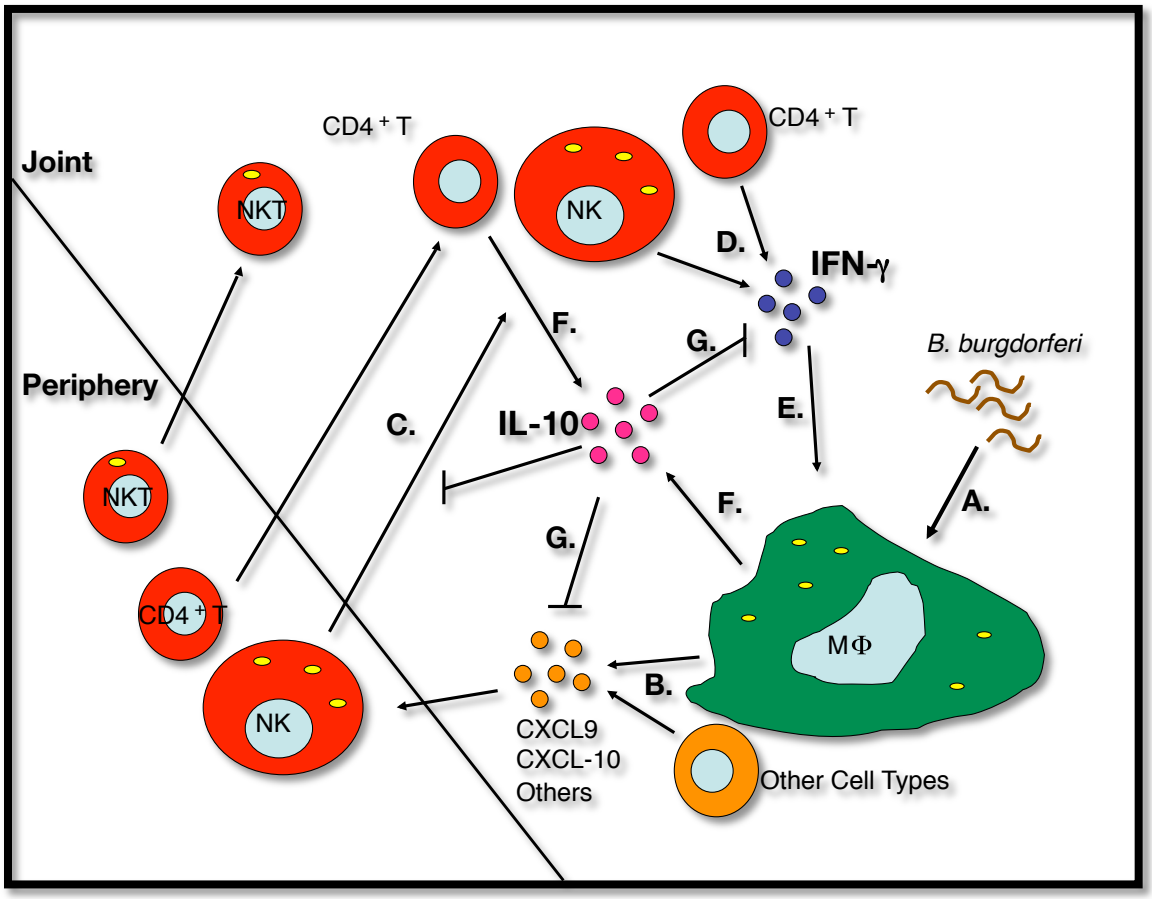
Discussion

The IL-$10^{-/-}$ mouse has proven to be a useful tool in the study of the inflammatory response to *B. burgdorferi* on both the arthritis resistant B6 and arthritis susceptible C3H backgrounds (36, 43). An important feature of this model is that the heightened inflammatory response permitted in the absence of IL-10 results in 5-to 10-fold fewer spirochetes in the joint tissue (36, 43), unlike many other immunodeficiencies, which result in higher levels of tissue spirochetes. Thus, a unique feature of this model is the observation that very low levels of antigen are capable of triggering an exuberant inflammatory response. Increased arthritis severity in these mice is hypothesized to be due to the collateral damage caused by a poorly regulated immune response. In the human Lyme disease patient population there are rare individuals who continue to display symptoms of infection even following a regimen of therapy expected to eradicate the spirochetes (5, 9). In some reports, these individuals are treated with anti-inflammatory drugs used for rheumatoid arthritis (49), after which, most symptoms resolve. Hence, we hypothesize that the IL-$10^{-/-}$ mouse may provide insight into the involvement of

dysregulated response to the initial triggering effect of tissue infiltrating *B. burgdorferi*, and that this may result in delayed resolution of pathological development.

Previous gene expression analysis of joint tissue from *B. burgdorferi* -infected mice revealed an interferon-inducible response that correlated to increased arthritis severity in C3H and B6 IL-$10^{-/-}$ mice (39). While this response was early (1 week of infection), and evanescent in C3H mice, it was delayed (2 weeks) and sustained (4 weeks) in B6 IL-$10^{-/-}$ mice. Importantly, only the IL-$10^{-/-}$ mouse displayed induced expression of any interferon gene, with IFN-γ transcripts increased by 16- and 22-fold at 2 and 4 weeks of infection, respectively (39). A final critical difference between C3H and IL-$10^{-/-}$ mice was that the most robustly upregulated transcripts in C3H mice were for a group of GTPases, whereas in IL-$10^{-/-}$ mice, the chemokines *CXCL-9* and *CXCL-10* were most prominent. In the present study, systemic production of IFN-γ was also observed in IL-$10^{-/-}$ mice (Figure 2.4A), which suggested arthritis developed in these mice could be due to failed regulation of IFN-γ expression in the infected joint tissue. Indeed, neutralization of IFN-γ reduced arthritis severity (Figure 2.4A, Table 1), suppressed the expression of the interferon-inducible transcripts *CXCL-9* and *CXCL-10* (Figure 2.5A, B), and limited the early infiltration of NK and NKT cells into the infected joints of IL-$10^{-/-}$ mice (Figure 2.6C, D; Figure 2.9). Joint histopathology also demonstrated a reduced mononuclear infiltrate in IL-$10^{-/-}$ mice treated with anti-IFN-γ. The observation that infected IL-$10^{-/-}$ mice harbor more macrophages in their joint tissue than WT mice (Figure 2.6B, F) provides a possible explanation for the enhanced host defense observed in IL-$10^{-/-}$ mice, and supports the conclusion made by Lazarus *et al.,* that innate immunity is responsible for the enhanced host defense seen in IL-$10^{-/-}$ mice

Figure 2.9. *Model of Lyme arthritis development in B6 IL-10$^{-/-}$ mice. B. burgdorferi* spirochetes are detected by macrophages and other resident cell types in the joint (A). *B. burgdorferi* stimulated production of CXCL-9, CXCL-10, and other chemokines is exaggerated in IL-10$^{-/-}$ mice (B). NK cells, NKT cells, CD4^{+} T cells, and other cell types are recruited to the infected joint through the influence of multiple chemokines (C). NK cells and CD4^{+} T cells produce IFN-γ (D). Macrophages and other cell types in the joint are activated by IFN-γ, and produce a second wave of chemokines, including CXCL-9 and CXCL-10, completing a feedback loop (E). Macrophages and CD4^{+} T cells produce IL-10 (F). IL-10 regulates the expression of IFN-γ, and subsequent production of CXCL-9 and CXCL-10 (G).


Joint
Periphery
NKT
NKT
CD4 + T
CD4 + T
CD4 + T
NK
NK
NK
D.
IFN-γ
F.
G.
B. burgdorferi
C.
IL-10
E.
F.
A.
G.
MΦ
B.
CXCL9
CXCL-10
Others
Other Cell Types

(50). This enhanced host defense phenotype was partially abrogated in mice receiving anti-IFN-γ (Figure 2.4C). While the upstream events that culminate in sustained production of IFN-γ were not identified, mediation by IL-12 is a possibility as *B. burgdorferi* stimulated IL-10$^{-/-}$ macrophages produced 5 to 10 fold more of this cytokine than WT B6 macrophages (unpublished observation).

These findings highlight a critical difference in arthritis development between C3H and B6 IL-10$^{-/-}$ mice. Whereas arthritis development in C3H mice is dependant on Type I interferon and occurs in the absence of IFN-γ (16, 21), our results demonstrate that IFN-γ is required for arthritis development in B6 IL-10$^{-/-}$ mice. Thus we conclude that

both Type I and Type II interferon pathways can influence arthritis development, given the correct circumstances. Although many of the downstream effectors are identical between Type I and Type II interferon signaling, the mechanisms for arthritis development between C3H and B6 IL-10$^{-/-}$ mice appears to be distinct. Of interest was the previous finding that the addition of exogenous IL-10 via adenoviral delivery failed to suppress arthritis development in C3H mice (43). This may reflect failure of IL-10 to regulate the Type I IFN pathway inherent in the C3H mouse, and further suggests that Type I IFN masks pro-arthritic contributions of IFNγ in infected C3H mice (16, 21, 39). This supports the observation that Lyme arthritis can occur via multiple mechanisms as assessed by genetic linkage analysis, where several pathways have been implicated in Lyme arthritis development (51, 52).

B. burgdorferi induction of IFN-γ has been observed both *in vitro* and *in vivo* for multiple mouse strains and cell types, as well as in human PBMC's (53-55). In C3H mice, depletion of NK and NKT cells resulted in reduced expression of most interferon-

inducible genes, in a near identical fashion to IFN-γ neutralization in C3H mice (21). Other studies have linked NKT cells to IFN-γ in murine Lyme carditis and arthritis (42, 47). In carditis, IFN-γ signaling limits pathology and serves to activate local macrophages and induce killing of *B. burgdorferi* spirochetes (42). Our finding indicate that $CD4^+$ T cells, but not NK/NKT cells, are responsible for the high levels of IFN-γ in the serum of infected IL-$10^{-/-}$ mice (Figure 2.7A), whereas both $CD4^+$ T cells and NK cells are the major sources of IFN-γ in the joint tissue of infected IL-$10^{-/-}$ mice (Figure 2.8A; Figure 2.9).

The critical role of IL-10 in suppressing tissue specific induction of interferon-inducible genes and Lyme arthritis development in B6 mice suggested that early and consistent local production of IL-10 would be necessary to suppress arthritis severity. A variety of cell types are capable of producing IL-10, including components of both the innate and adaptive immune response and other cell types associated with tissue function and vasculature (56). In this study, two approaches were employed to link IL-10 to its cellular source in the infected joint. Both radiation chimeras and the IL-10 reporter mouse demonstrated that leukocytes were the sources of IL-10 expression in the infected joint. Furthermore, both $CD4^+$ T cells and $F4/80^+$ macrophages were found to express IL-10 in infected joint tissue with comparable contributions of the detectable GFP reporter (Figure 2.2D-F; Table I; Figure 2.9). Although the subset of $CD4^+$T cell responsible for IL-10 production was not indentified in this study, we suspect involvement of T_H2, T_{reg}, or ever the newly identified T_r1 subset of $CD4^+$ T cells. The finding that $FoxP3^+$ T_{reg} cells in the joint were significantly fewer than the total IL-10-producing $CD4^+$ T cells suggested that the $CD4^+$ T cell subset(s) responsible for IL-10 expression consist of many

non-T_{reg} cells. The peak of IL-10 expression in the joint occurs at 11 days post infection, and prior to the development of arthritis. This suggests that IL-10 has a dampening effect on localized inflammation, and serves to prevent pathological development, rather than resolve it. The early suppressive effects of IL-10 are robust and pleiotropic, as they limit expression of interferon-inducible genes, pro-inflammatory cytokines, and cellular infiltrates into the joint through regulation of IFN-γ-dependant and IFN-γ-independent pathways. The primary function of IL-10 in arthritis development is to suppress expression of IFN-γ, which is required for arthritis development in IL-$10^{-/-}$ mice.

The IL-$10^{-/-}$ mouse model provides a much-needed model for studying the role of IFN-γ in Lyme arthritis development. While complete IL-10 deficiency represents an extreme example of dysregulated inflammation, a host of factors could influence a prolonged inflammatory response. An important finding of the current study is demonstration that $CD4^+$ T cells in the infected joint can produce both pro and anti-arthritic cytokines, and that loss of the anti-inflammatory signal results in increased disease severity. This observation suggests a dominant effect of anti-inflammatory signaling, and that any number of events could combine to disrupt a delicate balance between bactericidal and pathologic inflammation. Interestingly, a loss of function polymorphism in the human *il10r1* gene has recently been reported (57-60), and is associated with risk for extra-pulmonary tuberculosis and rheumatoid arthritis (57, 60). While it is unlikely that treatment-refractory Lyme arthritis patients test positive for these polymorphisms, the end results among these patients and B6 IL-$10^{-/-}$ mice are similar, in the elevated expression of IFN-γ and IFN-γ-inducible chemokines, and possibly represent the result of an insufficient regulatory response.

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CHAPTER 3

SKIN AS A MAJOR SOURCE OF IL-10 DURING *B. burgdorferi* INFECTION OF MICE

Introduction

Lyme disease describes symptoms that occur following infection with the spirochete *B. burgdorferi*, which is transmitted to humans and animals through the bite of infected ticks of the genus *Ixodes* (1-4). These bacteria cause lesions in the tissues they preferentially colonize, namely the skin, heart, nervous system, and joints (4). The hallmark symptom associated with *B. burgdorferi* infection is the development of a 'bull's eye' rash at the site of the tick bite, termed erythema migrans (EM) (5). This rash develops within 1 to 2 weeks following infection in the majority of patients, and is thought to arise due to a localized and infiltrating inflammatory response, produced by innate and adaptive immune cells, as the spirochetes disseminate outward and downward from the site of inoculation and associate with the dermal microvasculature (6-10). Induced blister fluid extracted from EM lesions demonstrated the presence of multiple pro-inflammatory cytokines in the skin (8).

The skin is an important tissue for the life cycle of *B. burgdorferi*. Not only is it the first tissue to encounter the spirochetes when they are deposited into the dermis by the feeding *Ixodes* tick, but it is also the tissue through which larval ticks acquire the infection, rendering them capable of further disease transmission. It is reasonable to assume that a robust inflammatory response that significantly reduces the number of *B. burgdorferi* in the host would make it difficult for these bacteria to be acquired in subsequent tick feeding, and ultimately, future bacterial transmission to additional hosts. Indeed, a cadre of pro-inflammatory cytokines are produced within the EM lesions of patients, including IFN-γ, TNF-α, IL-2, IL-4, and IL-6, and it is thought that this response is beneficial to the host (8, 11, 12). Despite this *B. burgdorferi*-elicited pro-

inflammatory response in the initial stages of infection, the spirochetes survive and go on to infect and replicate within multiple other tissues (5).

Interestingly, high levels of the anti-inflammatory cytokine IL-10 were also reported within the EM lesions of patients (8). This cytokine has been reported to limit the expression and activity of many pro-inflammatory cytokines, including those listed above (13, 14). Multiple infection models in mice have demonstrated that IL-10 deficiency results in a reduced pathogen burden, however this is often at the expense of host-mediated tissue damage (15). This is also the case with *B. burgdorferi* infection of IL-$10^{-/-}$ mice, where IL-10 deficiency results in 5-10 fold fewer bacteria in the joint tissue, but arthritis severity is increased (16). Thus, the induction of IL-10 is beneficial to *B. burgdorferi*, and this may be a mechanism utilized by the spirochete to dampen, and evade the full brunt of the host immune response.

Previous microarray analyses of infected joint tissue from arthritis resistant B6 mice and arthritis susceptible C3H and B6 IL-$10^{-/-}$ mice identified IL-10 transcripts to be up-regulated only in B6 mice at 2 weeks of infection (17). Thus, resistance to arthritis development in B6 mice was correlated with induction of IL-10. Microarray analyses of infected joint tissue from B6 mice demonstrated a very modest induction of IL-10 expression, whereas the suppressive effects on *CXCL-9* and *CXCL-10* transcripts in the joint tissue was robust. It was therefore hypothesized that tissues outside the joint may also contribute to the production of anti-arthritic IL-10. This chapter explores skin as a direct source of IL-10 during *B. burgdorferi* infection.

Materials and Methods

Mice and infection

C57BL/6 (B6), B6.129P2-*IL-10tm1Cgn*/J (IL-$10^{-/-}$), (B6.129S6-*Il10*^{tm1Flv}/J (*tiger),* mice were purchased from The Jackson Laboratories. C3H/HeN mice were purchased from Charles River Laboratories. TLR2-deficient mice were provided by Tularik (San Francisco, CA) and generated by Deltagen (Redwood City, CA). All mice were housed in the University of Utah Animal Research Center (Salt Lake City, UT) and strict adherence to institutional guidelines were followed. To avoid colitis development, IL-$10^{-/-}$ mice were kept on antibiotic water (trimethoprim and sulfamethoxazole) until one day prior to infection. Mice were infected with 2×10^4 bacteria of the clonal *Borrelia burgdorferi* strain N40 as described. Infection was confirmed in mice sacrificed prior to 14 days of infection by culturing bladder tissue in BSK II media containing 6% rabbit serum, phosphamycin, and rifampicin. ELISA quantification of *B. burgdorferi*-specific IgM and IgG concentrations were used to confirm infection in mice euthanized at and after 14 days of infection as described.

Preparation of single cell suspensions from mouse tissue

Single cell suspensions were prepared from skin surrounding the rear ankle joint, by placing skin into Petri dishes containing 3 ml digestion buffer. The buffer consisted of endotoxin-free Liberase TM (Roche) at 0.2 mg/ml in RPMI 1640. Collagenase digestions were incubated for 1 hour at 37° C. After incubation, digestion reactions were filtered through a 100 μm cell strainer, centrifuged, and the red blood cells lysed. Cells were resuspended in 200μl of flow cytometry buffer prior to antibody labeling. Blood was

obtained by cheek puncture, and collected in Eppendorf tubes containing acid citrate dextrose (ACD). Blood leukocytes were isolated as described (18).

Flow cytometry

All flow cytometry data was analyzed using BD CellQuest Pro software. FACS data were collected on a BD FACS Canto II flow cytometry. 7'AAD (eBioscience) was used in all experiments, and dead cells were excluded from analyses, as were doublet cells. All antibodies used for flow cytometry were purchased from either BioLegend or eBioscience. Unconjugated F_c blocking antibody (clone 93 eBioscience) was included in all antibody-labeling experiments. Fluorochrome-conjugated antibodies used in this study were as follows: FITC conjugated α-B220 (RA3-6B2); PE conjugated α-CD3ε (145-2C11), α-F4/80 (BM8); PE-Cy-7 conjugated α-CD4 (GK1.5), α-CD11b (M1/70); APC conjugated α-CD11c (N418), αCD45.1/Ly5.1 (A20), α-TCR-β (H57-597); Pacific Blue conjugated α-CD45.2 (104).

Isolation of RNA and quantitative RT-PCR

For all experiments examining gene expression in skin tissue, mice were killed, and the skin was removed from the tibiotarsal joints, or a small section of the back where needle inoculation had occurred. Skin samples were placed in Eppendorf tubes, and frozen immediately in dry ice/ethanol, and stored at -80°C. Isolation of total RNA from skin tissue was performed by acid guanidine extraction as described. Five micrograms of skin RNA were reverse-transcribed as described. Primers sequences used in this study were: *CXCL-10* Forward (5'-GAA ATC ATC CCT GCG AGC CTA TCC-3'), Reverse

(5'-GCA ATT AGG ACT AGC CAT CCA CTG GG-3'; *IL-10CD* (does not recognize sterile transcript produced by IL-$10^{-/-}$ mouse) Forward (5'-GCT CTT ACT GAC TGG CAT GA-3'), Reverse (5'-TTC CGA TAA GGC TTG GCA AC-3'. Primer sequences for *β–actin* (17), *CXCL-9* (19), and the *B. burgdorferi 16s rRNA* (17) used in this study can be found with their respective citations. All RT-PCR experiments were conducted on a Roche LC-480.

Generation of radiation chimeras

IL-$10^{-/-}$ mice 5 weeks of age were lethally irradiated with 2 doses of 525 cGy 3 hours apart using a GE Isovolt Titan. Twenty-four hours after irradiation; splenocytes were harvested from donor mice (either IL-$10^{-/-}$ Ly5.2^{+}, or WT congenic B6 mice that were Ly5.1^{+}). Irradiated mice were then injected *i.v.* with 2×10^{7} splenocytes in a 200µl volume. Chimerism was determined at 2, 4, 6, and 14 weeks after irradiation by flow cytometry analysis of blood leukocytes. After full reconstitution, B cells and myeloid lineage cells were found to be >90% donor-derived, while T cells were approximately 60% donor-derived (see Appendix A).

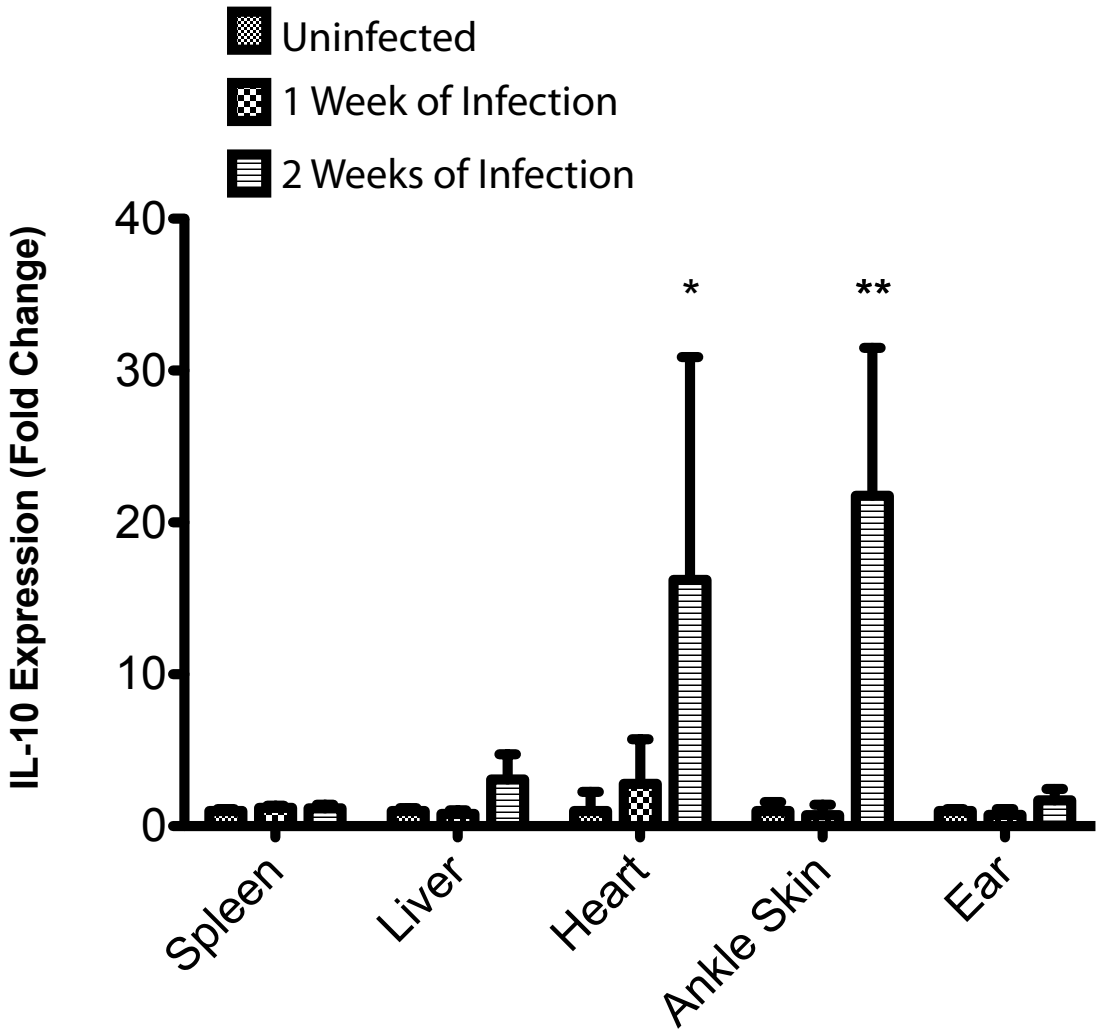
Results

To determine the relative contribution of IL-10 provided by non joint tissues, B6 mice were infected and expression of IL-10 was assessed at day 7 and 14 of infection in the spleen, liver, heart, ear, and in the skin surrounding the tibiotarsal joint. IL-10 transcripts were detected in all tissues except the ear at day 14 of infection (Figure 3.1). While the spleen and liver likely represent major sources of IL-10 as whole tissues,

infection-induced expression of IL-10 transcripts was not robust. In contrast to these findings, both the heart and skin from the ankle joint displayed robustly induced IL-10 transcript expression relative to uninfected controls, and represent regions of highly rich IL-10 expression at 2 weeks of infection (Figure 3.1).

Previous experiments utilizing macrophages derived from both arthritis resistant B6 mice and arthritis susceptible C3H mice demonstrated an unequal ability to produce IL-10 (16). In addition to limited IL-10 production, C3H macrophages also produced higher quantities of pro-inflammatory cytokines (16). Finally, microarray analyses of infected joint tissue failed to demonstrate induction of IL-10 in arthritis-susceptible C3H mice, suggesting that a failure to produced IL-10 may account for the differences in arthritis severity seen in B6 and C3H mice (17). Additionally, B6 and C3H mice lacking Toll-like receptor 2 (TLR2) developed considerably more severe arthritis than WT mice of their respective strains (20, 21). Arthritis development in these mice has also been correlated to extremely high expression of the chemokines CXCL-9 and CXCL-10, as well as other interferon-inducible transcripts (20). The robust induction of interferon-inducible genes is also correlated to increased arthritis severity in both the C3H and B6 IL-$10^{-/-}$ strains, with the latter strain also possessing robust induction of CXCL-9 and CXCL-10 (17). Furthermore, macrophages stimulated with *B. burgdorferi* lipoproteins produced IL-10 in a TLR2-dependent fashion (21). Therefore it was hypothesized that a failure to induce IL-10 expression in the arthritis susceptible C3H and B6 TLR$2^{-/-}$ mouse strains accounted for the increased arthritis severity in *B. burgdorferi*-infected mice. To

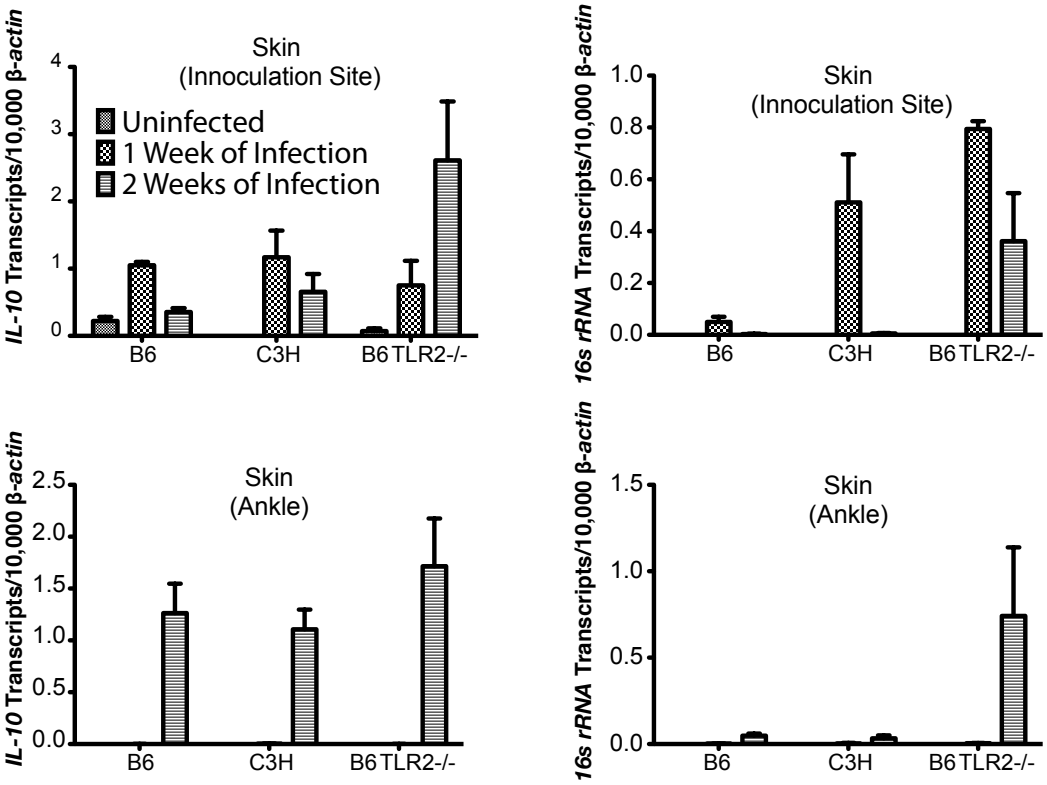
Figure 3.1. *Induced expression of IL-10 is highly enriched in the infected heart and skin at 2 weeks after B. burgdorferi infection.* RT-PCR analyses of selected tissues from uninfected mice, and *B. burgdorferi* infected mice at 1 and 2 weeks of infection. cDNA was prepared from tissues as described in Materials and Methods. N=3 mice per timepoint. Bars represent the mean fold change ± calculated S.E.M, which includes error associated with β-actin normalization, and fold change normalization. Values were calculated by diving each value by the mean obtained from uninfected samples. Statistical significance is indicated by (*) or (**), and represents p values of $p>.05$ and $p>.01$, respectively from one-way ANOVA and the Bonferoni post test for pair-wise comparison.


Uninfected
1 Week of Infection
2 Weeks of Infection
IL-10 Expression (Fold Change)
40
30
20
10
0
*
**
Spleen
Liver
Heart
Ankle Skin
Ear

test this hypothesis, B6, C3H and B6 TLR2$^{-/-}$ mice were infected with *B. burgdorferi*, and IL-10 expression in the skin (an IL-10-rich tissue) was quantified by RT-PCR at 7 and 14 days of infection, with the understanding that a failure to produce IL-10 in the skin (both skin from the inoculation site and the skin from the ankle joint) would be correlated to increased arthritis severity in both the C3H and B6 TLR2$^{-/-}$ mouse strains.

In skin taken from the inoculation site, IL-10 expression peaked at 7 days of infection in B6 and C3H mice, and receded by 2 weeks of infection in B6, C3H, but was elevated in B6 TLR2$^{-/-}$ mice (Figure 3.2). The fact that IL-10 expression was at similar levels at all time points between B6 and C3H strains suggests that C3H mice are not incapable of producing IL-10. Furthermore, the induction of IL-10 in the skin is not dependent on *B. burgdorferi* lipoproteins signaling through TLR2, as IL-10 expression was present in the skin of TLR2$^{-/-}$ mice. Interestingly, in skin samples from the inoculation site of B6 and C3H mice, IL-10 expression reflected the presence of *B. burgdorferi* in the tissue, which returned to low levels by 2 weeks of infection. In contrast, *B. burgdorferi 16s rRNA* was easily detected in samples from TLR2$^{-/-}$ mice at 2 weeks of infection (Figure 3.2). Similar findings were obtained in skin surrounding the ankle joint, with comparable levels of IL-10 expression between B6, C3H, and B6 TLR2$^{-/-}$ mice, however, unlike the results obtained from skin from the inoculation site, IL-10 expression was only detected at 2 weeks of infection at this more distant site (Figure 3.2). As in skin samples taken from the inoculation site, IL-10 expression in skin from the ankle joint also reflected the presence of spirochetes (Figure 3.2-D). Taken together, these data demonstrate that the arthritis susceptible C3H strain in perfectly capable of producing IL-10 at levels comparable to that seen in B6 mice, which suggests IL-10

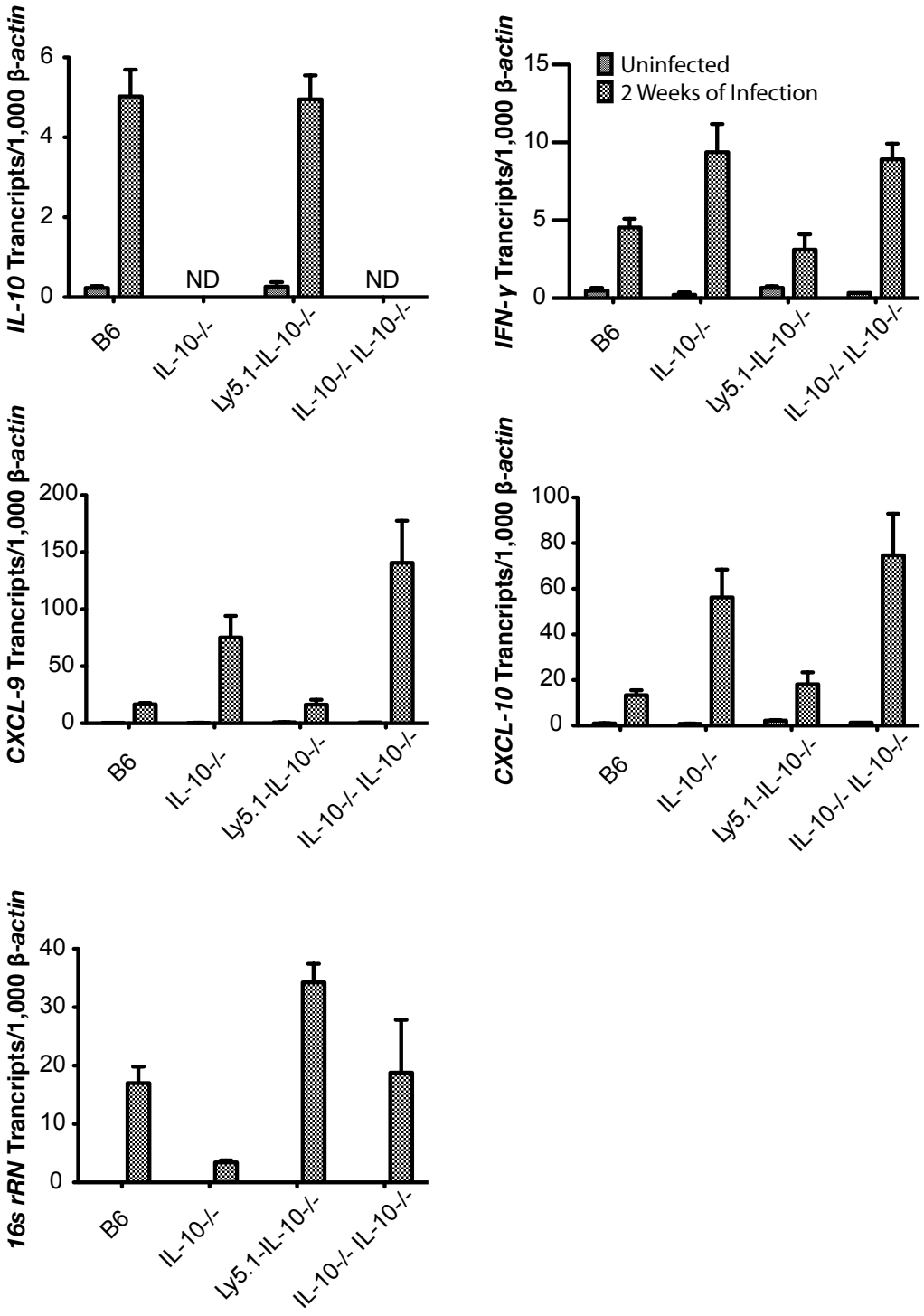
Figure 3.2. *IL-10 Expression in skin from infected B6, C3H, and B6 TLR2$^{-/-}$ mice correlates to the presence of B. burgdorferi.* RT-PCR analyses of skin from either the site of inoculation, or from around the tibiotarsal (ankle) joint. Graphs on the left column display IL-10 transcripts over time, while graphs on the right column show the presence of *B. burgdorferi 16s rRNA* over time. N=3 mice per group for each time point. Bars represent the mean expression normalized to β-actin ± S.E.M.


Skin
(Innoculation Site)
Uninfected
1 Week of Infection
2 Weeks of Infection
IL-10 Transcripts/10,000 β-actin
16s rRNA Transcripts/10,000 β-actin
Skin
(Ankle)
B6
C3H
B6 TLR2-/-

production is not a differentiating factor between B6 and C3H mice, consistent with published findings (22). Furthermore, the increased arthritis severity seen in B6 TLR2$^{-/-}$ mice is not due to an inability of these mice to produce IL-10 during *B. burgdorferi* infection.

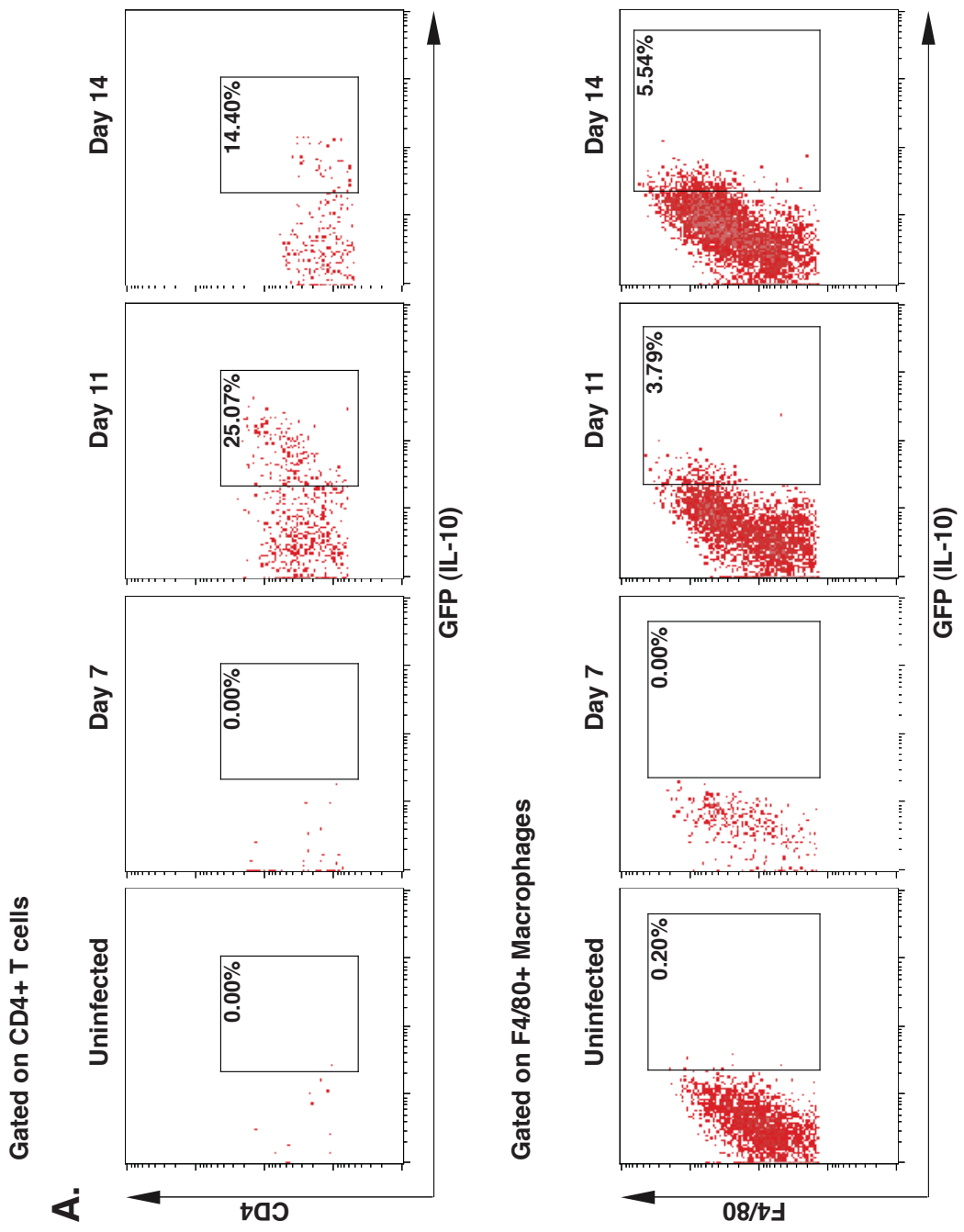
In IL-10$^{-/-}$ mice, *B. burgdorferi* infection results in increased arthritis severity and a profound induction of interferon-induced transcripts, not present in WT B6 mice. The demonstration that skin from the ankle joint was enriched for IL-10 expression highlighted a need to characterize the cellular source of IL-10. To determine if the source was hematopoietic in nature, radiation chimeras were constructed by lethally irradiating IL-10$^{-/-}$ mice (Ly5.1^{-}), followed by transplantation of WT, Ly5.1^{+} splenocytes. The efficiency of engraftment over time is shown in Appendix A. At 12 weeks after transplantation, chimeras and control mice were infected with *B. burgdorferi*, and *IL-10* transcripts were quantified by RT-PCR at 2 weeks of infection. In the skin from the ankle joint, IL-10 expression was detected at high levels in the skin, and was similar between unirradiated B6 mice and Ly5.1→IL-10$^{-/-}$ chimeras, suggesting a hematopoietic source of IL-10 in the skin of *B. burgdorferi* infected mice (Figure 3.3). Interestingly, the presence of IL-10 in the skin also reflected higher levels of *B. burgdorferi 16s rRNA*, suggesting that leukocyte-derived IL-10 permits a higher bacterial burden in the skin (Figure 3.3). The ability of IL-10 to suppress the expression of *IFN-γ*, and the interferon-inducible transcripts *CXCL-9* and *CXCL-10* was also assayed by RT-PCR. Expression of both *CXCL-9* and *CXCL-10* was significantly reduced in Ly5.1→IL-10$^{-/-}$ chimeras, as compared to control chimeras transplanted with autologous, IL-10$^{-/-}$ splenocytes. Together, these data support the conclusion that a hematopoietic source of

Figure 3.3. *A hematopoietic source of IL-10 regulates expression of interferon-inducible transcripts in the skin*. RT-PCR analysis of ankle skin at 2 weeks after infection with *B. burgdorferi* from WT B6 (N=4), B6 IL-$10^{-/-}$ (N=4), WT LY5.1→IL-$10^{-/-}$ chimeras (N=4), and IL-$10^{-/-}$→IL-$10^{-/-}$ chimeras (N=3). Expression of *IL-10, IFN-γ, CXCL-9, CXCL-10, and B. burgdorferi 16s rRNA* was normalized to *β-actin*. The term 'ND' indicates that IL-10 transcripts were not detected.

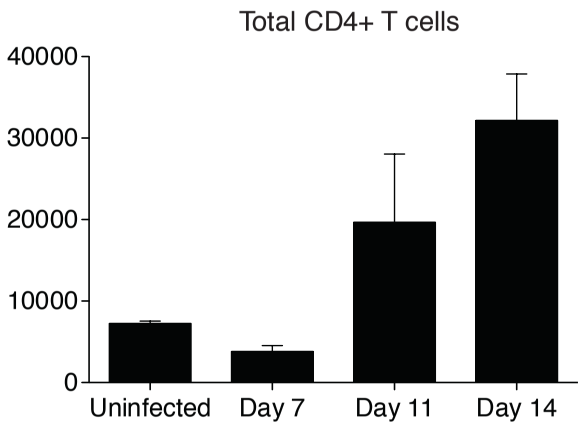

IL-10 Trancripts/1,000 β-actin
6
4
2
0
ND
ND
B6
IL-10-/-
Ly5.1-IL-10-/-
IL-10-/- IL-10-/-
IFN-γ Trancripts/1,000 β-actin
15
10
5
0
Uninfected
2 Weeks of Infection
B6
IL-10-/-
Ly5.1-IL-10-/-
IL-10-/- IL-10-/-
CXCL-9 Trancripts/1,000 β-actin
200
150
100
50
0
B6
IL-10-/-
Ly5.1-IL-10-/-
IL-10-/- IL-10-/-
CXCL-10 Trancripts/1,000 β-actin
100
80
60
40
20
0
B6
IL-10-/-
Ly5.1-IL-10-/-
IL-10-/- IL-10-/-
16s rRN Trancripts/1,000 β-actin
40
30
20
10
0
B6
IL-10-/-
Ly5.1-IL-10-/-
IL-10-/- IL-10-/-

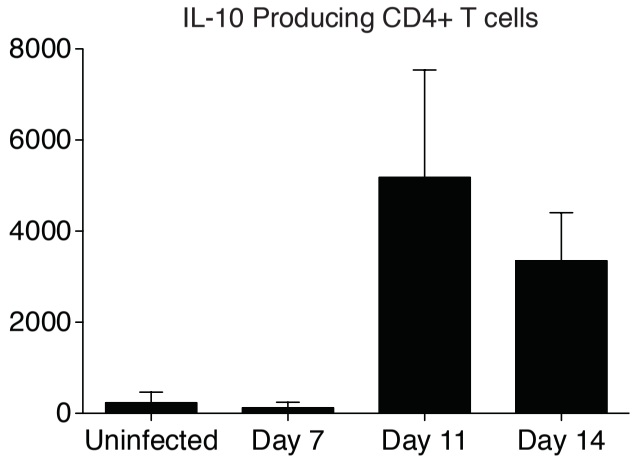
IL-10 limits expression of pro-inflammatory interferon-inducible genes in the skin of *B. burgdorferi* infected mice, similar to what was observed for joint tissue. To further characterize the cellular source of IL-10 in the skin of infected mice, a kinetic analysis of IL-10 expression was performed using the 'tiger' IL-10 reporter mouse strain. These mice contain an IRES-GFP reporter cassette in the 3' UTR region of the IL-10 gene (23). These mice were infected with *B. burgdorferi*, and ankle skin from infected tiger mice was harvested at 7, 11, and 14 days of infection. As in the previous chapter, which identified F4/80$^+$ macrophages and CD4$^+$ T cells as the primary sources of IL-10, these cell types were also the only cell types associated with IL-10 (GFP) expression in the skin from the ankle joint. Indeed, numerous CD11c$^+$ cells, which broadly labels dermal dendritic cell subsets and epidermal Langerhan's cells, were detected in skin samples, however, none were positive for GFP (data not shown). GFP (IL-10) was not detected in uninfected mice, nor was it found at 7 days after infection. However, as was seen in joint tissue, at day 11 of infection, there was a robust infiltration of both CD4$^+$ T cells and F4/80$^+$ macrophages (Figure 3.4A, B). As a population, IL-10 (GFP)$^+$ CD4$^+$ T cells were at a higher frequency than were macrophages, although there were more total IL-10 (GFP)$^+$ macrophages than there were CD4$^+$ T cells (Figure 3.4A, B). Additionally, the mean fluorescence intensity for IL-10 (GFP) appeared to be higher in T cells than in macrophages, as observed in the joint (Chapter 2, Table 2.1). Interestingly, the frequency and total number of IL-10 (GFP)$^+$ CD4$^+$ T cells decreased at day 14 of infection, whereas, both the frequency and total number of IL-10 (GFP)$^+$ macrophages increased from day 11 of infection to day 14 of infection (Figure 3.4A, B).

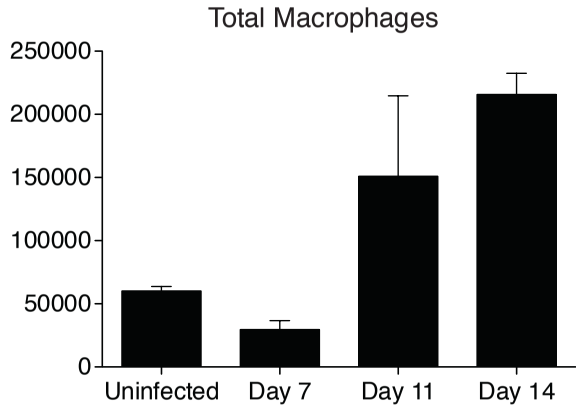
Figure 3.4. *CD4+ T cells and macrophages are the major sources of IL-10 in the skin of B. burgdorferi-infected mice*. Control and infected tiger mice (N=3 per group) were analyzed for IL-10 (GFP) producing $CD4^+$ T cells and macrophages in skin tissue from the joint. Flow cytometry analysis of IL-10 expression in skin $CD4^+$ TCR-β^+ T cells from uninfected mice, at day 7, day 11, and 14 of infection (A-top). Flow cytometry analysis of IL-10 expression in skin $F4/80^+$ macrophages from uninfected mice, at day 7, day 11, and day 14 of infection (A-bottom). Total skin $CD4^+$ TCR-β^+ T cells and total IL-10 $(GFP)^+$ $CD4^+$ TCR-β^+ T cells (B-top). Total skin $F4/80^+$ macrophages and total IL-10 $(GFP)^+$ macrophages (B-bottom).

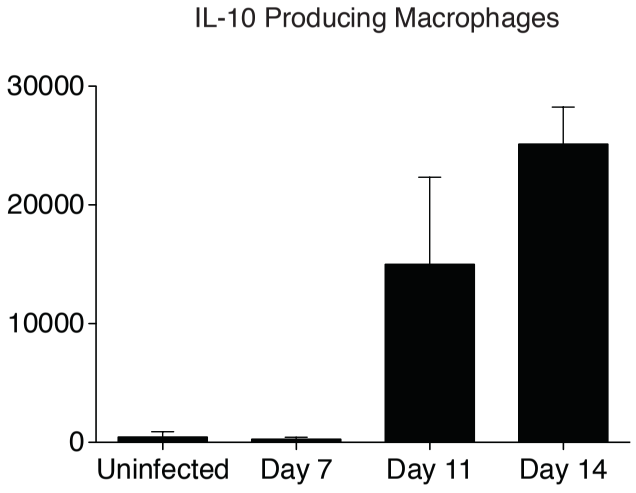

A.
Gated on CD4+ T cells
Uninfected
Day 7
Day 11
Day 14
0.00%
0.00%
25.07%
14.40%
CD4
GFP (IL-10)
Gated on F4/80+ Macrophages
Uninfected
Day 7
Day 11
Day 14
0.20%
0.00%
3.79%
5.54%
F4/80
GFP (IL-10)

B.


Total CD4+ T cells
40000
30000
20000
10000
0
Uninfected
Day 7
Day 11
Day 14


IL-10 Producing CD4+ T cells
8000
6000
4000
2000
0
Uninfected
Day 7
Day 11
Day 14


Total Macrophages
250000
200000
150000
100000
50000
0
Uninfected
Day 7
Day 11
Day 14


IL-10 Producing Macrophages
30000
20000
10000
0
Uninfected
Day 7
Day 11
Day 14

Discussion

Initial studies in *B. burgdorferi*-stimulated macrophages derived from arthritis susceptible C3H mice and arthritis resistant B6 mice comparing cytokine production, and found increased production of pro-inflammatory cytokines and nitrite among the arthritis susceptible strain. Additionally, macrophages from the arthritis resistant B6 strain produced much higher levels of IL-10 than C3H-derived macrophages (16). Previous microarray analyses of infected joint tissue from these two strains also revealed a robust interferon-inducible response that correlated to increased arthritis severity in C3H mice. A similar interferon-induced profile was also observed in B6 IL-$10^{-/-}$ mice. Finally, no induction of *IL-10* transcripts were observed in the infected joint tissue of C3H mice (17). With the demonstration that *B. burgdorferi* lipoproteins induce IL-10 production in macrophages by signaling through TLR-2, that B6 and C3H mice lacking Toll-like receptor 2 (TLR-2) developed considerably more severe arthritis than WT mice of their respective strains, and that arthritis development in these mice has also been correlated to extremely high expression of interferon-inducible transcripts, IL-10 was a prime candidate for explaining differences in arthritis severity among B6, C3H and TLR-$2^{-/-}$ mouse strains (20, 24). The finding that IL-10 expression was robustly induced in the skin from the ankle joint allowed the formation of the hypothesis that IL-10 expression was reduced or absent in the skin from infected C3H and B6 TLR-$2^{-/-}$ mice. RT-PCR analysis of infected skin derived from infected B6, C3H, and B6 TLR-$2^{-/-}$ mice demonstrated similar expression levels of IL-10, suggesting that signaling through TLR-2 is not necessary for IL-10 induction in the skin. Interestingly, the infected joints of TLR-$2^{-/-}$ mice have been reported to contain a high number of T cells, which could be a major

source of IL-10 in the skin of TLR-2$^{-/-}$ mice. Production of IL-10 by macrophages stimulated with *B. burgdorferi* lipoproteins was shown to be dependant on TLR2 signaling, although IL-10 production was only partially reduced when macrophages were stimulated with sonicated *B. burgdorferi* (21). This suggests that cells of the innate immune system are capable of producing IL-10 through TLR-2-independent pathways, thus the full measure of IL-10 expression in the skin is likely due to enhanced T cell produced IL-10, with a smaller contribution by macrophages. The finding that expression of IL-10 was similar in skin from B6 and C3H mice further indicates that a failure to produce IL-10 is not the reason for differing arthritis phenotypes between B6 and C3H mice. This conclusion is consistent with a report that showed retroviral delivery of exogenous IL-10 failed to reduce arthritis severity in infected C3H mice (22). Additionally, none of the QTL identified by genetic linkage analysis between B6 and C3H intercross population contained the *il10* gene (25).

A variety of cell types are capable of producing IL-10, including components of both the innate and adaptive immune response and other cell types associated with tissue function and vasculature (14). In these studies, two approaches were employed to link IL-10 to its cellular source in the infected skin. Both radiation chimeras and an IL-10 reporter mouse demonstrated that leukocytes were the sources of IL-10 expression in the infected skin. Furthermore, both CD4^{+} T cells and F4/80^{+} macrophages were found to express IL-10 in infected and skin tissues with comparable contributions of the detectable GFP reporter. The infiltration of these IL-10 producing cells was first detected at 11 days after *B. burgdorferi* infection, similar to what was seen in the joint (Chpater 2). Similar to what was found for IL-10 (GFP) expressing cells in the joint tissue, the GFP MFI was

greater in $CD4^+$ T cells than in macrophages within the infected skin tissue.

B. burgdorferi induced of IL-10 by cells of both the innate and adaptive immune system is beneficial to both the spirochete and the host. IL-10 produced by the host dampens the inflammatory response that would otherwise cause more severe tissue damage, resulting in pathology such as arthritis. The benefit to the spirochete is that, in the presence of IL-10, the bacteria are more able to infect the host, and reach greater densities in the tissue (16, 22). Because the skin plays a central role in both the transmission of spirochetes through the bite of infected ticks, and the acquisition of *B. burgdorferi* by feeding ticks, the induction of IL-10 is a possible mechanism utilized by the spirochete, to ensure its own propagation and survival (2).

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CHAPTER 4

DISCUSSION

Overview

Interleukin-10 is a potent anti-inflammatory cytokine with pleiotropic activity. As in other infection models with the IL-10$^{-/-}$ mouse, the heightened inflammatory response to *Borrelia burgdorferi* permitted in the absence of IL-10 results in 5-to 10-fold fewer bacteria in the joint tissue, but infection result in a higher degree of lesion severity. Thus, a unique feature of this model is the observation that very low levels of bacterial antigens are capable of triggering an exuberant inflammatory response. Previous microarray analyses of infected joint tissue from IL-10$^{-/-}$ mice demonstrated an enhanced expression of pro-inflammatory cytokines, most notably IFN-γ. In the absence of IFN-γ signaling, arthritis is ameliorated in IL-10$^{-/-}$ mice, but mice harbored more spirochetes in their joint tissue. Thus, the phenotypes associated with IL-10 deficiency are dependent on IFN-γ, and labels this pro-inflammatory cytokine as the primary target of IL-10 during arthritis development. Because of the relationship between these two cytokines, the focus of this dissertation was to characterize the cellular sources of the anti-arthritic IL-10, and the pro-arthritic IFN-γ. Utilizing an IL-10 reporter mouse, CD4^{+} T cells and macrophages were shown to be the primary sources of IL-10 within the infected joint and skin tissues, linking these cell types to an anti-arthritic activity. Furthermore, enrichment of IFN-γ transcripts were found in NK cells and CD4^{+} T cells isolated from the joint tissue in infected IL-10$^{-/-}$ mice, formally linking these cells types to a pro-arthritic activity. The finding that CD4^{+} T cells are capable of both promoting and suppressing arthritis development illustrates the importance of a highly regulated inflammatory response to limit tissue damage during the host response to invading pathogens.

Role of IFN-γ in Lyme Arthritis

The role of IFN-γ in Lyme disease has long been a topic of debate. In human patients, early production of IFN-γ in the EM lesion is thought to be beneficial to the host (1, 2), but in the late stages of disease, IFN-γ is thought to be associated with pathologies such as arthritis (3-5). T_H1 polarized $CD4^+$ T cells have been postulated to the primary source of the late, pro-arthritic IFN-γ, although γ/δ T cells have also been found in Lyme arthritis patients (6-8). Interestingly, the production of IFN-γ in Lyme arthritis patients is most prominent among those with post-treatment Lyme disease, where high levels of IFN-γ were found the synovial fluid or arthritis joints after antibiotic therapy (5).

As discussed in Chapter 1, $CD4^+$ T cell polarity in murine Lyme arthritis, particularly, the T_H1 polarized subset, has thoroughly been examined in inbred strains of mice. Although early studies using IFN-γ-neutralizing antibody reported reduced ankle swelling in *B. burgdorferi*-infected C3H mice, these reports were not repeated in subsequent studies using a similar method, nor using C3H IFN-$\gamma^{-/-}$ mice (9, 10). A key component in this discrepancy is the lack of histopathological assessment of arthritis in the early studies (11). In addition to the finding that IFN-γ was not required for arthritis development, additional findings have shown that this cytokine is also not required for arthritis resistance, nor is it necessary for the normal host defense against *B. burgdorferi* (9, 12).

Previous gene expression analysis of joint tissue from *B. burgdorferi* -infected mice revealed an interferon-inducible response that correlated to increased arthritis severity in C3H and B6 IL-$10^{-/-}$ mice (13). While this response was early (1 week of infection), and evanescent in C3H mice, it was delayed (2 weeks) and sustained (4

weeks) in B6 IL-10$^{-/-}$ mice. Importantly, only the IL-10$^{-/-}$ mouse displayed induced expression of any interferon gene, with IFN-γ transcripts increased by 16- and 22-fold at 2 and 4 weeks of infection, respectively (13). Further analyses of B6 IL-10$^{-/-}$ mice revealed high concentrations of IFN-γ in the serum at 2 weeks of infection. Taken together, the localized (joint) and systemic (serum) IFN-γ responses led to the hypothesis that IFN-γ was driving arthritis development in B6 IL-10$^{-/-}$ mice. To test this hypothesis, B6 IL-10$^{-/-}$ mice were injected with PBS, a control antibody, or IFN-γ-neutralizing antibody over a 4-week infection with *B. burgdorferi*. IL-10$^{-/-}$ mice treated with the anti-IFN-γ, but not PBS or control antibody, developed significantly less severe arthritis as assessed by ankle swelling and histopathology. A major finding in this dissertation is that IFN-γ promotes arthritis development in B6 IL-10$^{-/-}$ mice, and suggests that the dysresulation of IFN-γ, whether by lack of IL-10 or some other mechanism, has the potential to promote the development of Lyme arthritis. Thus, while former murine models of Lyme arthritis were unable to link IFN-γ to Lyme arthritis development, the IL-10$^{-/-}$ mouse provides an opportunity to measure the arthritic potential of this cytokine.

Production of IFN-γ during *B. burgdorferi* infection has been observed both *in vitro* and *in vivo* for multiple mouse strains and cell types, as well as in human PBMC's (2, 14, 15). In an effort to identify possible cellular sources of IFN-γ, single cell suspensions of joint tissue were prepared from infected B6 and B6 IL-10$^{-/-}$ mice, and were analyzed by flow cytometry. Among the populations found within the infected joint previously associated with IFN-γ production were NK cells, NKT cells, and α/β T cells, which were later found to consist primarily of CD4^{+} T cells (14, 16-18). Depletion studies in *B. burgdorferi*-infected IL-10$^{-/-}$ mice indicate that CD4^{+} T cells, but not

NK/NKT cells, are responsible for the high levels of IFN-γ in the serum of infected IL-$10^{-/-}$ mice. Expression of interferon-inducible transcripts in the infected joint was not significantly reduced following NK/NKT cell depletion, and only a modest reduction in expression was observed following $CD4^+$ T cell depletion. This finding suggested either an incomplete depletion of target cell populations in the joint, or the possibility that multiple cell types produce IFN-γ within the infected joints of IL-$10^{-/-}$ mice. To address the latter issue, an assay was designed where NK cells, NKT cells, and $CD4^+$ T cells were sorted from single cell suspensions of joint tissue from infected IL-$10^{-/-}$ mice, followed by RT-PCR quantification of IFN-γ transcripts from the sorted fractions of cells. Enrichment of IFN-γ transcripts above unfractionated controls was observed in the NK cell and $CD4^+$ T cell fractions, but not in the NKT cell fraction of sorted cells. This suggests that NKT cells do not have an IFN-γ-mediated pro-arthritic activity in the joint tissue of IL-$10^{-/-}$ mice. An important distinction between this study and previous studies seeking to determine cellular sources of IFN-γ is that previous studies measured IFN-γ production in cells re-stimulated *in vitro* or observed missing effects of IFN-γ signaling after cellular depletion (7, 10, 16, 19-22). The current study directly linked IFN-γ mRNA to NK cells and $CD4^+$ T cells by sorting these populations directly from the infected joint tissue of IL-$10^{-/-}$ mice, followed by RT-PCR quantification of IFN-γ mRNA transcripts. Interestingly, NKT cell have been reported to have an anti-arthritic activity in the BALBc mouse strain (17). In addition, NKT cell-derived IFN-γ was also linked to the suppression of Lyme carditis (16). The perceived contradiction that IFN-γ promotes pathological development in one tissue (joint), but prevents it in another (heart), is easily explained by the high expression of IL-10 in the infected heart (Chapter 3). This suggests

that production of IFN-γ may cause excessive tissue damage only if it is improperly regulated.

Signaling of IFN-γ through its receptor results in the transcription of multiple interferon-inducible genes. Two of these genes, *Cxcl9* and *Cxcl10* were among the most highly induced transcripts in the joint tissue of *B. burgdorferi*-infected IL-10$^{-/-}$ mice. These chemokines signal through CXCR3, which is expressed on T cells, NK cells, and monocytes (23). Both *Cxcl9* and *Cxcl10* were initially characterized as IFN-γ inducible genes. Interestingly, expression of both of these chemokines was significantly diminished in *B. burgdorferi*-infected IL-10$^{-/-}$ mice injected with the IFN-γ-neutralizing antibody. With the understanding that arthritis is driven by IFN-γ, and the induction of CXCL-9 and CXCL-10 is dependent on IFN-γ, there is likely a positive feedback loop that begins with early production of IFN-γ, followed by the production of CXCL-9 and CXCL-10 by IFN-γ-responsive cells. These chemokines recruit NK cells and possibly T cells to the infected joint, whereon they produce more IFN-γ, and thus, begin the cycle anew. The finding that IFN-γ, CXCL-9, and CXCL-10 were present at high level in the synovial fluid of patients with post-treatment Lyme arthritis suggests this feedback loop may also be present in humans, as does not require a high burden of antigen to develop, as antibiotic therapy is designed to eradicate the bacteria. Thus, the low antigenic burden, high level of IFN-γ, CXCL-9, and CXCL-10 in *B. burgdorferi*-infected IL-10$^{-/-}$ mice closely resembles the symptoms associated with human post-treatment Lyme disease.

Induction of IL-10 in During *B. burgdorferi* Infection

Initial studies in *B. burgdorferi*-stimulated macrophages derived from arthritis-susceptible C3H mice and arthritis-resistant B6 mice compared cytokine production, and found increased production of pro-inflammatory cytokines and nitrite among the arthritis susceptible strain. Additionally, macrophages from the arthritis resistant B6 strain produced much higher levels of IL-10 than C3H-derived macrophages (24). Previous microarray analyses of infected joint tissue from these two strains also revealed a robust interferon-inducible response that correlated to increased arthritis severity in C3H mice (13). A similar interferon-induced profile was also observed in B6 IL-$10^{-/-}$ mice. Finally, induction of *IL-10* transcripts was not observed in the infected joint tissue of C3H mice (13). With the demonstration that *B. burgdorferi* lipoproteins induce IL-10 production in macrophages by signaling through TLR-2, that B6 and C3H mice lacking Toll-like receptor 2 (TLR-2) developed considerably more severe arthritis than WT mice of their respective strains, and that arthritis development in these mice has also been correlated to extremely high expression of interferon-inducible transcripts, IL-10 was a prime candidate for explaining differences in arthritis severity among B6, C3H and TLR-$2^{-/-}$ mouse strains (25, 26). The finding that IL-10 expression was robustly induced in the skin from the ankle joint allowed the formation of the hypothesis that IL-10 expression was reduced or absent in the skin from infected C3H and B6 TLR-$2^{-/-}$ mice. RT-PCR analysis of infected skin derived from B6, C3H, and B6 TLR-$2^{-/-}$ mice demonstrated that all three of these strains are capable of IL-10 gene expression at similar levels from both the ankle skin, and skin taken from the inoculation site. This finding is consistent with a report that showed retroviral delivery of exogenous IL-10 failed to reduce arthritis

severity in infected C3H mice, indicating the underlying mechanism for arthritis development in C3H mice is not regulated by IL-10 (27).

Role of IL-10 in Lyme Arthritis

The critical role of IL-10 in suppressing tissue specific induction of interferon-inducible genes and Lyme arthritis development in B6 mice suggested that early and consistent local production of IL-10 would be necessary to suppress arthritis severity (13, 24). A variety of cell types are capable of producing IL-10, including components of both the innate and adaptive immune response and other cell types associated with tissue function and vasculature (28). In these studies, two approaches were employed to link IL-10 to its cellular source in the infected joint. Both radiation chimeras and the IL-10 reporter mouse demonstrated that leukocytes were the sources of IL-10 expression in the infected joint and skin. Furthermore, both $CD4^+$ T cells and $F4/80^+$ macrophages were found to express IL-10 in infected joint and skin tissues with comparable contributions of the detectable GFP reporter. Interestingly, the GFP mean fluorescence intensity (MFI) among $CD4^+$ T cells from the joint tissue of infected *tiger* reporter mice was significantly higher than in macrophages at 11 and 14 days of infection (Chapter 2, Table 1). This signifies that T cells produce more IL-10 on a single cell basis than macrophages. This observation has been reported elsewhere, and the differences likely represents differing upstream signaling events that culminate in IL-10 gene expression (29). Indeed, much data suggests that TLR signaling pathways in macrophages induce transcription factors such as NF-κB, C/EBP, and others to bind enhancer regions within the IL-10 promoter region (30-35). In $CD4^+$ T cells, IL-10 production is dependent on TCR signaling which

culminates in the activation and binding of c-Jun, among other transcription factors, to the IL-10 promoter (29). Additionally, multiple DNaseI hypersensitivity sites have been identified within the IL-10 locus, but few of these enhancer containing regions are shared between $CD4^+$ T cells and macrophages (29). Thus, differences in the magnitude of IL-10 expression between macrophages and $CD4^+$ T cells during *B. burgdorferi* infection are hypothesized to be due to a unique usage of transcription factors, and epigenetic markers associated with cell lineage differentiation. The finding that there are more IL-10 $(GFP)^+$ macrophages present in the infected joint, especially at 14 days after infection suggests that, while $CD4^+$ T cells produce more IL-10 on an individual basis, the overall contribution by both populations is comparable. Although the subset of $CD4^+$ T cell responsible for IL-10 production was not indentified in this study, we suspect involvement of T_H2, T_{reg}, or ever the newly identified T_r1 subset of $CD4^+$ T cells. The finding that $FoxP3^+$ T_{reg} cells in the joint were significantly fewer than the total IL-10-producing $CD4^+$ T cells suggested that the $CD4^+$ T cell subset(s) responsible for IL-10 expression consist of many non-T_{reg} cells. The peak of IL-10 expression by $CD4^+$ T cells in the joint and surrounding skin occurs at 11 days after infection, and prior to the development of arthritis. This suggests that IL-10 has a dampening effect on localized inflammation, and serves to prevent pathological development, rather than to resolve it. Interestingly, no $CD11c^+$ cells were found to express the GFP reporter in the skin and joint during *B. burgdorferi* infection of tiger mice, despite the high likelihood of dermal dendritic cells and Langerhans cells interacting with the invading spirochetes. Dendritic cell subsets and Langerhans cells, both of which stain positive for the CD11c marker, have been postulated to have regulatory roles in other models of inflammation; however

any anti-inflammatory role filled by these cell types is independent of IL-10 during *B. burgdorferi* infection (36-39).

The early suppressive effects of IL-10 are robust and pleiotropic, as they limit expression of interferon-inducible genes, pro-inflammatory cytokines, and cellular infiltrates into the joint through the regulation of IFN-γ-dependant and IFN-γ-independent pathways. The primary function of IL-10 in arthritis development is to suppress expression of IFN-γ, which is required for arthritis development in IL-$10^{-/-}$ mice. Interestingly, the accumulation of macrophages in the joint tissue of infected mice was 3-4 fold greater in IL-$10^{-/-}$ mice when compared to WT controls, but depletion of IFN-γ in IL-$10^{-/-}$ mice did not reduce the total number of macrophages in the infected joint. This suggests that the robust macrophage infiltration of joint tissue in IL-$10^{-/-}$ mice is not a major component of arthritis development. Interestingly, treatment of IL-$10^{-/-}$ mice with the IFN-γ-neutralizing antibody resulted in a modest increase in joint spirochetes. With the understanding that macrophages are capable of *B. burgdorferi* phagocytosis in the absence of opsonizing antibody, and the fact that there are greater numbers of macrophages in the infected IL-$10^{-/-}$ joint, it is reasonable to conclude that the large populations of macrophages within the infected joints of IL-$10^{-/-}$ mice is responsible for the enhanced pathogen clearance seen in IL-$10^{-/-}$ mice, consistent with other findings (40-42). It is also likely that IFN-γ serves to maintain and influence the activation state of these macrophages, and that in the absence of IFN-γ signaling, these macrophages are less able to kill the invading spirochetes.

Differences in Lyme Arthritis Development Between C3H and B6 IL-10$^{-/-}$ Mice

The finding that IFN-γ mediates arthritis development in B6 IL-10$^{-/-}$ mice highlights a critical difference in arthritis development between C3H and B6 IL-10$^{-/-}$ mice. Whereas arthritis development in C3H mice is dependant on Type I interferon and occurs in the absence of IFN-γ, results from Chapter 2 demonstrate that IFN-γ is required for arthritis development in B6 IL-10$^{-/-}$ mice (9, 10). Thus we conclude that both Type I and Type II interferon pathways can influence arthritis development, given the correct circumstances. Although many of the downstream effectors are identical between Type I and Type II interferon signaling, the mechanisms for arthritis development between C3H and B6 IL-10$^{-/-}$ mice appears to be distinct. Of interest was the previous finding that the addition of exogenous IL-10 via adenoviral delivery failed to suppress arthritis development in C3H mice (27). This may reflect failure of IL-10 to regulate the Type I IFN pathway inherent in the C3H mouse, and further suggests that Type I IFN masks pro-arthritic contributions of IFNγ in infected C3H mice (16, 21, 39). It has often been though that maximal arthritis severity occurs in WT C3H mice. Interestingly, C3H IL-10$^{-/-}$ mice developed more severe Lyme arthritis than their WT counterparts, suggesting that IL-10 regulates a previously unrecognized pro-arthritic pathway, which possibly involves IFN-γ. This supports the observation that Lyme arthritis can occur via multiple mechanisms as assessed by genetic linkage analysis, where several loci have been implicated in Lyme arthritis development (43, 44).

The IL-10$^{-/-}$ Mouse as a Model of Persistent Lyme Arthritis

Most human patients with Lyme arthritis and other late stage symptoms respond to antibiotic therapy, after which arthritis eventually resolves (45). However, in some cases arthritis persists for many months after treatment designed to eradicate infection, suggesting that there is a subset of individuals that maintain a long-term inflammatory response in the absence of active infection (5, 45). This condition has been termed "antibiotic-refractory Lyme arthritis" of "Post-Treatment Lyme Disease," (46, 47). Although initial hypotheses sought to explain this phenomenon by infection-induced autoimmunity, the current study helps to elucidate the possibility that local immune response in the joint to the invading spirochetes is poorly regulated and results in sustained inflammation within the synovium (45). In support of this hypothesis, studies in mice have reported that robust antibiotic therapy does not always eliminate *B. burgdorferi* from the tissues (48, 49). A primary phenotype associated with the IL-10$^{-/-}$ mouse model of Lyme arthritis is that the heightened inflammatory response permitted in the absence of IL-10 results in 5-to 10-fold fewer spirochetes in the joint tissue (24, 27). Thus, a unique feature of this model is the observation that very low levels of antigen are capable of triggering an exuberant inflammatory response. In IL-10$^{-/-}$ mice, this inflammatory response most notably consisted of highly expressed *IFN-γ*, and the IFN-γ-inducible chemokines *CXCL-9* and *CXCL-10* at both 2 and 4 weeks of infection (13). Similarly, in human patients with antibiotic-refractory Lyme arthritis, the synovial fluid collected from arthritic joint contained high concentrations of IFN-γ, CXCL-9, and CXCL-10 (5). In addition to these similarities, arthritis in IL-10$^{-/-}$ mice persists beyond 4 weeks of infection, as ankle measurements taken at the peak of arthritis severity (4 weeks

of infection) did not differ significantly from measurements taken at 8 weeks of infection (Appendix B).

The IL-10$^{-/-}$ mouse model provides a much-needed model for studying the role of IFN-γ in Lyme arthritis development, as well as a new model of persistent arthritis. While complete IL-10 deficiency represents an extreme example of dysregulated inflammation, a host of factors could influence a prolonged inflammatory response. Interestingly, a loss of function polymorphism in the human *il10r1* gene has recently been reported (50-54), and is associated with risk for extra-pulmonary tuberculosis and rheumatoid arthritis (50, 53). While it is unlikely that treatment-refractory Lyme arthritis patients possess these genetic polymorphisms, the end results among these patients and B6 IL-10$^{-/-}$ mice are similar, in the elevated expression of IFN-γ and IFN-γ-inducible chemokines, and possibly represent the result of an insufficient regulatory response.

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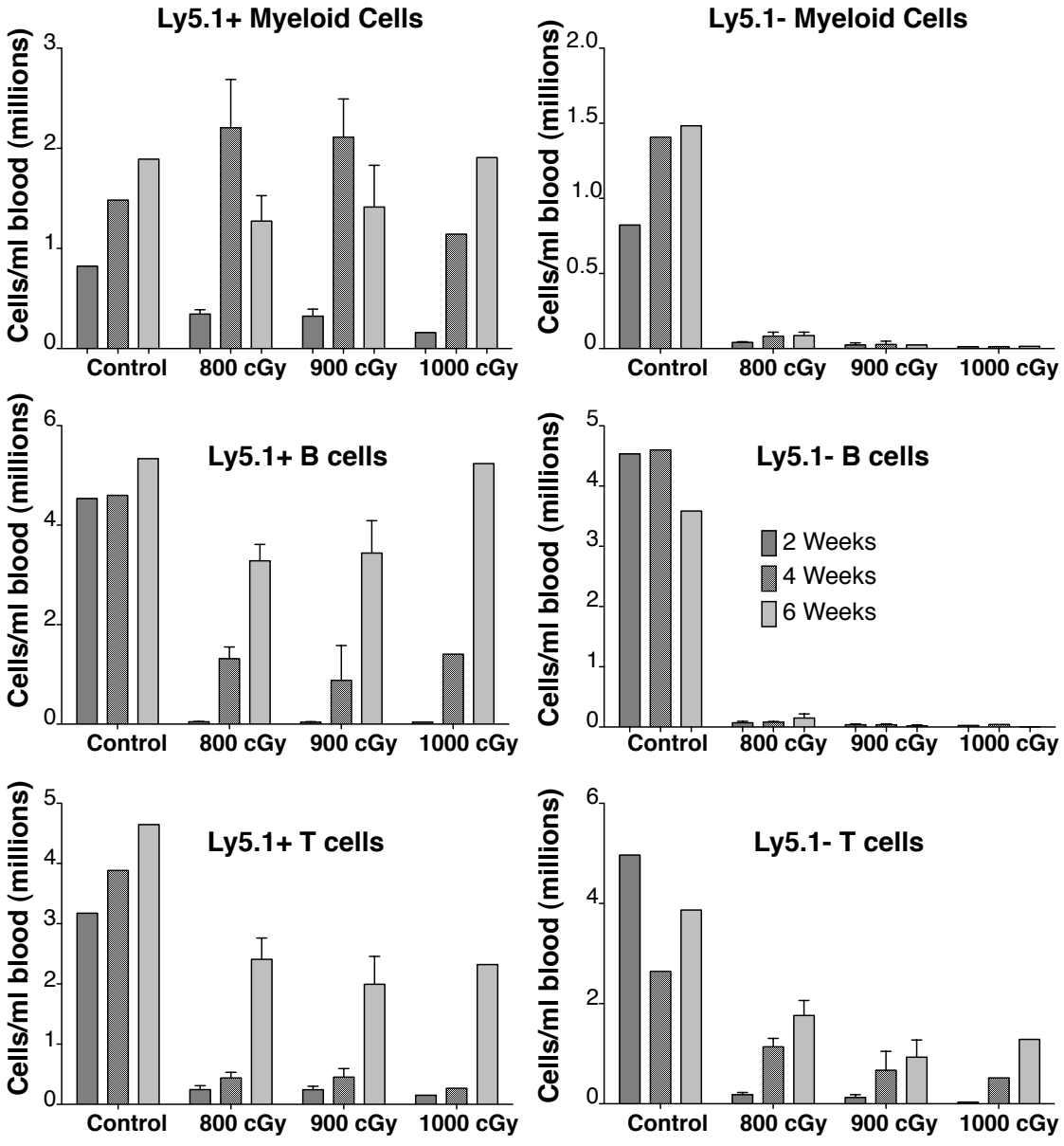
APPENDIX A

RECONSTITUTION EFFICIENCY OF LY5.1→IL-$10^{-/-}$ RADIATION SPLENIC CHIMERAS

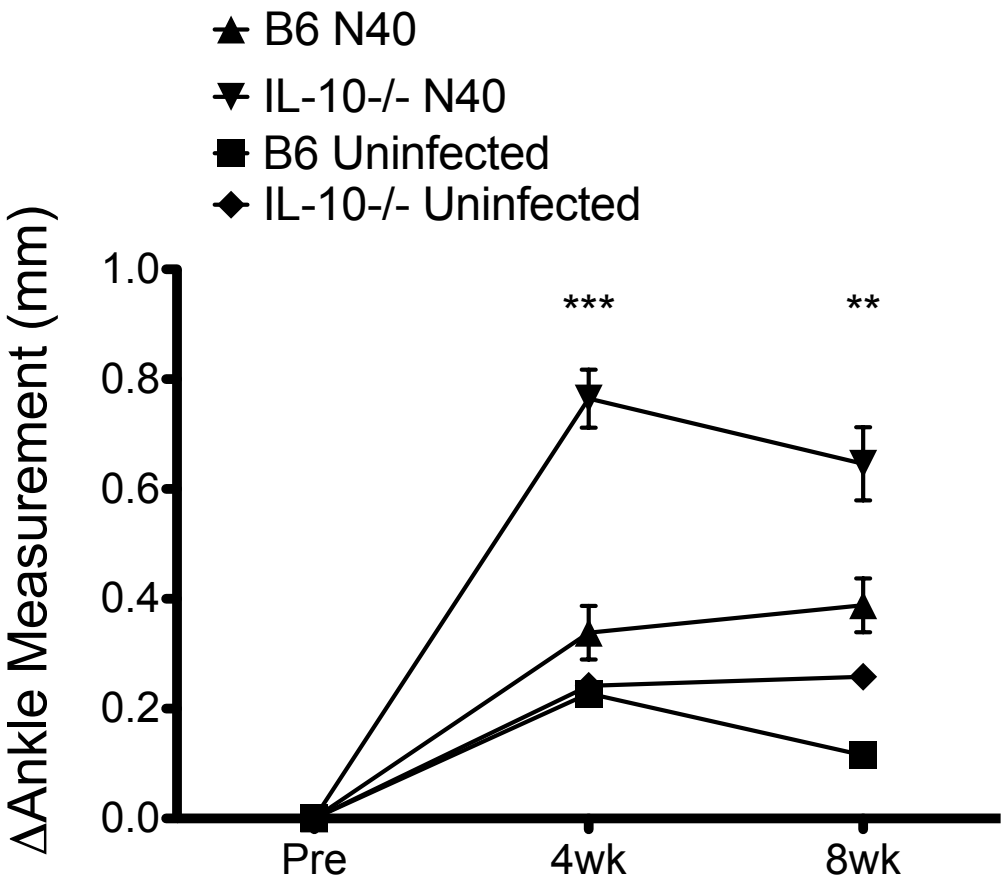
Figure A.1 IL-$10^{-/-}$ mice ($Ly5.1^{-}$) were lethally irradiated with the indicated dose of radiation, and transplanted with 2×10^{7} splenocytes 24 hours later. Control animals (one IL-$10^{-/-}$ $Ly5.1^{-}$ and one $Ly5.1^{+}$ WT) were not exposed to any radiation, but were used to indicate normal leukocyte numbers. Engraftment of donor-derived ($Ly5.1^{+}$) leukocytes was monitored at 2, 4, and 6 weeks following lethal irradiation by flow cytometry. Donor-derived blood leukocytes were identified as $Ly5.1^{+}$, while residual, IL-$10^{-/-}$ leukocytes were identified as $Ly5.1^{-}$. Lineage markers used to define myeloid cells, B cells, and T cells, were CD11b, B220, and CD3ε, respectively. Values for total cell numbers were calculated by multiplying the total leukocyte count by the frequency of each lineage. Bars represent the mean ± S.E.M.


Ly5.1+ Myeloid Cells
Ly5.1- Myeloid Cells
Ly5.1+ B cells
Ly5.1- B cells
Ly5.1+ T cells
Ly5.1- T cells
Cells/ml blood (millions)
Control
800 cGy
900 cGy
1000 cGy
2 Weeks
4 Weeks
6 Weeks

APPENDIX B

THE IL-10$^{-/-}$ MOUSE IS A MODEL OF SUSTAINED ARTHRITIS

Figure B.1 *Arthritis persist beyond 4 weeks in B. burgdorferi infected IL-10$^{-/-}$ mice*. Six week old B6 and IL-10$^{-/-}$ mice were infected with the clonal (N40) strain of *B. burgdorferi*, or sham infection. Immediately prior to infection, ankle measurements were obtained, and the value was subtracted from subsequent measurements to determine swelling. Swelling values from uninfected mice represent normal growth patterns. Ankle measurements were assessed in a blinded fashion. Points represent the mean change in ankle measurement ± S.E.M. Statistical significance obtained by one-way ANOVA and the Bonferoni post test is indicated by (*) and (**), and represent p values of > 0.05 and > 0.01 respectively. Ankle swelling at 8 weeks of infection was not significantly reduced or increased in infected IL-10$^{-/-}$ mice or B6 mice respectively.


B6 N40
IL-10-/- N40
B6 Uninfected
IL-10-/- Uninfected
ΔAnkle Measurement (mm)
1.0
0.8
0.6
0.4
0.2
0.0
***
**
Pre
4wk
8wk