Immunity and Tolerance in the K/BxN Model of Spontaneous Arthritis and Endocarditis

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DEDICATION

This dissertation is dedicated to:

My mother, Eva Cahill
You championed my every whim of higher education,
even when it became clear that I couldn't stop.
I'm profoundly thankful.

and

My husband, Dr. Steven Haasken
The sacrifices you made for my career and happiness
are a weight not many could grasp.
I marvel at how you hold on.

ABSTRACT

Immunological tolerance is achieved through multiple mechanisms, including the dominant tolerance exerted by regulatory T cells as well as the induction of anergy in potentially autoreactive lymphocytes. Here, we investigated the role of two molecules of interest, β2 integrins and the class a macrophage scavenger receptor (Msr1), in the pathogenesis of systemic autoimmune disease utilizing the K/BxN TCR transgenic mouse model of spontaneous autoimmune arthritis and endocarditis.

Genetic absence of β2 integrins had no effect on the severity of arthritis and unexpectedly increased the extent of cardiovascular pathology. The exaggerated cardiac phenotype of the β2 integrin-deficient K/BxN mice was accompanied by immune hyperactivation and was linked to a defect in regulatory T cells. These findings are consistent with a model in which the development of arthritis in K/BxN mice relies primarily on autoantibodies, whereas endocarditis depends on an additional contribution of effector T cells.

K/BxN mice lacking Msr1 had decreased incidence and severity of arthritis. Protection from disease was associated with undetectable levels of antibodies recognizing the key autoantigen in this model, GPI, despite the presence of a population of GPI-specific B cells. Msr1-deficient macrophages displayed impaired uptake of GPI, and Msr1-deficient mice had an elevated concentration of circulating GPI. These data suggest a model in which deficiency of Msr1 can lead to a higher concentration of a

soluble autoantigen, resulting in alterations in B cell tolerance and protection from autoimmune disease.

Together, these studies contribute to our understanding of the different layers of immunological tolerance responsible for protecting against spurious responses against self.

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CHAPTER ONE

Introduction

I. BENEFITS AND DANGERS OF A DIVERSE IMMUNE REPERTOIRE

The purpose of the immune system is to defend the host against an ever-evolving variety of pathogens. Such protection is achieved through the collaboration of innate and adaptive immune elements. Specifically, cells and molecules of the innate immune system swiftly detect and respond to potential threats by ligation of their pattern recognition receptors to a discrete set of highly conserved pathogen-associated molecular patterns. When innate immune attempts to neutralize the pathogen are prolonged or unsuccessful, the adaptive immune system is engaged. In contrast to their innate counterparts, adaptive immune cells have the capacity to generate antigen receptors of myriad specificities due to recombinase-activating gene (RAG) expression, as well as the ability to form immunological memory, which makes them formidable weapons in the immune arsenal. However, the consequence of having lymphocytes that can recognize such a broad scope of targets is the potential to elicit *horror autotoxicus* as a result of generating clones specific for self-antigens.

Multiple layers of protection against autoreactive lymphocytes exist. Indeed, as new immune cells are continually produced, the establishment and maintenance of self-tolerance is a lifelong necessity. As such, these safeguards are often categorized as either central or peripheral tolerance, where the former refers to security checkpoints in lymphocyte development and the latter denotes tolerance mechanisms outside the primary lymphoid organs. Unfortunately, the increasing prevalence of organ-specific and systemic autoimmune diseases is demonstrative that tolerance can and does fail [1-4]. A

more thorough understanding of the regulatory mechanisms governing immunological tolerance would benefit the development of therapies that seek to exploit the immune system for the treatment of autoimmune diseases, cancer, and infectious diseases.

II. LYMPHOCYTE DEVELOPMENT AND CENTRAL TOLERANCE

T cells

T cells and B cells arise from multipotent progenitors in the bone marrow. A subset of those progenitors will go on to settle in the thymus [5, 6], the anatomical site of T cell development and central tolerance induction. Upon gaining entry into the thymus at the cortico-medullary junction [7], developing T cells migrate to the cortex where cortical thymic epithelial cells (cTEC) provide survival signals to those thymocytes that have productively rearranged their T cell receptors (TCR), resulting in the recognition of peptides presented by major histocompatibility molecules (pMHC) class I and II, a process termed positive selection [8-10]. The majority of cortical thymocytes nevertheless fails to interact with pMHC and undergoes negative selection [11, 12], the induction of apoptosis due to receptor neglect.

Once the presence of a functional TCR is established, positively selected thymocytes migrate to the medulla [13], where they are interrogated for safety by medullary thymic epithelial cells (mTEC). A subset of mTEC has the unique ability to present tissue-restricted antigens on MHC I and II via expression of the autoimmune regulator (AIRE) gene [14], effectively allowing them to screen thymocytes for autoreactivity. Medullary thymocytes that bind to self pMHC with low affinity are allowed to mature further and comprise the effector T cell repertoire. In contrast, those developing T cells that bind strongly to self pMHC are either negatively selected as potentially dangerous clones [15], or are selected to become regulatory T cells (Tregs)

[16-18]. The AIRE-dependent process of negative selection is so paramount to the maintenance of immunological tolerance that defects in the AIRE gene cause autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in humans [19, 20] and similar organ-specific autoimmunity in mice [14, 21, 22].

However, there are circumstances under which cells can be rescued from negative selection. An example of this is receptor editing, in which a non-productive or autoreactive TCR is internalized, the antigen receptor gene rearranges further, and an altered TCR is displayed on the cell surface for another opportunity to be positively selected [23, 24]. Additionally, in a departure from Burnet's clonal selection theory which posited "one lymphocyte - one antigen receptor", it has been demonstrated that developing thymocytes may escape negative selection by the expression of alternate endogenous TCRα [25-27] and TCRβ [28] chains, which rearranged subsequent or concomitant to a negatively-selecting TCR. Such dual receptor T cells are a means by which self-reactive TCR could bypass negative selection and pose an autoimmune threat in the periphery.

B cells

The subset of multipotent progenitors fated to differentiate into the cell lineage that gives rise to B cells experiences development and central tolerance in the bone marrow [29]. In a process similar to that of T cells, developing B cells must undergo a series of RAG-mediated recombination events of B cell receptor (BCR) heavy and light chains [30, 31] as they migrate toward the central axis of the bone marrow. The final

stage of development from immature to mature B cell occurs in the secondary lymphoid organs. In contrast to thymocyte development, B cell development in the bone marrow is largely antigen-independent. Instead, this highly ordered process relies upon the adhesive contacts and survival signals provided by bone marrow stromal cells [32-34]. Only those B cells that have generated in-frame, productive BCR recombinations are allowed to proliferate and express the BCR gene product, whereas cells with non-productive BCR must either make additional rearrangements or apoptose [35]. Mutations in key BCR signaling elements highlight the importance of BCR signaling in the selection of a functional and safe repertoire. For example, deficiency of Bruton's tyrosine kinase (Btk) in humans are associated with the primary immunodeficiency, X-linked agammaglobulinemia, in which B cells are unable to make antibody and are enriched for autoreactive clones [36-38].

Central tolerance may be imposed upon immature B cells once surface expression of IgM is achieved, the outcome of which depends upon the type and concentration of antigenic stimuli [39-41]. For instance, the strong binding of multivalent self-antigen to suface IgM results in either the clonal deletion of the immature B cell, or the induction of receptor editing similar to that in developing thymocytes [42, 43]. Defects in receptor editing have been implicated as contributing factors in both systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [38, 44-46]. Conversely, immature B cells whose surface IgM crosslinks soluble autoantigen are allowed to migrate to the periphery in a state of unresponsiveness termed anergy [47, 48]. B cells anergized thus are unable to

become activated by their cognate antigen, even with the provision of T cell help, and are thought to have a compromised lifespan [49].

Such tolerance mechanisms are not imposed on immature B cells whose BCR have a low affinity for non-crosslinking self-antigen, which are permitted into the periphery in "clonal ignorance" [50]. This allowance for potentially autoreactive lymphocytes does pose a certain risk, but there is evidence suggesting that these B cells actually have polyreactive BCR, which could explain the utility of such clones. Specifically, a BCR with a low affinity for autoantigen may also have a relatively higher affinity for a pathogen and therefore contribute to host-defense [51, 52]. Interestingly, most polyreactive B cells in humans are thought to be purged from the repertoire at the checkpoint from pre-B to immature B cell [53], but patients with RA and SLE have been shown to have defects in the elimination of polyreactive clones at this transition [54, 55].

III. PERIPHERAL TOLERANCE

Once T and B lymphocytes emigrate from the primary lymphoid organs into the periphery, their activities are subject to regulation by the various mechanisms of peripheral tolerance. The subtlest means of peripheral tolerance is the maintenance of an environment that supports lymphocyte ignorance of particularly dangerous autoantigen. This is achieved by the careful orchestration of signals governing lymphocyte circulation and trafficking [56-58], the exclusion of lymphocytes from immune-privileged organs, discreet tissue-specific antigen presentation, and the clearance of apoptotic debris. Indeed, alterations in this environment can contribute to a breakdown in self-tolerance. Impaired apoptotic clearance, for example, has been implicated in the pathogenesis of SLE [59, 60].

The direct suppression of lymphocytes and antigen presenting cells by Tregs, a subset of CD4+ T cells defined by expression of the transcription factor Foxp3 [61, 62], is considered dominant tolerance. Natural Tregs (nTregs) are generated in the thymus, but Tregs may also be induced in the periphery under certain conditions (iTregs) [63-65]. The suppressive effects of Tregs are thought to be exerted both through direct contact with inhibitory molecules, such as CTLA-4 [66] and PD-1 [67, 68], as well as through secreted factors like TGFβ [69-71]. In addition to restraining spurious immune responses against self, Tregs serve to limit immune responses to pathogens and maintain a state of tolerance to commensal bacteria [72]. Mutations in the gene encoding Foxp3 result in the severe autoimmune disease, IPEX (immune dysregulation, polyendocrinopathy,

enteropathy, X-linked syndrome) in humans [73] or Scurfy in mice [74], which is phenotypically similar to APECED.

Deletional mechanisms of tolerance also exist in the periphery and are similar to those of central tolerance. Chronic engagement of self-pMHC or strongly crosslinking antigen can trigger clonal deletion in T and B lymphocytes, respectively [75, 76]. This occurs through a combination of binding of Fas by FasL and the Bim-dependent activation of mitochondrial death pathways [77, 78]. Defects in either of these apoptotic mechanisms are known to cause lupus-like disease in mice [79, 80], and humans bearing mutations in Fas/FasL develop autoimmune lymphoproliferative syndrome (ALPS).

As in the primary lymphoid organs, lymphocytes which encounter autoantigen in the periphery can also be rendered anergic. The fate of deletion versus anergy induction seems to depend upon the context in which the antigenic stimuli are perceived. Several lines of evidence support multi-step models of lymphocyte activation which hold that antigen receptor engagement (signal 1) and costimulation (signal 2) are required for an appropriate immune response [81, 82]. Thus, the reception of signal 1 in the absence of signal 2 is thought to induce the hyporesponsive state of anergy [49, 83-85].

IV. MODELS OF AUTOIMMUNITY

The use of animal models of autoimmune diseases, beginning in the late 1950s [86, 87], represents a critical advancement in our understanding of the underlying genetic, environmental, and effector mechanisms at work. Previous to this, there had been no means by which to reproducibly test the hypotheses arising from the clinical characterizations of human autoimmune diseases that had long since been described. The development of appropriate animal models of human diseases remains a contemporary barrier to the creation of therapeutic interventions, as none fully recapitulates the pathogenesis observed in humans.

The first murine models to approach the complexities of systemic autoimmune diseases such as RA were restricted to individual target tissues. These models include the widely-studied antigen-induced arthritis [88] and collagen-induced arthritis [89], which emerged around the 1980s, and the mouse model of rheumatic heart disease [90] that was described nearly two decades later. Although each was an invaluable stepping-stone in the advancement of the field at the time, these models also have a number of inherent limitations. Specifically, all rely upon the inoculation of animals with an antigen in the presence of an adjuvant to initiate disease and none of them involve multiple tissue types, which is the hallmark of systemic autoimmune diseases.

In contrast, the K/BxN TCR transgenic mouse model of RA represents the only known animal model that involves autoimmune pathology in both the synovial joints and the cardiovascular system, as is seen frequently in humans with systemic autoimmune

diseases [91-93]. Additional advantages of this model include the spontaneous nature of the autoimmunity and that the system is highly genetically manipulable, making it well-suited to gene knockout studies. Ostensibly, the transgenic monoclonal nature of the K/BxN model is the only artificial aspect, of which all experimental disease models have some, in an otherwise unique and powerful tool.

V. SUMMARY OF THIS DISSERTATION

The objective of the studies presented in this dissertation was to further dissect the means by which the immune system provokes tissue-specific pathology amid ongoing systemic autoimmune disease, with the long-term goal of identifying therapeutically targetable pathways. Examples of this phenomenon include human patients with RA and SLE who develop cardiovascular pathology concurrent to arthritis [94, 95]. To address the question of how lymphocytes fueled by chronic inflammation can exert such discreet and disparate effects, we took advantage of the genetically tractable K/BxN TCR transgenic mouse model of spontaneous autoimmune arthritis [91] and cardiac valve inflammation (endocarditis) [93]. First, we asked whether differential usage of the β2 integrin family of adhesion molecules could account for the trafficking of immune cells to one target organ (joints) versus the other (heart) by introducing different β2 integrin-null alleles into K/BxN mice (Chapter 2). Interestingly, we found that absence of all β2 integrins (CD18-/- K/BxN) greatly augmented the cardiac disease without affecting the course of arthritis. This phenotype was found to be due to defects in Tregs, which revealed a novel role for CD4+ T cells in the pathogenesis of endocarditis. Second, we explored the role of apoptotic clearance in the breakdown of tolerance in the K/BxN model by generating K/BxN lacking the class a macrophage scavenger receptor (Msr1-/- K/BxN) (Chapter 3). Surprisingly, we found that Msr1-deficiency greatly reduced the incidence and severity of arthritis in K/BxN mice. Our data suggest that this protection from autoimmunity was due to alterations in antigen-specific B cells arising from

unusually high levels of the soluble form of the K/BxN model autoantigen, glucose 6-phosphate isomerase (GPI), indicating that Msr1 normally acts to remove GPI from the circulation.

Together, the observations herein demonstrate the potency of some of the different layers of immunological tolerance as they drive either immunity or tolerance in a robust model of spontaneous autoimmune disease. A deeper understanding of the breakdown and maintenance of tolerance will be necessary in the successful design of immune-based therapies, which continue to garner favor in the scientific community.

CHAPTER TWO

Absence of β2 Integrins Impairs Regulatory T Cells and Exacerbates $CD4^+$ T cell-Dependent Autoimmune Carditis

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I. INTRODUCTION

Leukocytic infiltration of multiple tissue types is the hallmark of systemic autoimmune diseases. The cardiovascular system is frequently targeted in patients with systemic autoantibody-associated disorders, leading to increased morbidity and mortality. Well-known examples include the association of coronary artery inflammation with rheumatoid arthritis (RA) and systemic lupus (SLE) and the occurrence of cardiac valve inflammation (endocarditis) in patients with rheumatic fever, SLE, antiphospholipid antibody syndrome, and occasionally RA [94-98]. How is the cardiovascular system targeted for attack in these disorders? More broadly, in systemic autoimmune diseases, are different immunopathogenic mechanisms at work in the various target organs?

We have taken advantage of the co-existence of autoimmune endocarditis in the K/BxN TCR transgenic mouse model of arthritis to begin to address these questions [93]. In this model, T and B cell autoreactivity against the ubiquitously-expressed antigen glucose-6-phosphate isomerase (GPI) results in the sustained production of high-titer arthritogenic anti-GPI antibodies [91, 92]. Interruption of the immunologic events leading up to anti-GPI autoantibody production prevents inflammation in both the joints and the heart. For instance, mice lacking B cells develop disease in neither tissue [93]. However, the pathogenic effector mechanisms in the two target organs diverge downstream of autoantibody production. Specifically, arthritis in K/BxN TCR transgenic mice relies on complement component C5 but not activating Fc gamma receptors (FcγR), whereas endocarditis depends on activating FcγR but not C5. Additionally, although

arthritis can easily be transferred via injection of serum (containing anti-GPI antibodies) from a K/BxN TCR transgenic mouse into a naïve recipient, endocarditis cannot [93]. These findings support the notion that the immunopathogenic mechanisms responsible for end-organ inflammation in systemic autoimmune diseases can indeed vary between target organs in a single organism.

Particular attention has been paid to the role of $CD4^+$ T lymphocytes in the pathogenesis of rheumatic carditis. $CD4^+$ T cells are found in the valve lesions in humans and in animal models, and there is much interest in whether the self-antigens they recognize are structural mimics of bacterial peptides [97, 99]. Whether $CD4^+$ T cells are necessary effectors in the pathogenesis of autoimmune carditis, however, has not been clearly defined. The K/BxN mouse model has allowed us to test directly whether $CD4^+$ T cells are required for the development of autoimmune carditis.

Inflammation entails the recruitment of leukocytes from the circulation into tissues. Cell surface adhesion molecules mediate leukocyte attachment to the vascular endothelium, a critical step in the inflammatory cascade. The β2 integrins are a major family of adhesion molecules expressed by cells of the hematopoietic lineage. The common beta chain of β2 integrins, CD18, heterodimerizes with one of four alpha subunits (CD11a, b, c, or d) to form functional receptors capable of binding a number of endothelial ligands, including ICAM-1 (CD54), as well as molecules of the extracellular matrix. In addition to their role in leukocyte adhesion to vascular endothelium, the β2 integrins participate in hemostasis, the formation of stable synapses between leukocytes, and other processes [100-102]. Highlighting the importance of these molecules during an

inflammatory response, deficiency of CD18 in humans and mice causes leukocyte adhesion deficiency syndrome, characterized by leukocytosis, increased susceptibility to infections, and impaired wound healing [102, 103]. Likewise, the β2 integrins participate in inflammation in the context of autoimmune diseases. Studies using different animal models of autoimmunity have indicated a role for one or more of the β2 integrins in promoting the development of type I diabetes, lupus-like disease, collagen-induced arthritis, experimental autoimmune encephalomyelitis (EAE), colitis, and psoriasis [104-111]. Most relevant to the present study, mice lacking CD11a or CD18 are protected against the development of K/BxN serum-transferred arthritis [112]. Considered in sum, these studies suggest that differential usage of β2 integrin heterodimers directs leukocytes from the blood to different target tissues, the specificity of which might depend both on the anatomic site as well as the nature of the inflammatory stimulus.

Leukocyte adhesion to the vascular endothelium is not, however, the only mechanism by which β2 integrins contribute to autoimmune disease pathogenesis. Interestingly, the development and function of regulatory T cells (Tregs) is compromised in mice lacking β2 integrins, leading to impaired peripheral immunological tolerance. In a model of autoimmune colitis, deficiency of CD18 led to reduced numbers of Tregs in the secondary lymphoid organs and increased disease severity [113], a phenotype that others have suggested is due to absence of LFA-1 (CD11a:CD18) [114]. Furthermore, in a model of psoriasis, Tregs derived from mice expressing a hypomorphic variant of CD18 demonstrated decreased proliferation and impaired suppressive function, attributed to reduced cell-to-cell contact [110]. Understanding how β2 integrins contribute to the

pathogenesis of autoimmune diseases therefore requires consideration of not only their role in recruiting leukocytes to inflamed tissues, but also their capacity to help maintain immunological tolerance. Here we explored the contribution of $CD4^+$ T cells and β2 integrins to the development of arthritis versus endocarditis in the K/BxN mouse model.

II. MATERIALS AND METHODS

Mice. KRN T cell receptor (TCR) transgenic mice on the C57BL/6 background [91] were a gift from Drs. Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA) and the Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France); C57BL/6 mice congenic for $H2^{g7}$ (B6.g7) [93] were also a gift from Drs. Mathis and Benoist. CD18 null mice on the C57BL/6 background (*Itgb2*^{tm2Bay}, stock number 003329) [103], originally obtained from The Jackson Laboratory (Bar Harbor, ME), were a gift from Dr. Yoji Shimizu at the University of Minnesota; CD11a (*Itgal*^{tm1Bll}, stock number 005257) [115], CD11b (*Itgam*^{tm1Myd}, stock number 003991) [116], and *Rag1*-deficient mice (*Rag1*^{tm1Mom}, stock number 002216) on the C57BL/6 background were purchased from The Jackson Laboratory and bred in our specific-pathogen-free colonies. The β2 integrin-deficient "K/BxN" lines were created by breeding mice bearing the appropriate β2 integrin knockout allele on the C57BL/6 background to KRN/B6 mice and also to C57BL/6 mice congenic for the NOD-derived MHC, A^{g7}. The MHC is the only NOD-derived genetic region the mice retain; to simplify nomenclature, however, we refer to the mice as "K/BxN" throughout this study as we have previously [93]. Genotyping was performed by PCR and confirmed by flow cytometry. All studies were conducted in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Minnesota (protocol numbers 0611A96106 and 0909A72086).

Flow Cytometry. Monoclonal antibodies (mAbs) used for flow cytometry included anti-Vβ6 (clone RR4-7), CD4 (RM4-5), CXCR5 (2G8), CD138 (281-2), CD16/32 (2.4G2), CD162 (2PH1) and isotype control antibodies IgG1 (R3-34), IgG2a (R35-95), IgG2b (A95-1) purchased from BD Pharmingen, and CD44 (IM7), CD45R (RA3-6B2), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD278 (7E.17G9), and isotype control antibodies IgG2a (eBR2a), and IgG2b (eB149/10HS) from eBioscience. Intracellular staining for $CD25^+$ (PC61.5) $Foxp3^+$ (FJK-16s) cells was performed per the manufacturer's protocol (eBioscience). Flow cytometry was performed using a FACSCalibur and an LSRII (BD Bioscience) and cells were analyzed using FlowJo V7.6 software (Tree Star, Inc.).

Histology. Histological sections of cardiac valves were prepared using hearts snap-frozen in optimal cutting temperature (OCT) compound, and 10 μm cryosections were prepared using a Leica CM305 S cryostat. Tissues were stained with hematoxylin and eosin (H&E) using standard protocols and imaged with an Olympus BX51 microscope or (where noted) a Leica DM5500 B stitching microscope. Mitral valve thickness was determined for each heart by measuring the thickest point of the valve in serial sections, as described [93]. Aortic valve, pericardial, and coronary artery inflammation were assessed by examining serial sections containing each structure, and scored for the presence or absence of inflammation.

Immunohistochemistry. Following Fc receptor blocking with anti-CD16/32 (clone 93, eBioscience) and anti-CD64 (clone N-19, Santa Cruz Biotechnology) antibodies, heart sections were stained with biotinylated antibodies recognizing CD4 (L3T4), CD11a (M17/4), CD54 (ICAM-1, clone 3E2), and isotype control antibodies IgG2a (R35-95), IgG2b (A95-1), and IgG1 (A19-3) purchased from BD Pharmingen; anti-CD11c (N418), CD18 (M18/2), Foxp3 (FJK-16s), and IFN-γ (R4-6A2), were from eBioscience. Unconjugated anti-IL-17 (E-19) and secondary biotinylated IgG from Santa Cruz Biotechnology were used in combination. Biotinylated antibodies were detected using the Vectastain ABC-AP kit with either the Vector Red substrate I or the ImmPACT DAB substrate kit (Vector Labs) and counterstained with DAPI or hematoxylin to visualize nuclei. Imaging was performed using an Olympus BX51 fluorescent microscope equipped with a digital camera and DP-BSW software (Olympus).

Assessment of arthritis and IgG titers. Arthritis was measured and total serum IgG and anti-GPI titers as well as IgG subtypes were determined as described [117, 118].

$CD4^+$ T cell Depletion. Within two days of the onset of arthritis, K/BxN mice were treated with 30 or 60 μg of purified monoclonal anti-mouse CD4 (GK1.5) or IgG2b isotype control antibodies (eBioscience) via intraperitoneal injection for three consecutive days. Mice were then aged for four weeks and assessed for arthritis and carditis as described above. The different doses reflect two different experiments; no

difference in response was observed between the two doses, so the results were combined.

Serum-Transferred Arthritis. Pooled serum (100 μL/dose) from CD18-sufficient or –deficient K/BxN mice was injected intraperitoneally into 6-week-old C57BL/6 recipient mice on days 0 and 2. Recipient mice were allowed to develop arthritis for three weeks, after which time their hearts were harvested and assessed for carditis as described above.

Mixed Bone Marrow Chimeric Mice. 6-week-old *Rag1*-deficient mice were sublethally irradiated with 300 Rad. Four hours after irradiation, $10x10^6$ bone marrow cells from either K/BxN, CD18-deficient K/BxN, or a mixture thereof, were injected retro-orbitally. Bone marrow chimeric mice were maintained on sulfamethoxazole and trimethoprim administered in drinking water for the duration of the study. Two months following bone marrow transplantation, the lymphoid organs and hearts were harvested for analysis.

Statistical Analysis. Statistical differences between mean values for groups were calculated using the Student's 2-tailed t-test. One-way ANOVA followed by Tukey's multiple comparison test was used to assess differences in the extent of cardiac pathology between integrin-sufficient and -deficient mice.

III. RESULTS

K/BxN carditis depends on sustained presence of $CD4^+$ T cells

The predominant inflammatory cell types differ in the synovial joints versus the cardiac valves in K/BxN TCR transgenic mice. Whereas neutrophils are the main cell type found in the arthritic joints [91], the valve-infiltrating cells are comprised primarily of mononuclear cells including $CD4^+$ T lymphocytes [93]. This apparent difference in effector cell types led us to explore an additional requirement for $CD4^+$ T cells in the pathogenesis of endocarditis beyond the initiation of the autoimmune response. Previous studies have shown that depletion of $CD4^+$ T cells in K/BxN mice before the onset of arthritis prevents its development, whereas depletion of $CD4^+$ T cells after arthritis onset had no effect on its severity [91] (**Figure 2.1 A**). In stark contrast, we found that depletion of $CD4^+$ T cells after the onset of arthritis in K/BxN mice significantly decreased the severity of cardiac valve inflammation (**Figure 2.1 B**), demonstrating a critical requirement for a sustained $CD4^+$ T cell presence in the pathogenesis of endocarditis. The valve lesions were characterized by a prominence of the Th1 effector cytokine IFNγ, along with some IL-17 (**Figure 2.2**). We did not detect IL-4 in the valve lesions (not shown). Thus, whereas autoantibodies are sufficient to provoke arthritis in this model, our earlier results [93] and these, considered together, suggest that both autoantibodies and $CD4^+$ T cells are required to provoke the cardiac pathology.

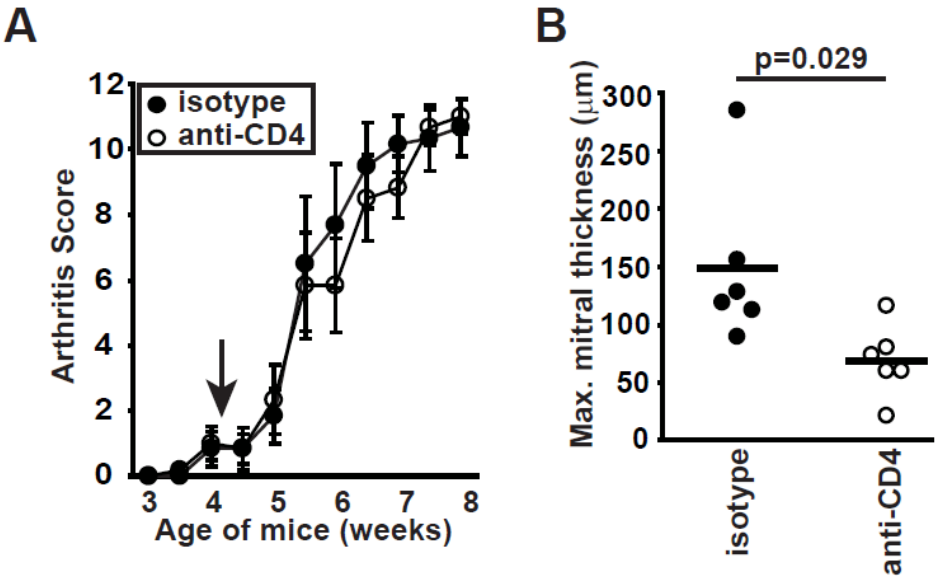


Figure 2.1. Depletion of CD4$^+$ T cells after arthritis onset protects K/BxN mice from endocarditis. (*A*) K/BxN mice were treated with purified monoclonal anti-mouse CD4 or isotype control antibodies starting within two days of the onset of arthritis (arrow) for three consecutive days. Mean arthritis scores (± SEM) are shown. (*B*) The maximal mitral valve thickness was determined via measurement of serial sections for each mouse. N=6 mice/group in two separate experiments.

Expression of β2 integrins and a common ligand in inflamed mitral valves of K/BxN mice

We were interested in determining how members of the β2 family of integrins contribute to cardiac pathology in K/BxN mice. Immunohistochemical analysis of inflamed mitral valves revealed the presence of CD18, CD11a, and CD11c (**Figure 2.3 A**), as well as CD11b expression [93]. Increased endothelial cell surface expression of specific ligands for β2 integrins occurs in the context of inflammation to facilitate leukocyte migration into the tissues. We next investigated whether the endothelium of K/BxN cardiac valves had an activated phenotype. Indeed, we observed increased expression of intercellular adhesion molecule-1 (ICAM-1), the primary counterreceptor for the β2 integrins, on inflamed K/BxN mitral valves compared with non-inflamed valves from TCR transgene-negative control mice (**Figure 2.3 B**). Thus, β2 integrins are expressed by the tissue-infiltrating leukocytes of K/BxN cardiac valves and under inflammatory conditions the endothelial cells lining the cardiac valves upregulate a main β2 integrin ligand. To determine the role of β2 integrins in the development of autoimmune endocarditis, we generated K/BxN TCR transgenic mice lacking CD18, CD11a, or CD11b.

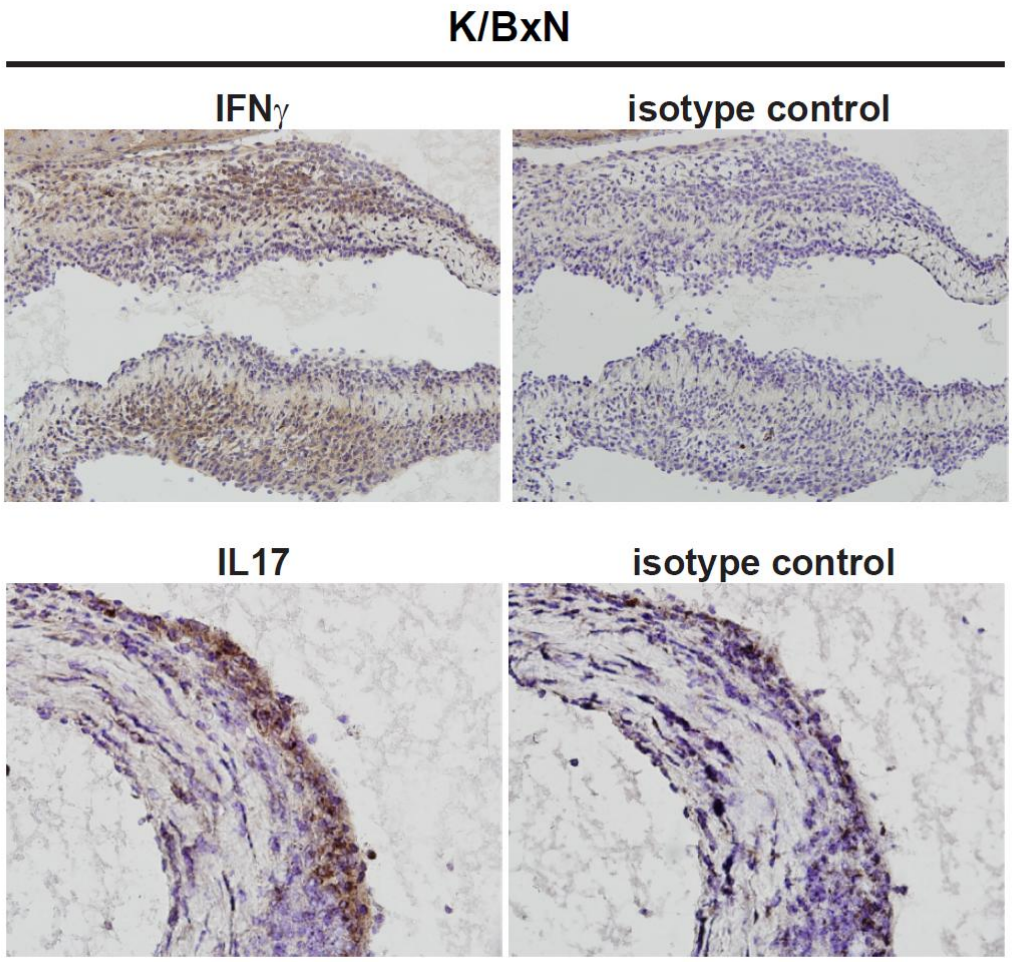


Figure 2.2. Valve-infiltrating CD4$^+$ T lymphocytes produce the effector cytokines, IFNγ and IL-17. Immunohistochemical staining of mitral valves of K/BxN mice demonstrates the presence of IFNγ (top) and to a lesser extent, IL-17 (bottom). Tissue sections were stained with biotinylated antibodies recognizing the indicated antigens (brown staining) present on tissue-infiltrating leukocytes, followed by hematoxylin (blue). Original objective: x40.

β2 integrins are dispensable for arthritis in K/BxN TCR transgenic mice

Based on the strict requirement for LFA-1 (CD11a:CD18) in the development of K/BxN serum-transferred arthritis, we anticipated that deficiency of CD18 in K/BxN TCR transgenic mice would similarly result in decreased arthritis severity [103, 112]. However, the absence of CD18, CD11a, or CD11b had no observable effect on the development of arthritis in the TCR transgenic mice (**Figure 2.4 A-F**). Moreover, we observed no differences in the pathogenic anti-GPI autoantibody titers (**Figure 2.4 G-I**). These findings suggest that deficiency of β2 integrins impairs neither the priming of KRN T cells nor their ability to provide help to GPI-specific B cells.

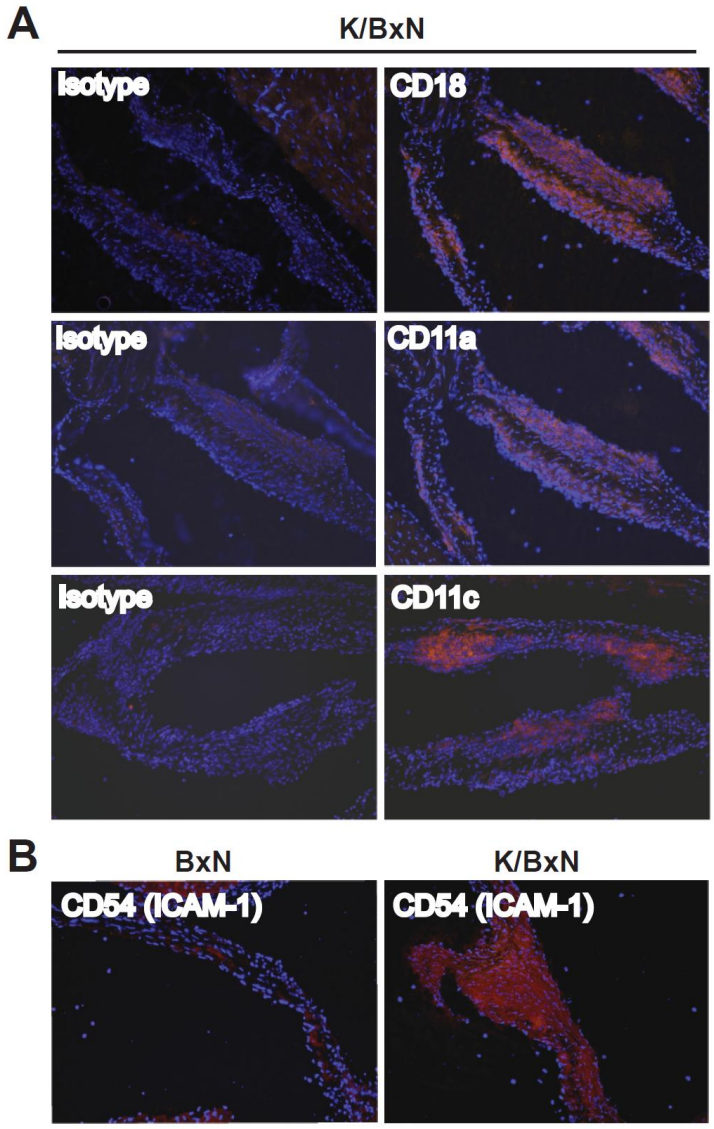


Figure 2.3. Expression of β2 integrins and ICAM-1 in the inflamed mitral valves of K/BxN mice. (*A*) Hearts of 8-wk-old K/BxN mice were sectioned longitudinally to expose the inflamed mitral valve. Tissue sections were stained with biotinylated antibodies recognizing antigens (red) present on tissue-infiltrating leukocytes or with appropriate isotype control antibodies as indicated, followed by DAPI to visualize nuclei (blue). Objective: x20. (*B*) Immunofluorescent comparison of ICAM-1 expression by normal control (BxN) versus inflamed (K/BxN) cardiac valves. Objective: x20.

Deficiency of CD18 exacerbates autoimmune carditis

Unexpectedly, we observed more severe cardiovascular disease in CD18-deficient K/BxN mice compared with CD18-sufficient littermates. The cardiac involvement in K/BxN mice is largely limited to the mitral valve with occasional aortic valve inflammation [93], consistent with what we observed in CD18-heterozygous K/BxN mice (**Figure 2.5**). Although the severity of mitral valve inflammation was not affected by CD18 deficiency (**Figure 2.7**), additional cardiac structures were inflamed. Specifically, we found that the absence of CD18 resulted in inflammation not only of the mitral valve (n=8/8 mice) but also the aortic valve (n=8/8), coronary arteries (n=5/8), and pericardium (n=5/8) (**Figure 2.5**). K/BxN mice lacking CD11a exhibited a similar yet less penetrant increase in the scope of carditis compared with CD11a-heterozygous littermates, whereas deficiency of CD11b did not increase the extent of the cardiac phenotype (**Figure 2.5 I**). Thus, although LFA-1 is essential for arthritis induced by the transfer of K/BxN serum [112], deficiency of β2 integrins in K/BxN TCR transgenic mice had no apparent effect on the course of arthritis and actually increased the extent of cardiovascular pathology, an effect attributable primarily to LFA-1. We next investigated the possible mechanisms by which the absence of CD18 augmented autoimmune endocarditis.

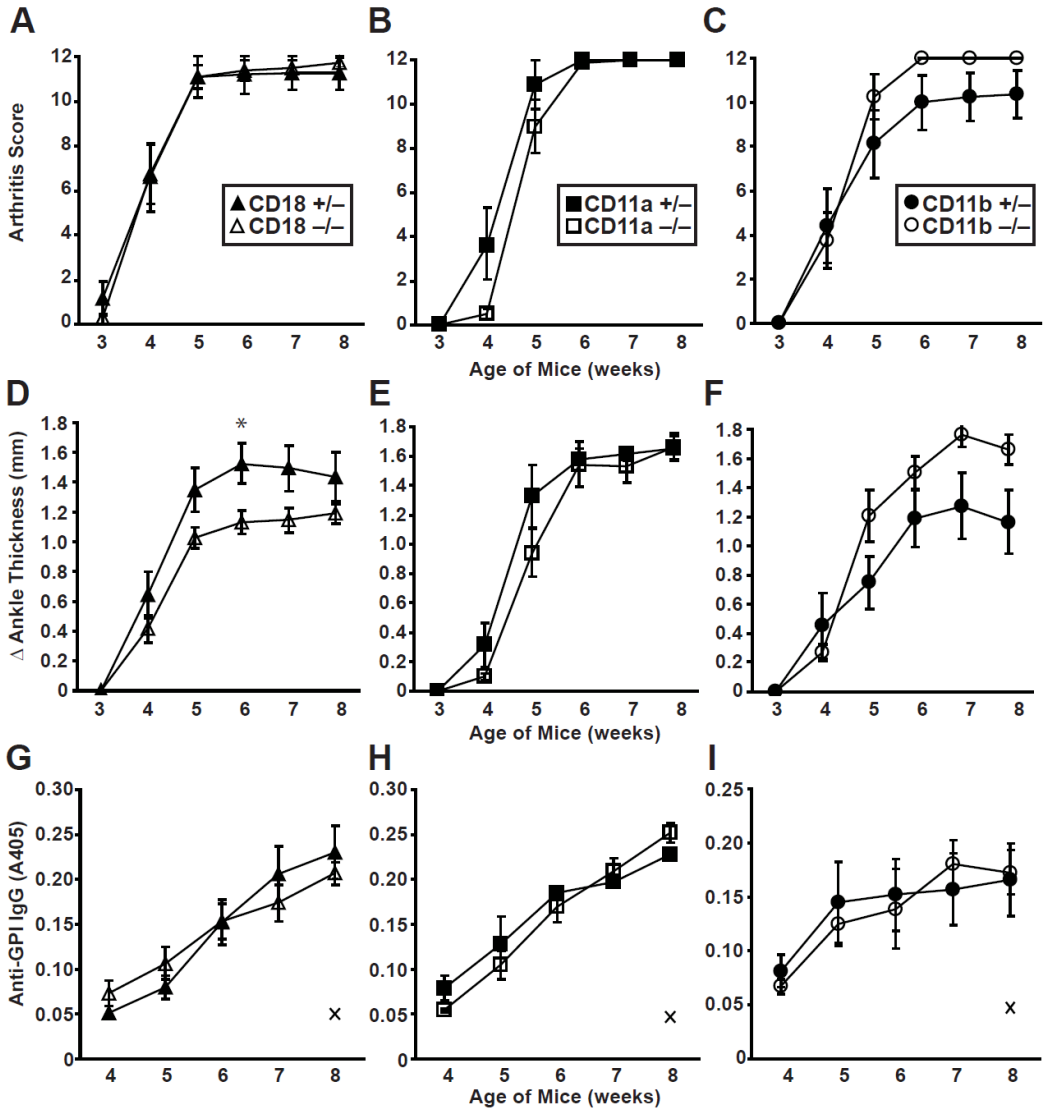


Figure 2.4. Arthritis development and autoantibody production are not impaired in K/BxN mice lacking β2 integrins. Clinical arthritis scores (*A-C*), changes in ankle thickness (*D-F*), and serum titers of anti-GPI IgG autoantibodies (*G-I*) were assessed in K/BxN mice expressing the heterozygous state (filled symbols) or lacking (open symbols) CD18 (*A, D, G;* n=11 mice/group), CD11a *(B, E, H;* n=8 mice/group), or CD11b (*C, F, I;* n=8 mice/group) at the indicated ages. Plotted values are mean ± SEM; *, $p<0.05$. The average anti-GPI titers at 8 weeks of age for non-arthritic integrin-deficient littermates lacking the KRN TCR transgene are provided as a reference and indicated by x in panels *G-I*.

Amplified immune response in the absence of β2 integrins

Given the critical role of $CD4^+$ T cells in the pathogenesis of K/BxN carditis, we first explored whether differences in T cell activation might underlie the more extensive cardiac involvement in β2 integrin-deficient K/BxN mice. Autoreactive $CD4^+$ T lymphocytes isolated from arthritic K/BxN mice have an activated phenotype consistent with their disease state [119]. We found that the expression of the T cell activation marker CD44 was further increased in the absence of CD18 (**Figure 2.6 A**). A similar hyperactivated state was noted among T cells from K/BxN mice lacking CD11a; however this phenotype was less apparent in CD11b-deficient mice (**Figure 2.6 B-C**). This increased expression of CD44 in CD18- and CD11a-deficient K/BxN mice echoed the exacerbated cardiovascular pathology noted in the same animals, potentially indicating a defect in T cell regulation in the absence of β2 integrins.

We also asked whether dysregulation of B cells could underlie the increased severity of the cardiovascular pathology seen in CD18-deficient K/BxN mice, as we have previously shown that activating Fcγ receptors (that bind IgG) are required specifically for the development of K/BxN endocarditis [93] and both K/BxN and CD18-deficient C57BL/6 mice are reported to have hypergammaglobulinemia [91, 103]. Indeed, the genetic combination of the KRN transgene and CD18-deficiency resulted in even more exaggerated hypergammaglobulinemia with levels of circulating total IgG were 4-5 times greater in CD18-/- K/BxN mice compared with CD18-sufficient K/BxN mice or with CD18-deficient KRN transgene-negative littermates (**Figure 2.6 D**). In contrast, mice lacking CD11a or CD11b had total serum IgG titers equivalent to control mice (**Figure**

2.6 E-F). Consistent with the fact that IgG1 is the predominant IgG subtype generated in K/BxN mice, we found that the elevated total IgG concentration in CD18-deficient K/BxN mice was due largely to increased levels of IgG1, although levels of IgG2b and IgG2c (the C57BL/6 variant of IgG2a [120]) were also increased relative to control CD18-sufficient K/BxN mice (**Figure 2.8 A**). Furthermore, although the titers of anti-GPI IgG were equivalent in CD18-deficient and –sufficient K/BxN mice (**Figure 2.4 G**), the distribution of IgG subtypes within the GPI-reactive antibody pool was altered in the CD18-deficient mice, with a shift toward IgG2b and 2c and away from IgG1 (**Figure 2.8 B**).

To address the possibility that these observed changes in IgG subtype distribution are responsible for the increased extent of cardiac disease in CD18-deficient K/BxN mice, we performed serum-transferred arthritis experiments using serum from CD18-sufficient or –deficient K/BxN mice. Mice that received CD18-/- K/BxN serum developed slightly more severe arthritis than those given the control K/BxN serum (**Figure 2.9 A-B**). Importantly, none of the mice developed cardiac pathology (**Figure 2.9 C**), suggesting that alterations in antibody production and/or subclass usage alone are not responsible for the increased extent of cardiac disease in CD18-/- K/BxN mice. These findings are consistent with the notion that autoreactive $CD4^+$ T cells are required in addition to autoantibodies for the pathogenesis of carditis (see **Figure 2.1**).

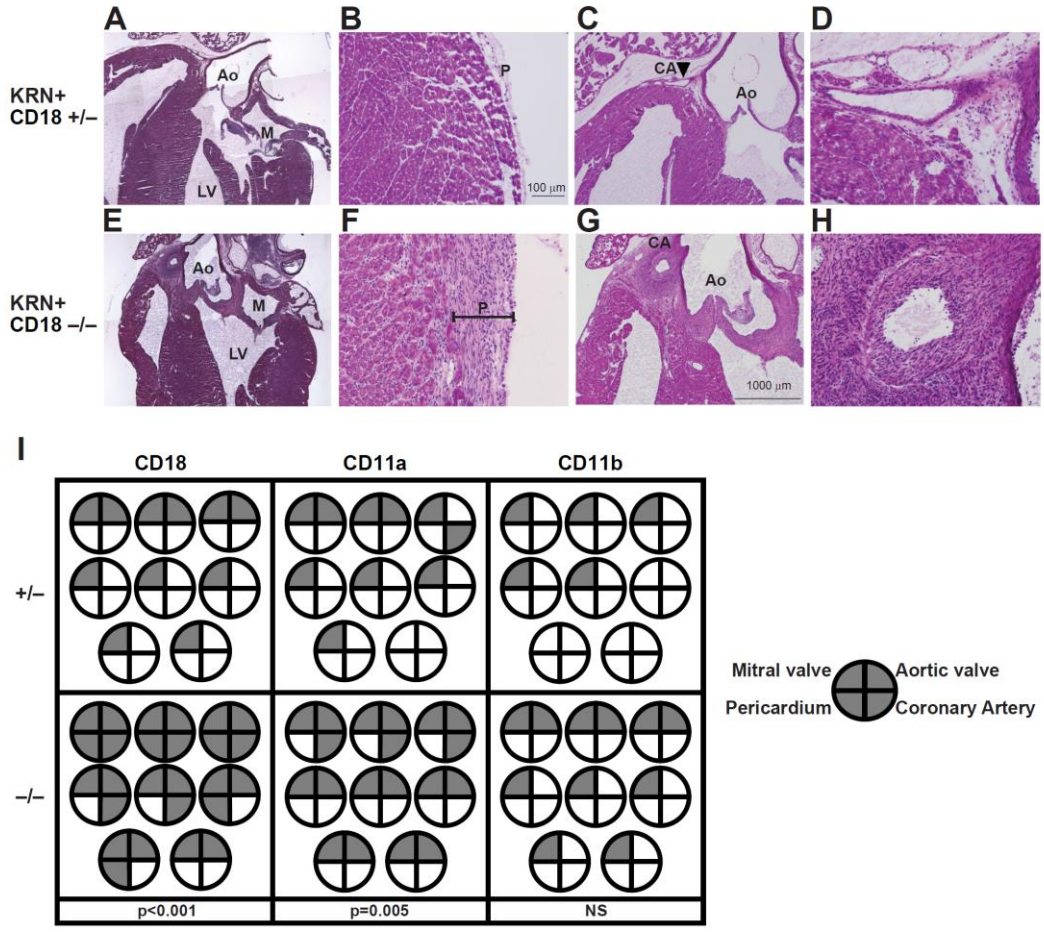


Figure 2.5. Augmented carditis in β2 integrin-deficient K/BxN mice. Histological analysis of the extent of cardiovascular disease in (*A*) CD18-sufficient K/BxN mice reveals inflammation limited to the mitral valve (stitching microscope, objective: x5) but sparing the (*B*) pericardium (objective: x20), (*C*) aortic valve (objective: x4), and (*D*) coronary arteries (objective: x20). In contrast, (*E*) CD18-deficient K/BxN mice exhibit marked inflammation of additional structures (stitching microscope, objective: x5) including (*F*) pericarditis (objective: x20), (*G*) aortic valve inflammation (objective: x4), and (*H*) coronary artery inflammation (objective: x20). (*I*) Distribution of cardiac inflammation in the three integrin-sufficient (top row) versus –deficient (bottom row) K/BxN mouse lines, where each pie represents one mouse and each wedge indicates inflammation of the cardiac structure designated in the key. Tissue sections stained with hematoxylin and eosin. Ao, aortic valve; CA, coronary artery; LV, left ventricle; M, mitral valve; NS, not significant; P, pericardium.

Regulatory T cell defect in CD18-deficient K/BxN mice

The hypergammaglobulinemia and increased T cell activation observed in CD18-deficient K/BxN mice prompted us to explore whether a more generalized defect in immune regulation existed. Accordingly, we found that CD18-deficient K/BxN mice had significant splenomegaly with a corresponding increase in total splenocyte numbers relative to CD18-sufficient K/BxN mice (**Figure 2.10 A-B**). We investigated whether a deficiency of Tregs might underlie this phenotype. Indeed, both the percentage and absolute number of Tregs in the lymph nodes of K/BxN mice lacking CD18 were less than half of those found in CD18-sufficient mice (**Figure 2.10 C-E**). The percentage of Tregs was also decreased in the spleens of CD18-deficient animals, indicating a deficit in the total numbers Tregs/mouse (**Figure 2.10 D-E**). Furthermore, although $Foxp3^{+}$ cells were easily identified in the cardiac valves of CD18-sufficient mice, we observed fewer of these cells in the valves of CD18-deficient K/BxN mice (**Figure 2.11**).

CD18-deficient K/BxN mice shared many phenotypic characteristics reported recently in K/BxN mice lacking all Tregs, due to the *scurfy* mutation (31). In particular, as reported in K/BxN *scurfy* mice [121], we found that CD18-deficient K/BxN mice had increased numbers of follicular T helper (Th) cells and extrafollicular Th cells relative to K/BxN mice (**Figure 2.12 A**), along with an exaggerated accumulation of plasma cells in the secondary lymphoid organs and decreased expression of the inhibitory Fc receptor FcγRIIB on the plasma cells (**Figure 2.12 B-D**). These data are consistent with an intrinsic Tregs defect in CD18-deficient K/BxN mice.

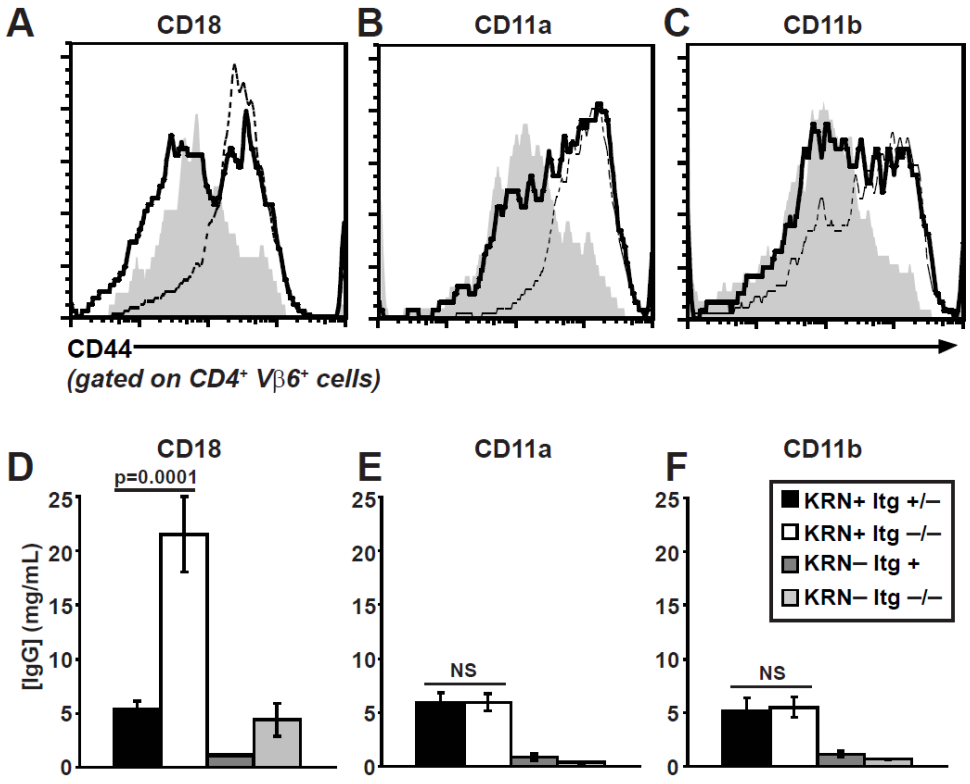


Figure 2.6. I**ncreased T cell activation and hypergammaglobulinemia in the absence of β2 integrins.** (*A-C*) Flow cytometric assessment of CD44 expression on $CD4^+V\beta6^+$ lymph node cells harvested from mice of the indicated genotypes (CD18-sufficient K/BxN, bold line; CD18-deficient K/BxN, dashed line; CD18-deficient KRN-, shaded). Data are representative of 3 independent experiments. Vβ6 is the KRN transgene-encoded TCRβ chain. (*D-E*) Serum IgG concentrations (mean ± SEM) were determined by ELISA performed on serum obtained from 8-week-old mice of the indicated genotypes. Data are representative of 8-12 mice/group, with the exception of KRN- Itg^+ for which there were 4 mice/group. P-values are indicated; NS=not significant; Itg, integrin.

To explore this possibility further, we generated mixed bone marrow chimeric mice. Specifically, we sought to determine whether wildtype (WT) K/BxN Tregs could exert dominant immunological tolerance and thereby constrain the increased cardiac pathology engendered by CD18 deficiency. Congruent with our genetic model, transplantation of WT K/BxN bone marrow alone resulted in less severe cardiovascular disease than did transplantation of CD18-deficient (KO) K/BxN marrow (**Figure 2.13 A**). Importantly, both the 50:50 (WT:KO) and 10:90 (WT:KO) mixed chimeras were protected from severe cardiac pathology relative to those mice that received only CD18 KO marrow (**Figure 2.13 A**), demonstrating a dominant tolerance-inducing effect of the WT bone marrow. These experiments also revealed a proliferative or survival disadvantage of the CD18-/- K/BxN T cells, as just a few hundred of the KO T cells were detected in the lymph nodes and spleens of the 50:50 chimeras and comprised only about 10% of the T cells in the 10:90 (WT:KO) chimeras (**Figure 2.13 B-C** *and data not shown*). Additionally, the presence of WT Tregs resulted in both the WT and KO effector T cell populations displaying a phenotype similar to WT K/BxN mice (higher CD62L, lower CD44) rather than the hyperactivated phenotype observed in CD18-deficient K/BxN mice (**Figure 2.13 D-E**), further supporting the dominant suppressive effect of the WT Tregs. These findings suggest that deficiency of CD18 impairs Tregs numerically and functionally, leading to increased activation of pathogenic effector $CD4^+$ T cells as well as hypergammaglobulinemia, and culminating in more extensive autoimmune carditis.

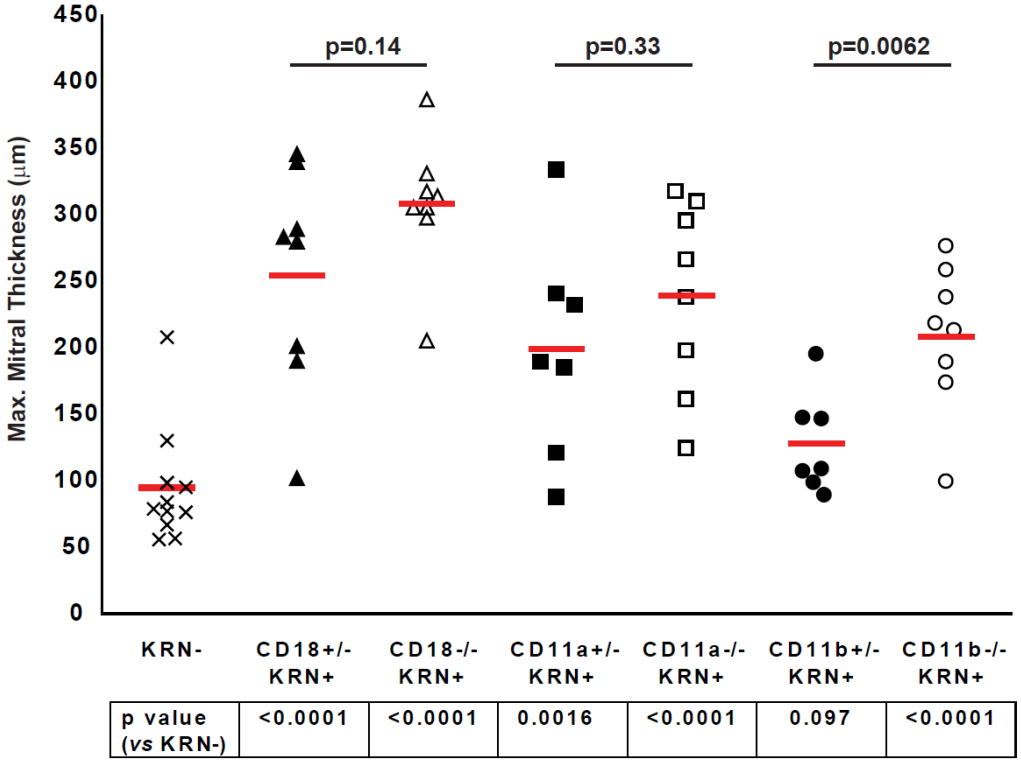


Figure 2.7. Absence of CD18 or CD11a does not increase thickness of mitral valve inflammation in K/BxN mice. Serial sections of mitral valves from mice of the indicated genotypes were measured at their thickest point, and the maximum valve thickness was plotted for integrin-deficient (open shapes) compared to integrin-sufficient K/BxN animals (filled shapes). The mitral valves of control non-arthritic BxN mice lacking both CD18 and the KRN transgene were also measured for reference (x). Each point represents one mouse; line indicates the mean. For each genotype, n=8. Note: the thickness of mitral valves of the CD11b-deficient K/BxN mice is statistically significantly greater than that of their CD11b-sufficient littermates because of less severe inflammation in the CD11b-sufficient animals; it is not greater than in the other strains, however.

IV. DISCUSSION

A better understanding of the disparate means by which systemic inflammation generates tissue-specific destruction is essential for the development of more directed therapeutic strategies for multi-organ autoimmune diseases. The genetic manipulability of the K/BxN TCR transgenic mouse model of spontaneous co-existing autoimmune endocarditis and arthritis affords the opportunity to dissect such mechanistic requirements. Here we have shown that $CD4^+$ T lymphocytes in addition to autoantibodies are required as effectors of autoimmune carditis. We have also shown that β2 integrins play a critical and unexpected role in limiting the extent of cardiovascular pathology via their influence on Tregs.

We have previously proposed that T cells contribute directly to the pathogenesis of endocarditis based on the finding that arthritis can be elicited by transfer of K/BxN serum, splenocytes, or bone marrow [91, 119], whereas endocarditis is only faithfully recapitulated with transplantation of bone marrow [93]. The present study supports this model. Specifically, depletion of $CD4^+$ T cells after the onset of arthritis protected K/BxN mice from endocarditis without affecting arthritis severity. Cumulatively, our findings suggest that autoantibodies alone are sufficient to induce arthritis, whereas the development of carditis in K/BxN mice depends on the additional sustained presence of effector $CD4^+$ T cells.

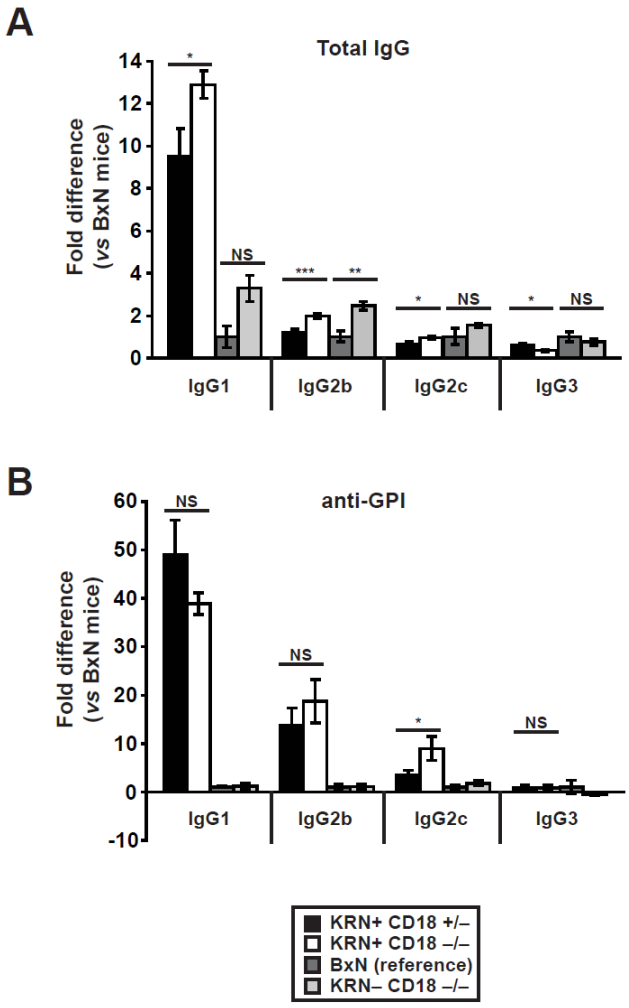


Figure 2.8. Total and GPI-specific IgG subtype production in CD18-deficient K/BxN mice. (*A*) Total serum IgG subtypes were analyzed by ELISA and normalized to BxN mice (dark grey, n=4). While IgG1 is the predominant isotype represented in CD18-sufficient K/BxN mice (black, n=12), those lacking CD18 (white, n=11), exhibited increases in IgG1, IgG2b, and IgG2c. Only levels of IgG2b were significantly increased in CD18-deficient KRN- (light grey, n=11) mice. (*B*) CD18-sufficient and –deficient K/BxN animals produced equivalent anti-GPI antibodies of the IgG1 and IgG2b isotypes, however, levels of anti-GPI IgG2c were significantly increased in CD18-deficient K/BxN mice. Values plotted are normalized means ± SEM; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; NS, not significant.

Our finding that CD18 deficiency resulted in more severe cardiac pathology was somewhat unexpected given the requirement for CD18 in the K/BxN serum-transferred arthritis model [112].

Although initial reports of CD18 null mice pointed to a potential defect in T cell priming [103], this appears not to impair the development of spontaneous autoimmunity in several models, now including K/BxN TCR transgenic mice. Rather, the β2 integrins seem to contribute critically to the development and/or function of Tregs in this and several other models of autoimmune or inflammatory diseases [110, 113, 114], resulting in exaggerated disease phenotypes. It is possible that deficiency of CD18 on effector T cells renders those cells less susceptible to Tregs-mediated inhibition, however the alteration in CD18-/- K/BxN effector T cell activation phenotype in the 10:90 (WT: KO) bone marrow chimera and less severe carditis indicates functional suppression of those cells. Tregs are critically required to exert control over the development of K/BxN arthritis [122], and genetic ablation of Tregs in the K/BxN TCR transgenic model (via introduction of the *scurfy* allele) results in more aggressive inflammatory arthritis [117], with a corresponding increase in follicular and extrafollicular Th cells, and an aberrant accumulation of plasma cells in the secondary lymphoid organs [121]. Although we did not observe increased arthritis severity in the absence of CD18, we did see similar increases in the Th subsets as well as the noted plasma cell abnormalities. That we observed no difference in arthritis severity due to CD18 deficiency likely reflects the fact that the *scurfy* mutation eliminates all $Foxp3^+$ Tregs, whereas the CD18-deficient mice retain some Tregs.

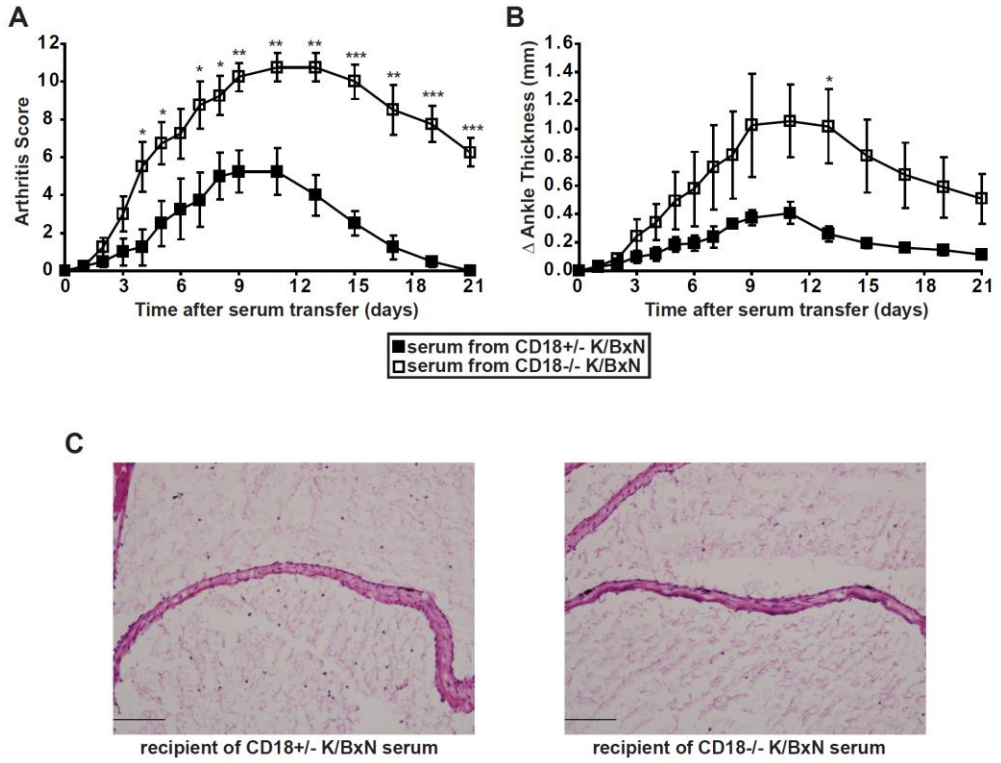


Figure 2.9. Transfer of serum from CD18-/- K/BxN mice does not generate cardiac pathology. Serum pooled from CD18-sufficient or –deficient K/BxN mice was injected into C57BL/6 mice on days 0 and 2. Arthritis was assessed via assignment of (*A*) clinical scores and (*B*) measurement of ankle thickness. Data plotted are mean ± SEM (4 mice/group); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (C) Representative photomicrographs of mitral valves from mice that received CD18-sufficient (left) and –deficient (right) K/BxN serum. Bar = 100 μm; Objective: x20.

The requirement for B cells, CD40, and FcRγ in the development of endocarditis suggests that autoantibodies contribute to the cardiac pathology via engagement of activating FcRs [98]. Although the majority of anti-GPI antibodies produced by K/BxN mice are of the IgG1 subtype [91], we were intrigued to find that deficiency of CD18 resulted in increased production of IgG2b and IgG2c along with more severe cardiac disease, potentially indicating a role for FcγRI and/or FcγRIV, the activating FcRs that bind these IgG subtypes preferentially, in the pathogenesis of endocarditis in this model [123]. However, it seems unlikely that hypergammaglobulinemia and altered IgG subtype usage alone are responsible for the more severe cardiovascular disease in the CD18-deficient K/BxN mice, because the CD11a-deficient K/BxN mice had more severe carditis without hypergammaglobulinemia, and transfer of CD18-/- K/BxN serum failed to provoke cardiac pathology. These findings underscore our interpretation that carditis in K/BxN mice depends on both autoantibodies and effector $CD4^+$ T cells.

That our results differ from the previous report that LFA-1 (CD11a:CD18) is required for the development of arthritis induced by injection of serum from K/BxN mice [112] highlights the inherent differences between the K/BxN TCR transgenic model and its derivative model, serum-transferred arthritis. We have previously reported a similar difference between the two models in mice lacking FcRγ, the signaling chain shared by activating Fc receptors: that is, complete protection from serum-transferred arthritis but no effect on arthritogenesis in the transgenic system [93]. These differences likely reflect the fact that the serum-transferred arthritis model interrogates the effector mechanisms linking autoantibodies to the development of arthritis, in the absence of an ongoing adaptive autoimmune response. In contrast, K/BxN TCR transgenic mice have a sustained adaptive autoimmune response characterized by the presence of autoreactive T and B lymphocytes and substantially higher titers of anti-GPI autoantibodies than those achieved in the serum transfer system. Thus, in the K/BxN serum transfer arthritis system, it is likely that absence of LFA-1 impairs neutrophil migration to the joints in response to the injected autoantibodies [112]. Indeed, LFA-1 expression by neutrophils was recently demonstrated to be crucial in the serum transfer model [124]. In contrast, in the K/BxN TCR transgenic animals, deficiency of β2 integrins results in impaired regulation of the ongoing adaptive autoimmune response, resulting in the hyperimmune state and more severe cardiovascular pathology we report. That arthritis develops in K/BxN TCR transgenic animals even in the absence of β2 integrins suggests that the inflammatory drive in these mice engages alternative mechanisms to recruit cells to the joints (and cardiac valves).

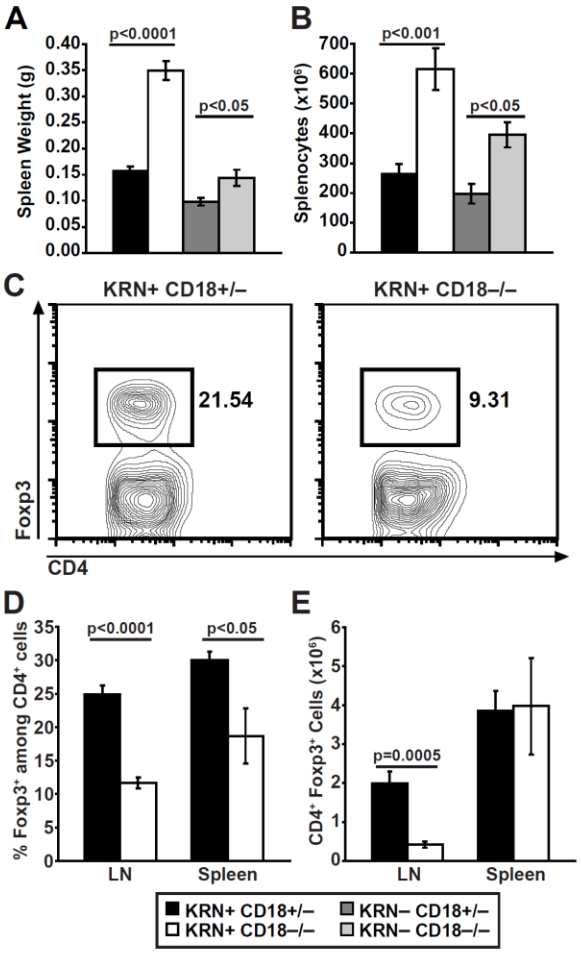


Figure 2.10. CD18 deficiency in K/BxN mice results in splenomegly and fewer peripheral Tregs **cells.** Measurement of spleen weights (*A*) and total spleen cellularity (*B*) in CD18-sufficient K/BxN mice (black, n=5), CD18-deficient K/BxN mice (white, n=6), and CD18-deficient KRN- mice (light grey, n=5) in comparison to non-arthritic CD18-sufficient KRN- littermate controls (dark grey, n=2). Values are mean ± SEM. (*C*) Representative flow cytometric assessment of the percentage of $CD4^+$ $Foxp3^+$ cells in lymph nodes from the indicated mice. $CD4^+$ $Foxp3^+$ cells also expressed CD25 (data not shown). Data are representative of four independent experiments. CD18-deficient K/BxN mice (white, n=6) have fewer than half as many $CD4^+$ $Foxp3^+$ regulatory T cells in their lymph nodes as compared to CD18-sufficient K/BxN mice (black, n=5) in terms of both (*D*) percentage and (*E*) absolute number. The percentage of Tregs in the spleens of K/BxN mice lacking CD18 was also reduced (*D*). LN, lymph node.

A critical difference between the heart and the joints is that dendritic cells (DCs), which are potent antigen-presenting cells, are well-described residents of normal cardiac valves [125], but not of the healthy synovium [126]. Thus, one plausible sequence of events leading to the development of endocarditis in this model is that circulating immune complexes bind to and activate the valve-resident DCs via Fcγ receptors, resulting in the recruitment and activation of pathogenic effector T cells [127, 128]. Tregs may be similarly recruited and activated, helping to constrain the inflammatory response [129, 130]. Interestingly, CD18- and CD11a-deficient Tregs have been reported to lose the ability to form aggregates around DCs, resulting in a functional inability of Tregs to produce suppressive cytokines [110, 131]. This may then allow the inflammatory process in the heart of CD18-deficient K/BxN mice to go unchecked and spread to additional cardiac structures, as we observed. It should also be noted that this working model leaves open the possibility that GPI might not be the crucial T or B cell autoantigen in the development of endocarditis – other antigenic specifities (or antigen non-specific immune complexes) could be at play. Importantly, a similar model, including T cell epitope spreading, has been proposed for the pathogenesis of rheumatic heart disease [97].

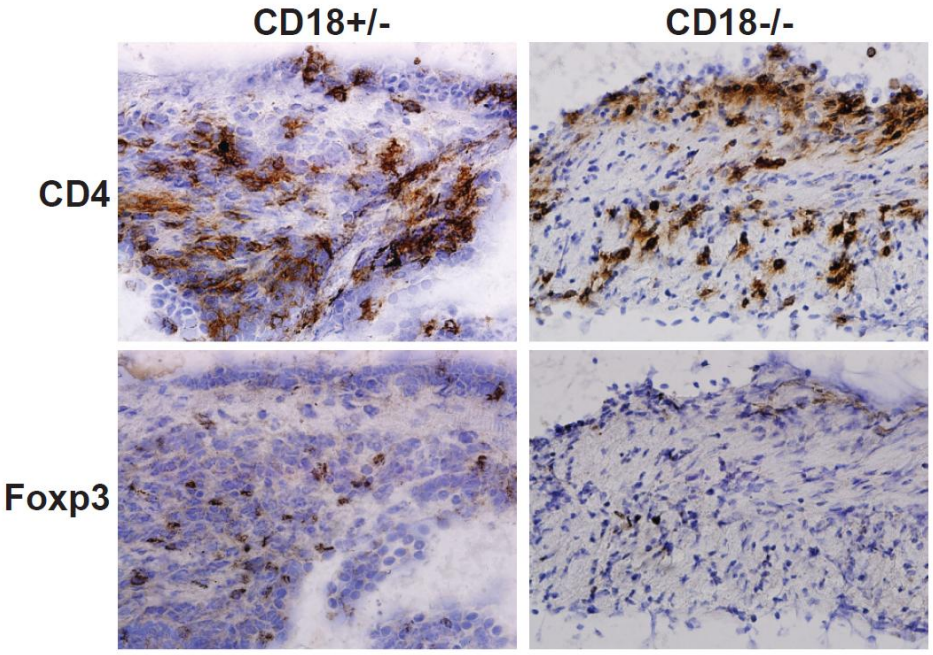


Figure 2.11. Paucity of Tregs **in the hearts of CD18-deficient K/BxN mice.** Immunohistochemical staining of mitral valves of CD18-sufficient (left) and CD18-deficient (right) K/BxN mice demonstrates similar numbers of $CD4^+$ infiltrating cells (top), with a reduction in the number of $Foxp3^+$ cells (bottom) in CD18-deficient K/BxN mice. Tissue sections were stained with biotinylated antibodies recognizing the indicated antigens (detected with DAB, brown) present on tissue-infiltrating leukocytes, followed by hematoxylin (blue). Photomicrographs are representative of a total of 5 mice/group in two separate experiments. Objective: x40.

How do our findings inform our understanding of human diseases? Cell adhesion molecules have been attractive targets of therapeutic intervention for a variety of diseases, based on the notion that such interference would impede trafficking of leukocytes to sites of inflammation (10-12). For instance, monoclonal antibodies specific for integrins have been approved for the treatment of autoimmune diseases in humans, such as natalizumab (anti-α4 integrin) for multiple sclerosis and efalizumab (anti-CD11a) for psoriasis. Similarly, a small molecule inhibitor of LFA-1 has recently been shown to reduce the severity of collagen-induced and anti-collagen antibody-induced arthritis in mice (42). However, our data suggest that one must also consider the effect that β2 integrins exert on the maintenance of peripheral tolerance, and that interference with such pathways might sometimes provoke autoimmune pathology. This may explain the reported occurrence of immune-mediated hematologic cytopenias and other immune-mediated disorders in some patients taking efalizumab (43-46), as well as the seemingly paradoxical occurrence of juvenile arthritis in some patients with leukocyte adhesion deficiency syndrome (47).

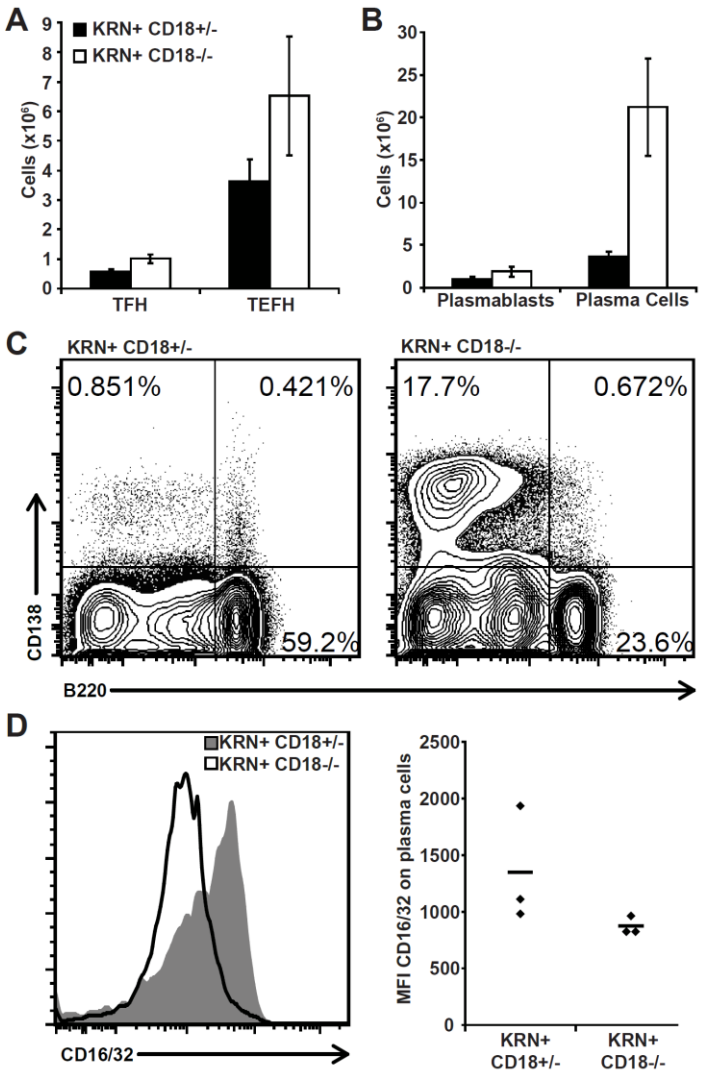


Figure 2.12. K/BxN mice lacking β2 integrins share characteristics with Tregs-**deficient K/BxN *scurfy* mice.** (*A*) Absolute numbers of follicular (TFH: $CD4^+$ $CD25^-$ $CXCR5^+$ $ICOS^+$) and extrafollucular (TEFH: $CD4^+$ $CD44^{hi}$ $CD62L^{lo}$ $PSGL\text{-}1^{lo}$) Th cells from spleen. (*B*) Absolute numbers of plasmablasts ($CD138^+$ $B220^{hi}$) and mature plasma cells ($CD138^+$ $B220^{lo}$) from spleen. (*C*) Representative flow cytometric assessment of plasmablasts and plasma cells in the indicated mice. (*D*) Representative histogram (left) and MFI (right) of the expression of the inhibitory Fcγ receptor Fc□RIIB (detected by anti-CD16/32 antibody) on plasma cells from the indicated mice. Data are representative of a total of 4 mice/group in two separate experiments. MFI, mean fluorescence intensity.

The data presented here offer new insights into the complex orchestration of the cells and molecules involved in the two related, yet distinct, diseases of the K/BxN TCR transgenic mouse. Autoreactive T lymphocytes provide the requisite B cell help to stimulate the production of high-titer autoantibodies necessary for the induction of both endocarditis and arthritis [91, 92, 119]; however the immune perpetrators responsible for pathogenic inflammation in the heart and joints differ. Once autoantibodies are formed, they are sufficient to provoke arthritis. In contrast, the cardiac pathology depends on an additional ongoing contribution of effector $CD4^+$ T cells. Deficiency of CD18 impairs Tregs, resulting in hyperimmune activation and more severe cardiac pathology.

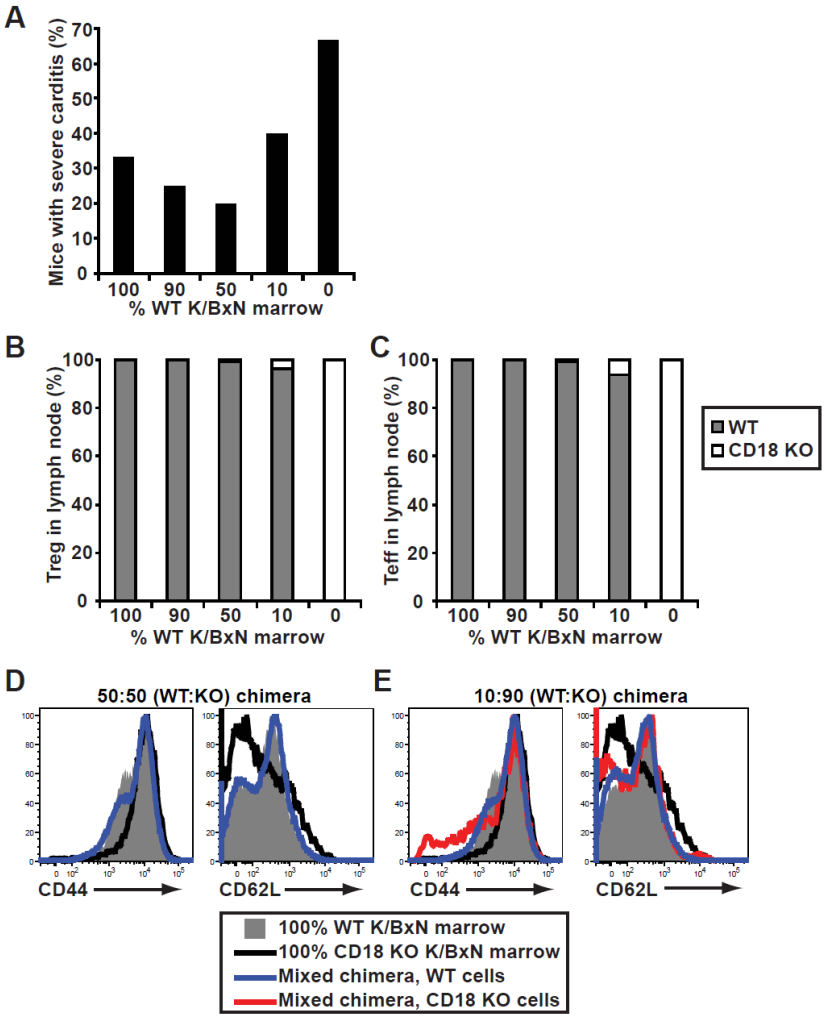


Figure 2.13. Mixed bone marrow chimeric mice show dominant tolerance-inducing effect of WT K/BxN bone marrow on CD18 KO bone marrow. (*A*) Hearts from the indicated mixed bone marrow chimeric mice were scored 1-4 based on the presence of inflammation of the mitral valve, aortic valve, coronary arteries, and pericardium. The percent of mice with a score of 3 or greater is depicted. Percentage of lymph node Tregs (*B*) and effector T cells (*C*) derived from either the WT versus CD18 KO bone marrow donor (determined by expression of the congenic marker CD45.1) in the indicated chimeric mice. T cell activation phenotype of the 50:50 (WT:KO) chimeras (*D*) and 10:90 chimeras (*E*); blue and red lines represent WT and KO donor cells, respectively, concatenated from three mice/group. For comparison, histograms of T cells from mice reconstituted with 100% WT (grey shade) or 100% KO (black line) are overlaid in both D and E. Data are representative of a total of 3-6 mice/group in two separate experiments.

CHAPTER THREE

Macrophage scavenger receptor 1 (SR-A) promotes autoantibody-mediated arthritis by impairing B cell self-tolerance

Haasken S, Auger JL, Taylor JJ, Hobday PM, Titcombe PJ, Mueller DL, and Binstadt BA. 2012. Macrophage scavenger receptor 1 (SR-A) promotes autoantibody-mediated arthritis by impairing B cell self-tolerance. *(in revision for J Immunol)*

I. INTRODUCTION

The generation of autoreactive lymphocytes is a consequence of having a diverse cellular immune repertoire capable of responding to threats of a wide range of specificities. As such, both cell-intrinsic and –extrinsic modes of immunological tolerance exist to constrain clones that recognize self-antigens [55, 132]. For B cells, central tolerance occurs in the bone marrow, where most self-reactive clones are either deleted or undergo receptor editing [42, 133, 134]. In the periphery, autoreactive B cells can be rendered tolerant (anergic) or induced to undergo apoptosis [49]. Autoimmunity can arise when these tolerance mechanisms fail.

The class A scavenger receptor, macrophage scavenger receptor 1 (Msr1, SR-A, CD204, encoded by the murine gene, *Msr1*), is a multifunctional receptor that is expressed primarily on cells of the myeloid lineage and that binds modified self- and pathogen-associated antigens [135]. Several lines of evidence support a role for Msr1 in peripheral tolerance. For example, one group reported a role for Msr1 in the maintenance of peripheral tolerance via tonic cross-presentation of self-antigens "nibbled" from the membranes of adjacent cells to CD8+ T cells [136]. Additionally, Msr1 may sequester sources of danger signals present among autoantigens by taking up apoptotic cellular debris. Of note, impaired clearance of apoptotic antigens has been implicated in the breakdown of tolerance in systemic lupus erythematosus (SLE) [137]. *Msr1*-deficient mice do not develop spontaneous autoimmune diseases, however, suggesting that these

reported “housekeeping” functions of Msr1 are not essential to maintain immunological tolerance and highlighting the fact that multiple layers of peripheral tolerance exist [138].

The K/BxN T cell receptor (TCR) transgenic mouse model of spontaneous autoimmune arthritis is well-suited to address questions regarding mechanisms of immunological tolerance. In this model, CD4+ T cells bearing the KRN transgene-encoded TCR recognize peptides derived from the ubiquitously expressed glycolytic enzyme, glucose 6-phosphate isomerase (GPI), presented on the MHC class II molecule I-A^{g7}. These activated T cells provide help to GPI-specific B cells, leading to the sustained production of high-titer arthritogenic anti-GPI autoantibodies [91, 92]. Joint pathology arises via antibody-mediated activation of the innate immune system, and transfer of K/BxN serum to naïve recipient mice is sufficient to provoke transient arthritis (termed K/BxN serum-transferred arthritis) [119]. Arthritis in K/BxN TCR transgenic mice develops reliably between 3-4 weeks of age, when autoreactive KRN CD4+ T cells begin to emerge from the thymus and when anti-GPI autoantibody production can be detected [91, 92], demonstrating that both T cell and B cell tolerance are breached in K/BxN mice.

Here we investigated how Msr1 impacts immunological tolerance in the K/BxN mouse model of spontaneous autoimmune arthritis.

II. MATERIALS AND METHODS

Mice. KRN TCR transgenic mice on the C57BL/6 (B6) background [91] were a gift from Drs. Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA) and the Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France); B6 mice congenic for H-2^{g7} (B6.g7) [93] were also a gift from Drs. Mathis and Benoist. *Msr1*-null B6 (*Msr1*^{tm1Csk}, stock no. 006096)[138], NOD/Lt (stock no. 001976) [139], *Rag1*-deficient B6 (*Rag1*^{tm1Mom}, stock no. 002216) [140], TCR-α-deficient B6 (*Tcra*^{tm1Mom}, stock no. 002116) [141], and μMT (*Ighm*^{tm1Cgn}, stock no. 002288) [142] mice were purchased from The Jackson Laboratory and bred in our specific-pathogen-free colonies. *Msr1*-deficient "K/BxN" mice (*Msr1*-/- K/BxN) were generated by breeding mice bearing the *Msr1* knockout (KO) allele on the B6 background to KRN/B6 mice and also to B6.g7. For ease of nomenclature, we refer to the KRN+ H-$2^{b/g7}$ mice as "K/BxN". Genotyping was performed by PCR. All studies were conducted in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Minnesota (protocol nos. 0611A96106 and 0909A72086).

Flow cytometry. Monoclonal antibodies used for flow cytometry were purchased from eBioscience unless otherwise noted, including anti-CD16/32 (clone93), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), B220 (RA3-6B2), IgM (eB121-15F9), Gr-1 (RB6-8C5), F4/80 (BM8), CD11c (N418), CD11b (M1/70), CD90.1 (HIS51), H-$2K^b$ (AF6-88.5.5.3), H-$2K^d$ (SF1-1.1.1), Vβ6 (RR4-7, BD Pharmingen), CD90.2 (53-2.1, BD

Pharmingen), and IgG1 (RMG101, Invitrogen). Intracellular staining using anti-Foxp3 (FJK-16s) and IgG (H+L) $F(ab')_2$ (Invitrogen) was performed using intracellular permeabilization/fixation reagents (eBioscience) per the manufacturer's protocol. GPI-PE and GPI-AF647-PE tetramers have been described previously (JJT and DLM, submitted for publication). Flow cytometry was performed using an LSRII and an LSRFortessa (BD Biosciences), and cells were analyzed using FlowJo v8.8.7 software (Tree Star).

Ag-specific B cell enrichment. GPI-specific B cells were enriched as described (JJT and DLM, submitted for publication). Briefly, pooled lymph node cells and splenocytes were incubated with the Ag-specific GPI-PE and Ag-nonspecific C5A-AF647-PE (control) tetramers, followed by incubation with anti-PE magnetic microbeads, after which the cells were passed through a magnetic column and both the Ag-specific B cell-enriched (bound) and polyclonal (unbound) fractions were collected. Both fractions were then labeled with a cocktail of fluorescent B cell and non-B cell markers for flow cytometric analysis.

Assessment of arthritis and IgG titers. Arthritis was assessed via clinical scoring and ankle measurements, and total serum IgG and anti-GPI titers were determined as described [117, 118].

Serum-transferred arthritis. Pooled serum (150 uL/dose) from K/BxN mice was injected intraperitoneally into recipient mice on days 0 and 2 [119]. The mice were monitored for the development of arthritis for 2 weeks as described above.

GPI capture ELISA. Levels of serum GPI from were measured as described [143]. *Msr1*-deficient mice are known to have increased levels of IgM specific for self- and commensal-derived antigens [144, 145], including GPI (not shown). Therefore, μMT-/- mice were used to allow determination of the GPI concentration in the absence of competing anti-GPI antibodies in the serum. The concentration of GPI was determined using a standard curve of recombinant GPI [146]. The plate was read on a Biorad Model 680 Microplate reader at 415 nm.

Anti-GPI IgG ELISPOT. Splenocytes were analyzed via ELISPOT to examine autoantibody production on a per-cell basis as previously described [147]. ELISPOT plates were read using an ImmunoSpot (Cellular Technology, Ltd).

In vitro lymphocyte activation. T cells were activated with plate-bound costimulatory antibodies. 96-well round-bottom plates (Sarstedt) were coated with 1 μM anti-CD3 (clone 145-2C11, BD Pharmingen) and anti-CD28 (37.51, eBioscience) antibodies in PBS overnight at 4°C. The following day, the plate was washed and lymphocytes were labeled with 5 μM CFSE (Molecular Probes), negatively enriched for CD4+ T cells per manufacturer's instructions (Millipore), plated at a density of $5x10^5$ cells/well in

complete media, and incubated at 37°C/5% CO_2. Cells were harvested at various timepoints post-incubation, stained with fluorescent antibodies specific for CD4+ T cells, and analyzed via flow cytometry.

Ag uptake by macrophages. Peritoneal macrophages were elicited by intraperitoneal injection of 1 mL Brewer thioglycollate medium (Fluka Analytical). Macrophages were collected 5 days later via peritoneal lavage, incubated with 5 μg GPI or BSA labeled with AF647 (Life Technologies) in complete media for 20 minutes at 37°C/5% CO_2, washed, and analyzed by flow cytometry.

Western blotting. Serum samples were separated by SDS-PAGE and transferred to Immobilin-FL membranes (Millipore). GPI was detected using serum from K/BxN mice, followed by peroxidase-conjugated goat anti-mouse IgG1 (Jackson Immunoresearch), developed with ECL Prime Western Blotting Detection Reagent (GE Healthcare), and imaged quantitatively on an ImageQuant LAS4000 workstation (GE Healthcare).

Adoptive transfers. Lymphocytes were either negatively enriched for CD4+ T cells ($1.5x10^6$ donor cells/mouse) (Miltenyi Biotech) or labeled with CFSE ($5x10^6$ donor cells/mouse) and injected intravenously into recipient mice heterozygous for H-$2^{b/g7}$. At the experimental endpoints, the lymphocytes were analyzed by flow cytometry and serum autoantibody titers were measured by ELISA.

Mixed bone marrow chimeric mice. *Rag1*-deficient recipient mice were sublethally irradiated with 300 rad. The following day, $10x10^6$ bone marrow cells from *Msr1*-sufficient or –deficient K/BxN mice were combined with $5x10^6$ bone marrow cells from unirradiated *Rag1*-deficient mice and injected intravenously into recipient mice. Mixed bone marrow chimeric mice were maintained on sulfamethoxazole and trimethoprim administered in drinking water for the duration of the study. Arthritis was measured for 10 weeks post-transplantation, after which the animals were sacrificed and serum and lymphoid organs were harvested for analysis.

Statistical analysis. Statistical differences between mean values for groups were calculated using the Student's two-tailed *t*-test. Relative risk was used to calculate differences in the incidence of arthritis between the genetic model and mixed bone marrow chimeric mice. P values ≤ 0.05 were considered significant.

III. RESULTS

K/BxN mice lacking Msr1 are protected from arthritis and endocarditis

To address how Msr1 might affect the development of autoantibody-associated arthritis, we generated K/BxN TCR transgenic mice lacking *Msr1* (*Msr1*-/- K/BxN). Surprisingly, half of the *Msr1*-/- K/BxN mice developed subtle to no arthritis by eight weeks of age (**Figure 3.1 A-D** and **Figure 3.2**). Some of the *Msr1*-heterozygous K/BxN mice *(Msr1*+/- K/BxN) were also protected from arthritis, although to a lesser extent, indicating a gene dose effect. Protection from arthritis did not segregate by gender or co-habitated littermates. The *Msr1*-/- K/BxN mice were also protected from mitral valve inflammation (endocarditis) (**Figure 3.2 A**), an additional site of inflammatory attack in this model [93]. To exclude the possibility that arthritis was simply delayed, we followed non-arthritic *Msr1*-/- K/BxN for 4 months, during which time the arthritis severity either remained consistent with the 8-week timepoint, or resolved (**Figure 3.2 B**).

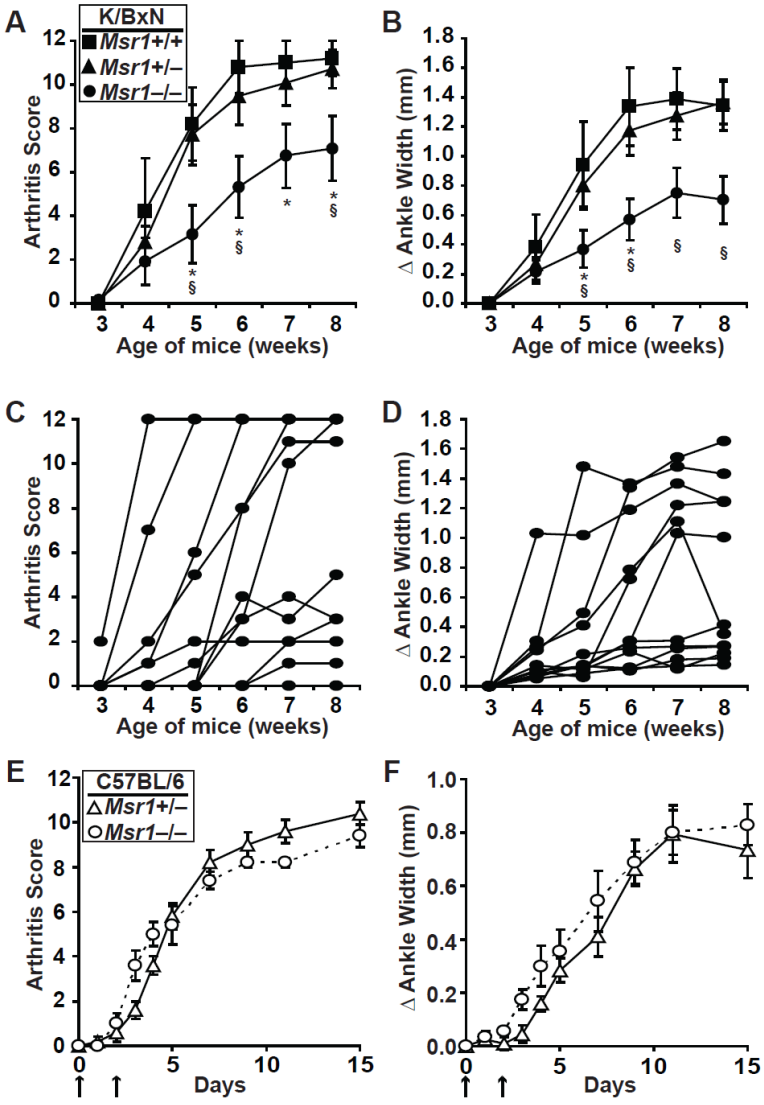


Figure 3.1. *Msr1*-deficient K/BxN mice are protected from arthritis and endocarditis. (**A** and **B**) Arthritis severity in *Msr1*-sufficient (filled squares, $n = 5$), -heterozygous (filled triangles, $n = 14$), and –deficient (filled circles, $n = 12$) K/BxN mice was assessed at the indicated ages and is depicted as (**A**) a qualitative score and (**B**) quantitative measurement of ankle thickness. Panels **C** and **D** show these same parameters in more detail for the Msr1-deficient group of K/BxN mice; each line represents one mouse. (**E** and **F**) Serum-transferred arthritis was induced in *Msr1*-heterozygous (open triangles, $n = 5$) and *Msr1*–deficient (open circles, $n = 5$) B6 mice by injection of arthritogenic K/BxN serum on days 0 and 2 (arrows). Plotted values are means ± SEM (A, B, E, F). The symbol "*" compares *Msr1*+/+ vs. *Msr1*-/- K/BxN and "§" compares *Msr1*+/- vs. *Msr1*-/- K/BxN. *, § indicates $p \leq 0.05$, §§ indicates $p \leq 0.01$.

The reduced incidence and severity of both arthritis and endocarditis suggested that *Msr1*-deficiency influenced the T and B cell-dependent initiation phase of autoimmunity in K/BxN mice. To exclude formally the possibility that *Msr1* deficiency interfered with the innate immune system-mediated effector phase of arthritogenesis, we asked whether *Msr1* deficiency affected the severity of arthritis induced by transfer of arthritogenic autoantibodies. Indeed, the absence of *Msr1* had no observable effect on the severity arthritis in the serum-transfer system (**Figure 3.1 E-F**). Taken together, these findings demonstrate that *Msr1* deficiency impacts the initiation phase of autoimmunity in K/BxN mice, not the effector phase. We therefore focused our attention on understanding how *Msr*1 deficiency affected T and B cell autoreactivity in K/BxN mice.

T cells are not affected by Msr1 deficiency

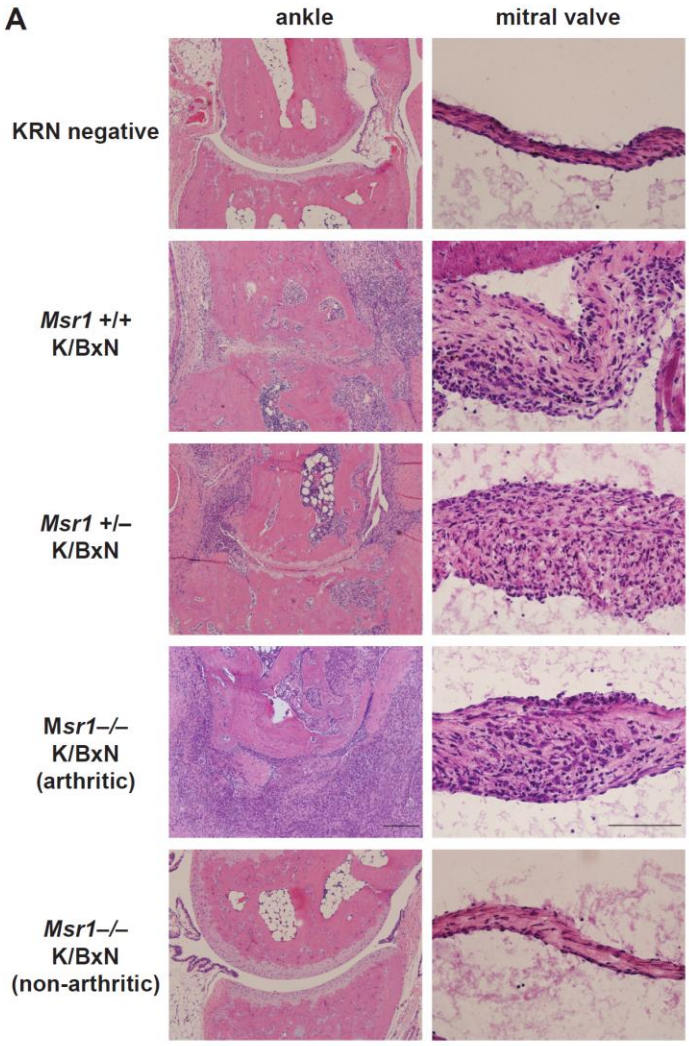
CD4+ T cells expressing the transgene-encoded KRN TCR are essential for autoimmunity to arise in K/BxN mice. Therefore, we first investigated how *Msr1* deficiency impacted T cell development and function. Although expression of Msr1 is predominantly restricted to innate immune cells [135, 148], it was possible that *Msr1* deficiency could affect T cell development in the thymus and thus impair the development of autoimmune disease in *Msr1*-/- K/BxN mice. Accordingly, we evaluated T cell subsets in the thymus and periphery from *Msr1*-sufficient and –deficient K/BxN mice, but found no difference in their numbers or relative frequencies (**Figure 3.3 A-B**). Expression of the T cell activation marker, CD44, on CD4+ T cells was also unaffected

in the *Msr1*-deficient K/BxN mice, regardless of whether or not the mice had arthritis, demonstrating that the activation of autoreactive CD4+ T cells was not impaired (**Figure 3.3 C**).

Msr1 has been reported perform a variety of functions including the capture and transfer of antigen for presentation, the clearance of host- and pathogen-derived components, and cell adhesion [135, 148]. Given the apparent block in the initiation of autoimmune disease, we next asked whether presentation of the relevant K/BxN autoantigen, a GPI-derived peptide presented by the MHC class II molecule I-A^{g7}, to KRN T cells was impaired in *Msr1*-deficient animals. CD4+ T cells from KRN/B6 mice proliferated with equivalent dynamics when adoptively-transferred into *Msr1*+/+ H-$2^{b/g7}$ or *Msr1*-/- H-$2^{b/g7}$ recipient mice (**Figure 3.3 D**). These findings suggest that *Msr1* deficiency does not alter presentation of GPI peptide to CD4+ KRN T cells.

The penultimate step in the initiation phase of K/BxN arthritis is the provision of T cell help to elicit the production of arthritogenic autoantibodies by B cells [119]. We therefore sought to determine whether T cells from *Msr1*-deficient mice were capable of driving autoreactive B cells to provoke arthritis. T cell-deficient (TCRα-/- H-$2^{b/g7}$) recipient mice developed arthritis equivalently following adoptive transfer of T cells from *Msr1*-sufficient or –deficient KRN/B6 donor mice and produced comparable levels of anti-GPI IgG (**Figure 3.3 E** and data not shown). This experiment demonstrates that *Msr1* deficiency in CD4+ KRN T cells does not impair their ability to become activated, provide productive B cell help, and provoke autoimmune arthritis. Together, these data

indicate that the T cell compartment is intact and functional, and is therefore not responsible for the protection from arthritis we observed in *Msr1*-/- K/BxN mice.


A
ankle
mitral valve
KRN negative
Msr1 +/+
K/BxN
Msr1 +/–
K/BxN
Msr1–/–
K/BxN
(arthritic)
Msr1–/–
K/BxN
(non-arthritic)

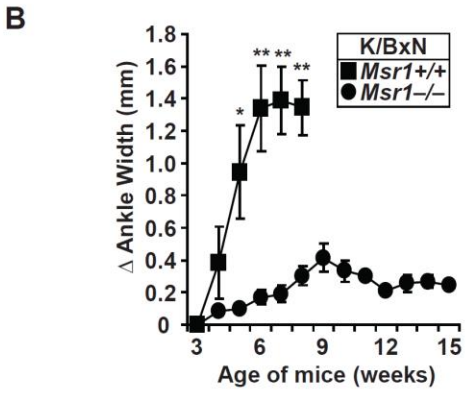

B
K/BxN
Msr1+/+
Msr1–/–
Δ Ankle Width (mm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
3
6
9
12
15
Age of mice (weeks)
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Figure 3.2. Decreased incidence and severity of arthritis and endocarditis in *Msr1*-/- K/BxN mice. (**A**) Histological analysis of ankle (left panels) and mitral valve (right panels) pathology in *Msr1*-sufficient, -heterozygous, and –deficient K/BxN mice demonstrates protection from both joint and cardiac valve pathology in non-arthritic *Msr1*-/- K/BxN mice. For ankles, original magnification = 10x, bar (in fourth row) indicates 200 microns. For mitral valves, original magnification = 40x; bar (in fourth row) indicates 100 microns. (**B**) Five non-arthritic *Msr1*-/- K/BxN mice (filled circles) were aged up to 15 weeks, during which any mild arthritis resolved or remained equivalent to the 8-week time point, as demonstrated by changes in ankle thickness. The data from the *Msr1*+/+ K/BxN mice (reproduced from **Figure 3.1 B**) are shown for comparison. Plotted values are means ± SEM. $*p \leq 0.05$, $**p \leq 0.01$.

Msr1 impairs B cell tolerance in K/BxN mice

As the T cell compartment in *Msr1*-/- K/BxN mice appeared intact, we next explored the B cell arm of K/BxN autoimmunity. *Msr1*-deficient and K/BxN mice had significantly less total serum IgG (**Figure 3.4 A**) and also significantly lower anti-GPI IgG titers (**Figure 3.4 B**) than *Msr1*-sufficient K/BxN mice. Interestingly, several of the arthritic *Msr1*+/- and *Msr1*-/- K/BxN mice had low levels of anti-GPI IgG, similar to the non-arthritic mice, implying that very little anti-GPI IgG is required to provoke arthritis. A previous study reported that mice treated with a pharmacological blocker of Msr1 generated a normal humoral immune response following immunization with tetanus toxoid, indicating that Msr1 insufficiency does not impair B cell responsiveness to a foreign antigen. Thus, the disparities in autoantibody titers in our model suggested that alterations in autoreactive B cells might underlie the reduced arthritis severity in Msr1-/- K/BxN mice [149]. Thus, the disparities in antibody titers in our model indicated that alterations in B cells might underlie the reduced arthritis severity in *Msr1*-/- K/BxN mice.

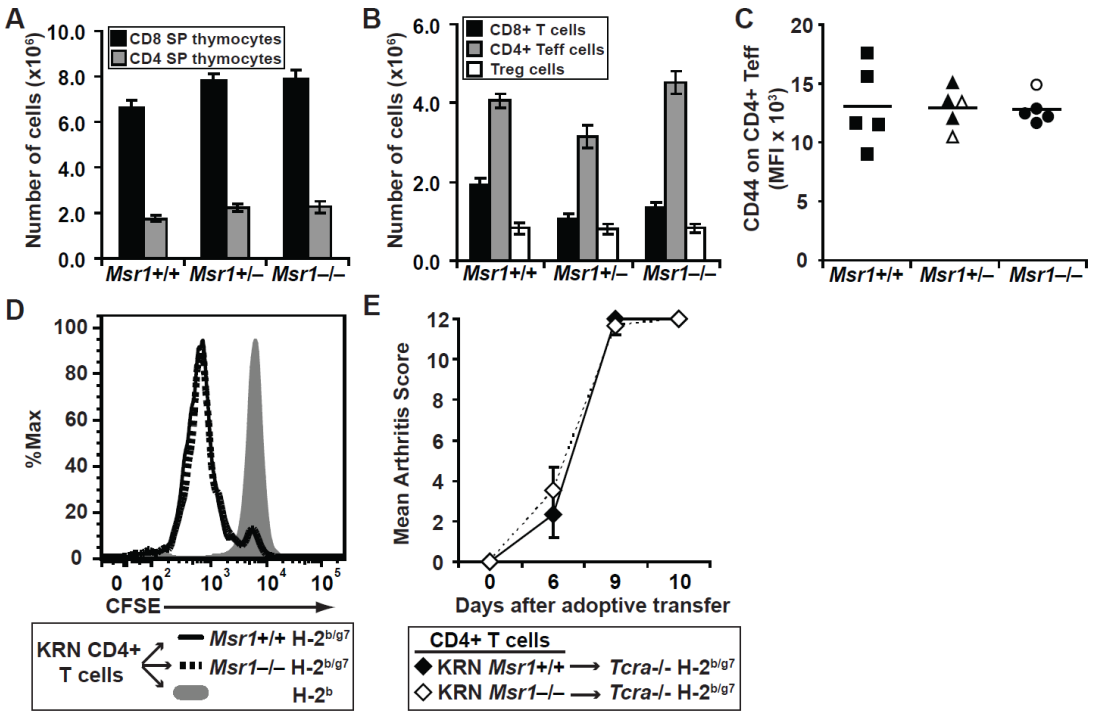


Figure 3.3. T cells are not affected by *Msr1* deficiency. T cell subsets from (**A**) thymus and (**B**) spleen were enumerated among K/BxN mice of the indicated *Msr1* genotypes. The values plotted are means ± SEM. (**C**) Surface expression of CD44 measure by mean fluorescence intensity (MFI) on splenic CD4+ effector T cells from *Msr1*-sufficient (squares), -heterozygous (triangles), and -deficient (circles) K/BxN mice was determined by flow cytometry (filled shapes indicate arthritic animals, open shapes indicate non-arthritic animals; n = 5 mice per genotype; bars represent mean values). (**D**) CFSE-labeled CD4+ T cells from KRN/B6 donor mice were adoptively-transferred into *Msr1*-sufficient (solid line) or -deficient (dashed line) H-2$^{b/g7}$-expressing recipient mice or *Msr1*-sufficient B6 (H-2^{b}) control mice (shaded histogram) and harvested 48 hours later for flow cytometric analysis. The data shown are representative of 3 separate experiments (n = 8 mice per group). (**E**) CD4+ T cells from KRN/B6 mice expressing *Msr1* (filled diamonds, n = 6 mice) or lacking *Msr1* (open diamonds, n = 7 mice) were adoptively transferred into T cell-deficient H-2$^{b/g7}$-expressing recipient mice and monitored for the development of arthritis. Plotted values are means ± SEM Data represent two separate experiments.

Next, we sought to determine if numerical differences in B cells could account for the lower anti-GPI IgG titers in *Msr1*-/- K/BxN mice. Despite having equivalent numbers of total B cells in the periphery (**Figure 3.4 C**), both arthritic and non-arthritic *Msr1*-/- K/BxN mice had significantly fewer anti-GPI IgG-secreting cells detected by ELISPOT than did *Msr1*-sufficient K/BxN mice (**Figure 3.5**), indicating either that GPI-specific B cells from *Msr1*-/- K/BxN mice were unresponsive or that they were being eliminated. To address these two possibilities, we utilized a recently developed B cell antigen-specific tetramer in combination with an antigen-nonspecific decoy tetramer strategy to detect GPI-specific B cells (JJT and DLM, submitted for publication). Mice lacking the KRN transgene contained very low numbers of GPI-specific B cells. In contrast, wildtype K/BxN mice averaged 300,000 GPI-specific B cells, whereas *Msr1*-/- K/BxN mice had roughly 80,000 (**Figure 3.4 D**). Notably, there was no difference in the number of GPI-specific B cells in arthritic versus non-arthritic *Msr1*-/- K/BxN animals. In agreement with the ELISA and ELISPOT data, GPI-specific B cells from *Msr1*-heterozygous and -deficient K/BxN mice expressed significantly less surface IgG1 (**Figure 3.4 E**), the predominant isotype of anti-GPI IgG, than did *Msr1*-sufficient K/BxN control mice. Considered in sum, these data strongly suggested that the pathogenic B cells from *Msr1*-/- K/BxN mice were being functionally restrained as measured by total and GPI-specific numbers, isotype switching, and autoantibody synthesis.

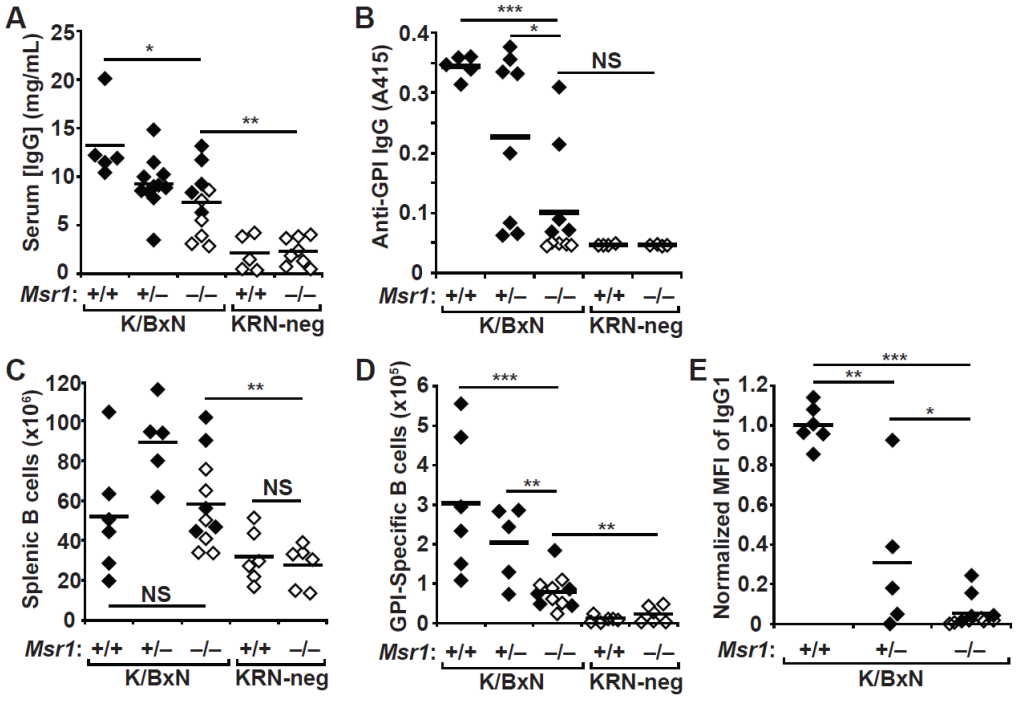


Figure 3.4. Msr1 impairs B cell tolerance in K/BxN mice. (**A**) Total serum IgG and (**B**) anti-GPI IgG were measured by ELISA in *Msr1*-sufficient, -heterozygous, and –deficient K/BxN mice, as well as KRN-negative *Msr1*-sufficient and –deficient mice. (**C**) Total B cells and (**D**) GPI-specific B cells from *Msr1*-sufficient, -heterozygous, and –deficient K/BxN mice, as well as KRN-negative *Msr1*+/+ and *Msr1*-/- mice were analyzed by flow cytometry using antigen-specific and antigen-nonspecific B cell tetramers. (**E**) Surface expression of IgG1, the predominant anti-GPI IgG isotype, was assessed on GPI-specific B cells. For plots in A, B, D, E, and F, each point represents one animal; filled diamonds indicate arthritic animals and open diamonds indicate non-arthritic animals; data were compiled from three separate experiments. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Msr1 deficiency promotes B cell anergy

The state of anergy, or hyporesponsiveness to antigenic stimuli, is a mechanism of peripheral tolerance in which potentially autoreactive cells can be silenced. In particular, B cells may be rendered anergic if they encounter their cognate antigen (signal 1) in the absence of TLR ligation or appropriate costimulation (signal 2) [49]. Although normally found in the cytoplasm, low levels of soluble GPI can also be detected in the serum of mice and humans [146, 150, 151]. Because Msr1 functions as a scavenger receptor, we hypothesized that Msr1 might bind and clear excess antigen (GPI) from the circulation. To address this, peritoneal macrophages from *Msr1*-sufficient, -heterozygous, and -deficient mice were incubated with fluorescently-labeled GPI or an irrelevant protein (albumin), using fluorescence as a measure of uptake. Macrophages lacking *Msr1* took up both GPI and albumin less efficiently than did wildtype macrophages and this defect appeared more pronounced for GPI (**Figure 3.6 A**). Correspondingly, we found a gene dose-dependent increase in circulating levels of GPI in the serum of *Msr1*-deficient mice (**Figure 3.6 B, C**), which ranged between 2-20 ug/mL by Western blot analysis. Although the GPI standard curve detected as little as 23 ng/mL, the serum GPI of *Msr1*-sufficient mice was below the level of detection for the assay, indicating that the concentration of GPI in the serum of *Msr1*-deficient mice is a log or more higher. These findings suggest that *Msr1* normally acts to maintain low levels of soluble GPI in the circulation.

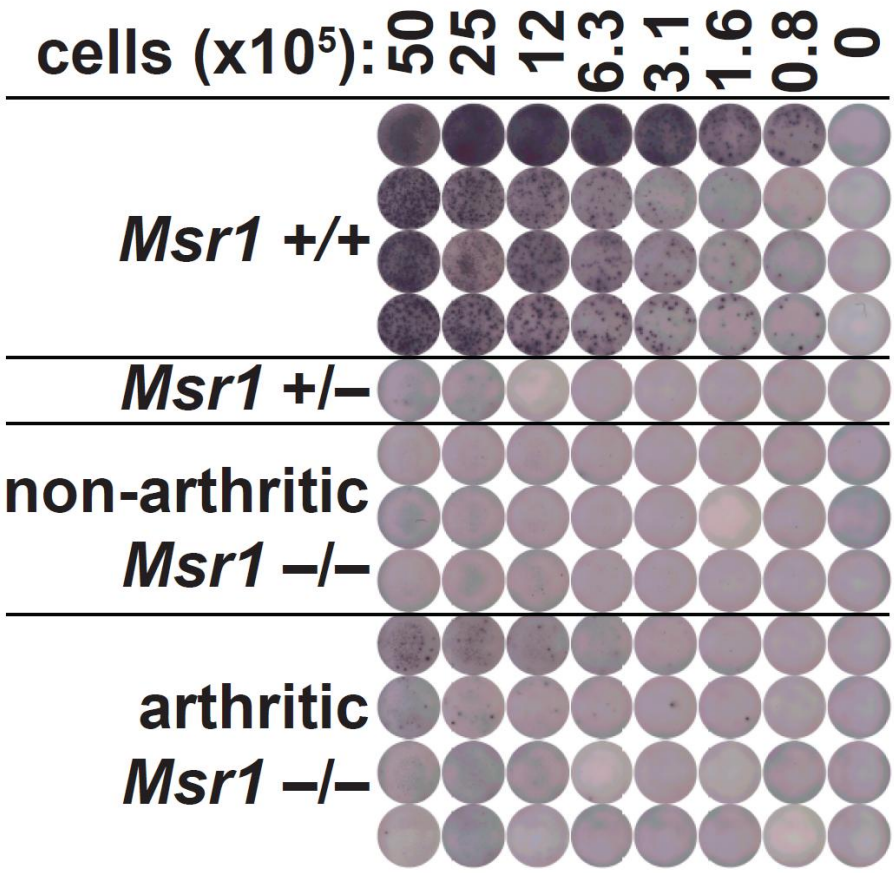
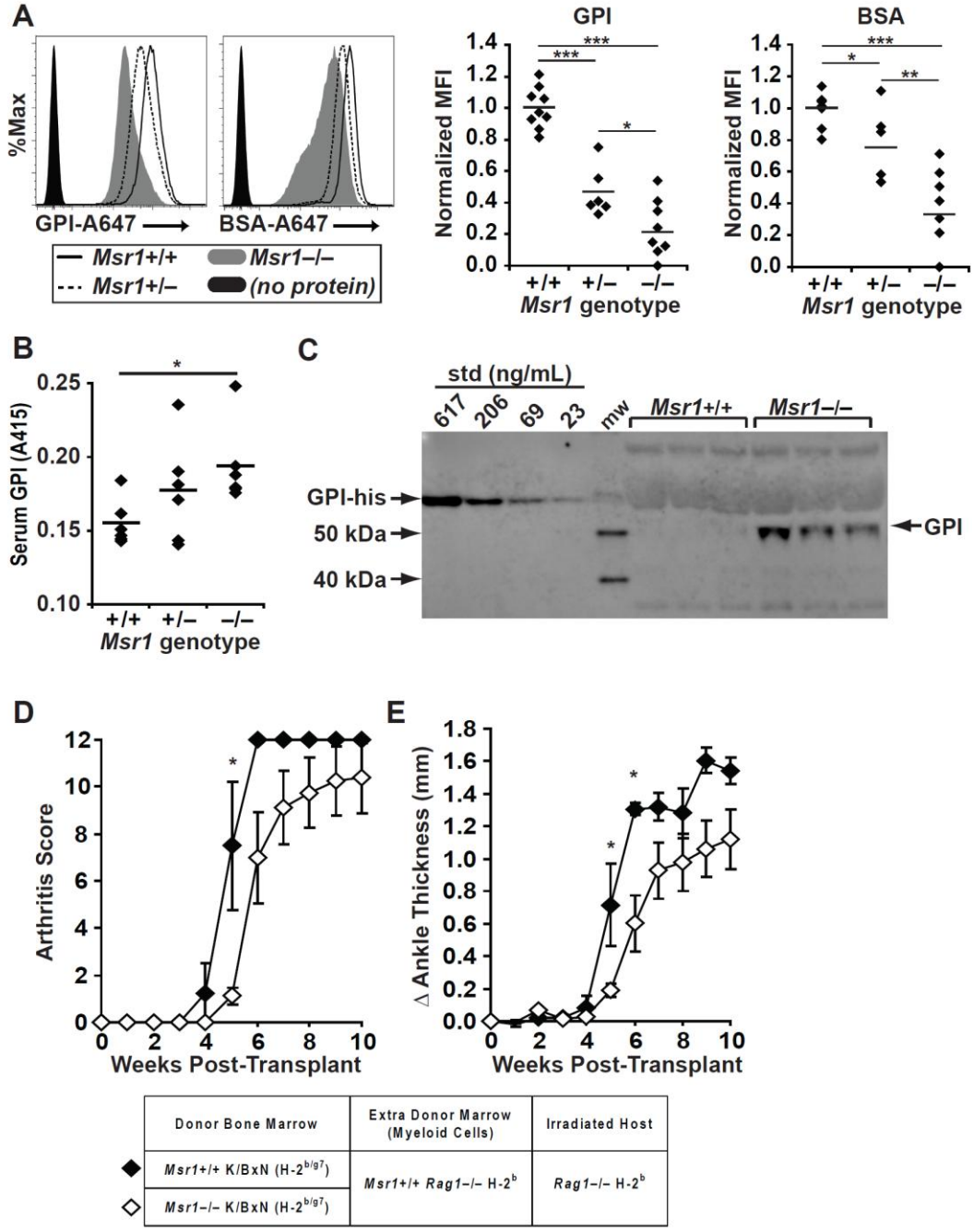


Figure 3.5. K/BxN mice lacking *Msr1* have infrequent anti-GPI IgG antibody-secreting cells. Anti-GPI IgG ELISPOT analysis of serially-diluted splenocytes from *Msr1*-sufficient, -heterozygous, and –deficient K/BxN mice demonstrates a paucity of anti-GPI IgG antibody-secreting cells even in arthritic *Msr1*-/- K/BxN mice. Data are representative of two separate experiments.

The finding that *Msr1*-deficient mice had increased circulating concentrations of GPI suggested that GPI-specific B cells were being anergized by this higher antigen concentration. We hypothesized that adding *Msr1*-sufficient antigen presenting cells to capture and thus "normalize" the concentration of GPI would allow GPI-specific B cells to avoid anergy induction. To test this, we created mixed bone marrow chimeric mice in which bone marrow from non-arthritic *Msr1*-/- K/BxN or arthritic *Msr1*+/+ K/BxN mice was mixed with bone marrow from *Rag1*-deficient mice (as a source of Msr1-expressing antigen presenting cells) and injected into sublethally-irradiated *Rag1*-deficient (Msr1-expressing) recipient mice. Indeed, mice transplanted with both *Msr1*-/- K/BxN marrow and *Msr1*-sufficient APCs developed arthritis essentially equivalently to control mice (**Figure 3.6 D, E**). Furthermore, the incidence of arthritis among the mice transplanted with *Msr1*-/- K/BxN bone marrow was significantly higher than the incidence among the *Msr1*-/- K/BxN TCR transgenic animals (87.5% vs. 52.5%, $p = 0.0014$). These findings suggest that *Msr1* deficiency in B cells per se is not responsible for the reduced arthritis severity in *Msr1*-/- K/BxN mice; rather it suggests that myeloid cells expressing Msr1 are able to regulate circulating levels of the autoantigen. In the case of GPI, *Msr1*-expressing mice have relatively lower circulating levels of GPI, GPI-specific B cells are not anergized, and autoimmunity ensues. In contrast, deficiency of *Msr1* impairs the uptake of GPI by macrophages, resulting in more effective B cell tolerance, and diminished autoimmunity.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BSA-A647
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Msr1+/–
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(no protein)
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BSA
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Msr1 genotype
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+/–
–/–
B
Serum GPI (A415)
C
std (ng/mL)
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69
23
mw
Msr1+/+
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GPI-his
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40 kDa
GPI
D
Arthritis Score
Weeks Post-Transplant
E
Δ Ankle Thickness (mm)
Donor Bone Marrow
Extra Donor Marrow (Myeloid Cells)
Irradiated Host
Msr1+/+ K/BxN (H-2b/g7)
Msr1–/– K/BxN (H-2b/g7)
Msr1+/+ Rag1–/– H-2b
Rag1–/– H-2b

Figure 3.6. *Msr1*-deficient mice have increased circulating GPI, which promotes B cell anergy. (**A**) Peritoneal macrophages from *Msr1*-sufficient (solid line), -heterozygous (dashed line), and –deficient (grey shade) B6 mice were incubated with fluorescently-labeled GPI (left panel), fluorescently-labeled irrelevant protein (BSA, right panel), or no protein (black shade) and fluorescent intensity was analyzed by flow cytometry as a measure of protein uptake. The left panels show representative plots. The right panel shows MFI values for individual mice, with bars indicating mean values. (**B**) Levels of soluble GPI from serum were evaluated by ELISA in *Msr1*+/+, *Msr1*+/-, and *Msr1*-/- μMT-/- mice. Each point represents one mouse and bars indicate means. (**C**) Serum GPI concentrations were determined by Western blot in μMT-/- mice of the indicated genotypes using a standard curve of GPI-His. Three mice from each genotype representing the range of absorbance values from the GPI ELISA (B) were chosen to quantify serum GPI concentrations. The rationale for using μMT-/- mice for (C and D) is explained in the Materials and Methods. (**D and E**) *Rag1*-/- recipient mice were sublethally irradiated and transplanted with a mixture of $10x10^6$ bone marrow cells from *Msr1*+/+ K/BxN mice (filled diamonds, $n = 4$) or *Msr1*-/- K/BxN mice (open diamonds, $n = 8$) plus $5x10^6$ bone marrow cells from Rag1-/- mice as a source of additional *Msr1*-sufficient myeloid cells. Arthritis was assessed via (D) clinical arthritis scores and (E) changes in ankle thickness. Plotted values are means ± SEM; data represent two separate experiments. For all panels, * indicates $p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

IV. DISCUSSION

Here, we report that *Msr1* deficiency is sufficient to prevent systemic autoimmunity in the K/BxN TCR transgenic mouse model of autoantibody-dependent arthritis. Specifically, in the absence of *Msr1*, circulating levels of the autoantigen GPI were increased; this correlated with fewer and less functional GPI-specific B cells and with reduced incidence and severity of arthritis. Our data suggest that *Msr1* impairs B cell tolerance by sequestering otherwise tolerogenic self-antigen.

The increased circulating concentration of GPI in *Msr1*-decificent mice could impact the function of GPI-specific B cells in a number of ways. For example, it could affect GPI-specific B cells directly through clonal deletion of high-affinity B cells or by the induction of anergy in GPI-specific B cells. Notably, B cells may be rendered anergic, or hyporesponsive to antigenic stimuli, if they encounter their cognate antigen (signal 1) in the absence of TLR ligation or appropriate costimulation (signal 2) [49]. Alternatively, it is possible that higher concentrations of circulating GPI could impact B cell activation indirectly by altering the cognate interactions between autoreactive CD4+ T cells and B cells. For instance, more widespread antigen presentation could hypothetically lead to more geographically diffuse T cell activation, thereby diminishing the likelihood that an activated T cell would encounter a GPI-specific B cell, with the net effect being a relative reduction in autoreactive B cell activation. Differentiating among these possible mechanisms will be the focus of future studies.

Our findings that *Msr1* impairs immunological tolerance are unexpected, considering that a main reported function of *Msr1* is the clearance of apoptotic debris, in conjunction with the accepted role of impaired apoptotic clearance in the breakdown of tolerance in SLE, another autoantibody-dependent disease [59, 137]. However, this discrepancy may be related more to timing, rather than to peculiarities of the model system. That is, *Msr1* deficiency early in lymphocyte development may result in an antigen-rich environment favoring the elimination or functional silencing of emerging autoreactive B cells, even in the presence of activated, autoreactive T cells. In contrast, in an organism with a mature immune system or amid established autoimmunity, inhibiting Msr1 and increasing the load of antigen might fuel autoreactivity and sustained inflammation [144, 152]. These scenarios are consistent with Mitchison's framework of "high zone tolerance" [153]. In agreement with this supposition and consistent with our findings, Mamula and colleagues recently noted decreased pathology and autoantibody titers in autoimmune-prone MRL mice genetically lacking *Msr1* [154], perhaps attributable to defects in Msr1-mediated trafficking of antigen from B cells to macrophages, resulting in decreased T cell activation [155]. However, our results suggest that the loss of *Msr1* does not appreciably impair T cell activation in our model system.

The observation that some *Msr1*-/- K/BxN mice developed arthritis while others did not is intriguing. A similar dichotomy in arthritis susceptibility has been reported in K/BxN mice lacking interleukin-4 and in K/BxN mice lacking the neonatal Fc receptor (FcRn) [118, 156]. In these studies and the present one, the development of arthritis correlated closely with the production of anti-GPI IgG autoantibodies, and even very low

titers of anti-GPI IgG were sufficient to provoke arthritis (see Fig. 3B). These findings are consistent with the notion that the threshold for developing arthritis can be crossed relatively easily, and that stochastic events likely impact if and when that crossing occurs. For instance, events such as B cell co-ligation of GPI and TLR agonists as well as GPI binding in proximity to an activated KRN T cell in the secondary lymphoid organs might account for those GPI-specific B cells that escaped tolerance induction in arthritic *Msr1*-/- K/BxN mice. This scenario is conceptually similar to the notion that TLR activation can promote the production of rheumatoid factor by autoreactive B cells [157].

The recent description of anti-Msr1 (anti-SR-A) antibodies among patients with SLE is notable. It has been suggested that such antibodies might interfere with the uptake of apoptotic debris, thereby promoting autoimmunity [144, 152]. Our findings suggest that understanding how anti-Msr1 antibodies might influence SLE disease pathogenesis will require detailed knowledge of the timing of when they appear and whether or how they affect the interaction of Msr1 with specific autoantigens.

The data presented in this study add to our understanding of the complex temporal and mechanistic balance of immunological tolerance. We demonstrate that the multifunctional receptor, Msr1, can regulate levels of GPI and that K/BxN mice lacking *Msr1* can be protected from autoimmunity via the alteration of GPI-specific B cell tolerance due to excess circulating autoantigen.

CHAPTER FOUR

Concluding statement

Autoimmune diseases are a growing health concern, particularly in developed countries [1-4]. Patients with systemic autoimmune diseases such as RA and SLE suffer higher mortality rates compared with the general population due in large part to cardiovascular complications [94-96], yet the immunological means by which disparate tissues are targeted thus remains unclear. Further exploration into the circumstances governing immunity versus tolerance is needed to identify more specific therapeutic strategies.

The results presented in this dissertation reveal novel insights into the immunological orchestration of systemic autoimmune pathology in a unique mouse model of inflammatory arthritis and endocarditis. Moreover, these data also highlight the many layers of immunological tolerance that one must consider when attempting to manipulate the immune system therapeutically. The observations in Chapter 2 contribute both to the literature on targeting adhesion molecules in autoimmunity as well as the role of Tregs in chronic inflammation. Leukocyte trafficking into the cardiac valve tissue during autoimmune endocarditis does not require the use of β2 integrins, potentially due to redundancy or compensatory mechanisms. The requirement for β2 integrins in normal regulatory T cell development exposed a previously unknown role for Tregs in the control of autoimmune-mediated cardiovascular inflammation, in addition to the restraint they exert on the pathogenesis of arthritis. Despite the risks outlined here and by other groups [158-161], integrin-targeting therapies continue to be studied and used because the benefits are thought to outweigh the risks, and alternatives are currently limited. Open areas of investigation include whether Tregs limit K/BxN carditis via the

suppression of antigen-presenting cells or at the level of the effector T cells, and if cardiac Tregs derive from natural or induced progenitors. Perhaps more important to our understanding of the breaches in tolerance at work in systemic autoimmune diseases is whether the effector T cells responsible for cardiac pathology have a distinct antigenic specificity from the arthritogenic T cells.

Scavenger receptors have long since been described as innate immune receptors responsible for the removal of a variety of antigens to avoid undesirable immune activation, but the data presented in Chapter 3 represent a novel situation in which absence of Msr1 lead to protection from K/BxN autoimmune disease due to tolerogenic effects on autoantigen-specific B cells. Such a powerful phenotype was unexpected, considering that the transgenic nature of the mouse model biases towards autoimmunity, not to mention the breadth of literature suggesting functional redundancy within the scavenger receptors [59, 135, 162-164]. That approximately 50% of Msr1-deficient K/BxN mice were spared from autoimmunity suggests a stochastic component in the timing and location of tolerance induction. Although it is unclear whether the loss of Msr1 exerted immunological tolerance on GPI-specific B cells via anergy induction, by clonal deletion, or by causing alterations in antigen-specific T- and B- cell interactions, these observations spark broader questions of application and teleology. For example, it would be of interest to explore whether Msr1 acts to limit all soluble antigens, and if so, why it might have evolved this way, given the protective effects observed in the K/BxN model of systemic autoimmunity. Or from a clinical standpoint, could Msr1 activity be induced therapeutically in an effort to decrease epitope spreading in a patient with

established autoimmunity, thus limiting future pathology? What is certain is that Msr1 merits a closer look in the context of immunological tolerance.

This thesis presents a body of work detailing the previously unidentified role for regulatory T cells in the control of autoimmune carditis, as well as a role for Msr1 in the regulation of tolerance in a unique mouse model of co-occurring autoimmune arthritis and endocarditis. These data have increased our understanding of the pathways governing the balance between tolerance and immunity, and provide the framework for future studies in the search for new therapeutic targets that can decrease the morbidity and mortality of patients with the complex clinical challenge that is systemic autoimmune disease.

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