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Macrophage Scavenger Receptor 1 (Msr1, SR-A) Influences B Cell Autoimmunity by Regulating Soluble Autoantigen Concentration

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The class A macrophage scavenger receptor Msr1 (SR-A, CD204) has been reported to participate in the maintenance of immunological tolerance. We investigated the role of Msr1 in a mouse model of autoantibody-dependent arthritis. Genetic deficiency of Msr1 in K/BxN TCR transgenic mice decreased the incidence and severity of arthritis because of decreased autoantibody production. Despite normal initial activation of autoreactive CD4+ T cells, potentially autoreactive B cells in Msr1−/− K/BxN mice retained a naive phenotype and did not expand. This was not due to an intrinsic B cell defect. Rather, we found that macrophages lacking Msr1 were inefficient at taking up the key autoantigen glucose-6-phosphate isomerase and that Msr1-deficient mice had elevated serum concentrations of glucose-6-phosphate isomerase. Arthritis developed normally when bone marrow from Msr1−/− K/BxN mice was transplanted into hosts whose macrophages did express Msr1. Thus, Msr1 can regulate the concentration of a soluble autoantigen. In this model, the absence of Msr1 led to higher levels of soluble autoantigen and protected mice from developing pathogenic autoantibodies, likely because of altered cognate interactions of autoreactive T and B cells with impaired differentiation of follicular Th cells. The Journal of Immunology, 2013, 191: 1055–1062.

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. Both cell-intrinsic and -extrinsic modes of immunological tolerance exist to constrain lymphocyte clones that recognize self-Ags (1, 2). For B cells, central tolerance occurs in the bone marrow, where most self-reactive clones are either deleted or undergo receptor editing (3–5). In the periphery, autoreactive B cells can be rendered tolerant (anergic) or induced to undergo apoptosis (6). Weakly autoreactive B cells can be maintained in a seemingly naive state of clonal ignorance if protected from cognate interactions with activated T cells (7). Autoimmunity can arise when these B cell 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 Ags (8). 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-Ags “nibbled” from the membranes of adjacent cells to CD8+ T cells (9). In addition, Msr1 may sequester sources of danger signals present among autoantigens by taking up apoptotic cellular debris. Of note, impaired clearance of apoptotic Ags has been implicated in the breakdown of tolerance in systemic lupus erythematosus (SLE) (10). 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 (11).

The K/BxN 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 (MHCII) molecule I-Aβ7. These activated T cells provide help to GPI-specific B cells, leading to the sustained production of high-titer arthritogenic anti-GPI autoantibodies (12, 13). Joint pathology arises via Ab-mediated activation of the innate immune system, and transfer of K/BxN serum to naive recipient mice is sufficient to provoke transient arthritis (termed K/BxN serum–transferred arthritis) (14). Arthritis in K/BxN TCR transgenic mice develops reliably between 3 and 4 wk of age when autoreactive KRN CD4+ T cells begin to emerge from the thymus and when anti-GPI autoantibody production can be detected (12, 13), demonstrating that both T cell and B cell tolerance are breached in K/BxN mice.

In this study, we investigated how Msr1 impacts immunological tolerance in the K/BxN mouse model of spontaneous autoimmune arthritis.

Materials and Methods

Mice

KRN TCR transgenic mice on the C57BL/6 (B6) background (12) were a gift from Drs. D. Mathis and C. Benoist (Harvard Medical School,
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Intracellular cytokine staining

Lymph node cells were isolated, stimulated with PCA and ionomycin, and stained intracellularly for IL-17 and IFN-γ as described previously (24).

Ag uptake by macrophages

Peritoneal macrophages were elicited by i.p. injection of 1 ml Brewer thioglycollate medium (Fluka Analytical). Macrophages were collected 5 d later via peritoneal lavage, incubated with 5 μg GPl or BSA labeled with AF647 (Life Technologies) in complete media for 20 min at 37°C/5% CO2, washed, and analyzed by flow cytometry.

Western blotting

Serum samples were separated by SDS-PAGE and transferred to Immobilon-FL membranes (Millipore). GPl was detected using serum from K/BxN mice, followed by peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories), developed with ECL Prime Western blotting Detection Reagent (GE Healthcare), and imaged quantitatively on an ImageQuant LAS4000 workstation (GE Healthcare). Because Ab H chain comigrates with GPl, Ab-deficient μMT−/− mice were used to allow detection of GPl.

Determination of serum albumin concentration

Serum albumin concentrations were determined in the same mice by ELISA, according to the manufacturer’s instructions (Bethyl Laboratories) The plates were read on a Bio-Rad Model 680 Microplate reader at 415 nm.

Cell purification and adoptive transfer

Cells were purified from spleens and lymph nodes. CD4+ T cells were purified by negative enrichment using a mixture of biotinylated Abs (CD4, CD8, CD11b, CD11c, F4/80, and GR-1) in combination with anti-biotin beads (Milteny Biotech). Lymphocytes were labeled with CFSE (where indicated) and injected intravenously into recipient mice. At the experimental end points, the lymphocytes were analyzed by flow cytometry.

Bone marrow chimeric mice

Rag1-deficient recipient mice were sublethally irradiated with 300 rad. The following day, 10×106 bone marrow cells from a Msr1-sufficient or -deficient K/BxN mice (combined with 5×106 bone marrow cells from unirradiated Rag1-deficient mice where indicated) and injected i.v. into recipient mice. The recipient mice were maintained on sulfamethoxazole and trimethoprim as described previously (24).

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 8 wk of age (Fig. 1A, 1B, Supplemental Fig. 1A). Some of the Msr1−/− K/BxN mice (Msr1−/− K/BxN) were also protected from arthritis, although to a lesser extent, indicating a gene dose effect (data not shown). Protection from arthritis did not segregate by gender or cohabited littermates. The Msr1−/− K/BxN mice were also protected from mitral valve inflammation (endocarditis) (Supplemental Fig. 1A), an additional site of inflammatory attack in this model (15). To exclude the possibility that arthritis was simply delayed, we aged anergic nonarthritic Msr1−/− K/BxN for 4 mo, during which time the arthritis severity either remained consistent with the 8-wk time point or resolved (Supplemental Fig. 1B).
that K/BxN mice lacking Msr1 had lower total IgG levels and lower anti-GPI IgG titers compared with controls. Furthermore, among the Msr1<sup>−/−</sup>/K/BxN mice, those with arthritis tended to have higher total IgG levels and anti-GPI titers than those without arthritis (Fig. 2), consistent with the tight link between autoantibody production and arthritis development in this model. We next sought to determine how Msr1 deficiency interfered with autoantibody production.

The T cell compartment is unaltered in Msr1-deficient K/BxN mice

Production of anti-GPI autoantibodies in K/BxN mice depends on the interaction of CD4<sup>+</sup> T cells expressing the transgene-encoded KRN TCR with GPI-specific B cells. We therefore asked whether the impaired autoantibody production in Msr1<sup>−/−</sup>/K/BxN mice was due to altered T cell development or activation, particularly in view of the restricted expression of Msr1 on macrophages, a cell type capable of presenting peptide:MHCII Ags to CD4<sup>+</sup> T cells (8, 25). We first evaluated T cell subsets in the thymus and periphery from Msr1-deficient and control K/BxN mice, but found no difference in their numbers or relative frequencies (Fig. 3A, 3B). Peripheral CD4<sup>+</sup> T cells from both groups of mice expressed comparable levels of the T cell activation marker, CD44, (Fig. 3D), demonstrating that the activation of pathogenic Th cells was not impaired (24, 26). Msr1 has been reported to perform a variety of functions, including the capture and transfer of Ag for presentation (8, 25). To address directly whether the absence of Msr1 led to reduced Ag presentation in vivo, we adoptively transferred CFSE-labeled naive CD4<sup>+</sup> T cells from KRN/B6 (H-2<sup>b</sup>) mice into Msr1<sup>+/+</sup> or Msr1<sup>−/−</sup> H-2<sup>b</sup>/K/BxN recipient mice and found that the transferred cells proliferated equivalently in both hosts (Fig. 3E), suggesting that the presentation of GPI peptide:1-A<sub>2</sub> complexes was not impaired. We also considered the possibility that CD4<sup>+</sup> T cells in Msr1<sup>−/−</sup>/K/BxN mice were anergic and therefore unable to provide sufficient B cell help. We found, however, that activated CD4<sup>+</sup> T cells isolated from Msr1<sup>+/+</sup> or Msr1<sup>−/−</sup> K/BxN mice proliferated indistinguishably when transferred together into H-2<sup>b</sup>/K/BxN hosts (Fig. 3F). Furthermore, there was no apparent difference in the expression of the CD4<sup>+</sup> T cell anergy markers CD73 and folate receptor 4 (FR4) in the two groups of mice (Fig. 3G) (27). Collectively, these findings suggest that the reduction in anti-GPI autoantibody production in Msr1<sup>−/−</sup>/K/BxN mice is not due to altered T cell development or activation.

Decreased autoantibody production in Msr1-deficient K/BxN mice

K/BxN mice have elevated levels of total serum IgG as well as high-titer, arthritogenic anti-GPI autoantibodies (13, 14). We found 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 initiation phase of autoimmunity in K/BxN mice (Fig. 1A) (27). Collectively, these findings suggest that the reduction in anti-GPI autoantibody production in Msr1<sup>−/−</sup>/K/BxN mice is not due to altered T cell development or activation.

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**FIGURE 1.** Msr1-deficient K/BxN mice are protected from arthritis and endocarditis. (A) Arthritis severity in Msr1<sup>+/+</sup> (●, n = 7) and Msr1<sup>−/−</sup> (○, n = 12) K/BxN mice was assessed at the indicated ages and is depicted as a qualitative score (left panel) and quantitative measurement (right panel) of ankle width. Plotted values are means ± SEM; p values are shown in the panels and were determined by repeated-measures ANOVA. (B) Same parameters as (A) in more detail for the Msr1<sup>+/+</sup>-versus Msr1<sup>−/−</sup> group of K/BxN mice; each line represents one mouse. (C) Serum-transferred arthritis was induced in Msr1<sup>+/+</sup> (●, n = 5) and Msr1<sup>−/−</sup> (○, n = 5) B6 mice by injection of arthritogenic K/BxN serum on days 0 and 2 (arrows). Plotted values are means ± SEM; p values determined by repeated-measures ANOVA were not significantly different.

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 of arthritis in the serum–transfer system (Fig. 1C). Taken together, these findings demonstrate that Msr1 deficiency impacts the initiation phase of autoimmunity in K/BxN mice and not the effector phase. We therefore focused our attention on understanding how Msr1 deficiency affected T and B cell autoreactivity in K/BxN mice.

**FIGURE 2.** Msr1 deficiency impairs autoantibody production. Total serum IgG (A) and anti-GPI IgG (B) were measured by ELISA in 8-wk-old Msr1<sup>+/+</sup> and Msr1<sup>−/−</sup>/K/BxN mice as well as KRN-negative Msr1<sup>+/+</sup> and Msr1<sup>−/−</sup> mice. Each point represents one animal; ●, arthritic animals; ○, nonarthritic animals; bars represent means. Data were compiled from three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
FIGURE 3. Msr1 deficiency does not affect KRN T cell activation. T cell subsets from the thymus (A) and spleen (B) were enumerated among K/BxN mice of the indicated Msr1 genotypes. The values plotted are means ± SEM; n = 5 mice/genotype. (C) Surface expression of CD44 measured by mean fluorescence intensity (MFI) on splenic CD4+ effector T cells from Msr1+/+ (squares) and Msr1−/− (circles) K/BxN mice was determined by flow cytometry. (D) The number of CD4+ lymph node T cells expressing intracellular IL-17 was determined by flow cytometry. In both (C) and (D), filled shapes indicate individual arthritic animals, open shapes indicate nonarthritic animals; bars represent mean values. (E) CFSE-labeled naive CD4+ T cells from KRN/B6 donor mice were adoptively transferred into Msr1+/+ (thick line) or Msr1−/− (thin line) H-2b/g7−expressing recipient mice or control B6 (H-2b) mice (shaded histogram) and harvested 48 h later for flow cytometric analysis. The data shown are representative of three separate experiments (n = 8 total mice/group). (F) CFSE-labeled, congenically marked CD4+ T cells (10^6) from Msr1+/+ K/BxN mice (thick line) or Msr1−/− K/BxN mice (thin line) were adoptively transferred together into H-2b/+–expressing hosts and harvested 3 d later for flow cytometric analysis. Data represent two experiments with a total of seven recipient mice. No CFSE dilution was seen in H-2b hosts (data not shown). (G) Expression of the T cell anergy markers CD73 and FR4 was evaluated among CD4+ splenocytes and lymph node cells in the indicated mice. Data represent two experiments with a total of three mice per group.

B cells remain relatively naive in Msr1−/− K/BxN mice

Genetic absence of Msr1 did not affect the total number of splenic B cells in K/BxN or B6 mice (Fig. 4A). We therefore used a recently developed tetramer enrichment strategy to enumerate Ag-specific (i.e., GPI-specific) B cells (20). We found that mice lacking the KRN transgene contained very low numbers of GPI-specific B cells. In contrast, Msr1+/+ K/BxN mice contained significantly more GPI-specific B cells relative to Msr1−/− K/BxN mice (300,000 versus 80,000 p < 0.001). Notably, there was no difference in the number of GPI-specific B cells in arthritic versus nonarthritic Msr1−/− K/BxN animals (Fig. 4B). In addition, we were unable to detect anti-GPI IgG-secreting cells by ELISPOT in either arthritic or nonarthritic Msr1−/− K/BxN mice, whereas they were easily detected in Msr1+/+ K/BxN mice (Fig. 4C). We therefore investigated the expression of B cell surface markers. We found that the expression of MHCII was essentially equivalent among GPI-specific B cells from Msr1+/+ and Msr1−/− mice. In contrast, GPI-specific B cell surface expression of IgM and IgD was substantially lower in Msr1+/+ K/BxN mice relative to Msr1−/− K/BxN mice, consistent with an activated B cell phenotype in the Msr1+/+ group and a more naive phenotype in the Msr1−/− group (Fig. 4D). Further analysis confirmed this impression. Specifically, more GPI-specific B cells in Msr1+/+ K/BxN mice demonstrated isotype switching as demonstrated by increased intracellular IgG1 expression and also contained a significantly larger population of GPI-specific IgM/CD38 GL7+ germinal center cells relative to Msr1−/− K/BxN mice (Fig. 4E, 4F) (28).

No intrinsic defect in Msr1-deficient B cells

We next asked why the GPI-specific B cells in Msr1−/− K/BxN mice remained relatively naive in the presence of apparently normally activated autoreactive CD4+ T cells. Two scenarios seemed likely defects that were either B cell–intrinsic or B cell–extrinsic. First, it was possible that Msr1−/− B cells have an intrinsic activation defect. Arguing against this possibility is the fact that Msr1 is not expressed by B cells, in addition to a prior study demonstrating normal T cell–dependent humoral immune responses in the presence of pharmacologic blockade of Msr1 (29). Nonetheless, we formally considered the hypothesis that the B cells from Msr1−/− mice were less efficient responders than wildtype B cells. To test this, we isolated congenically marked B cells from Msr1+/+ and Msr1−/− mice (H-2b+/+ and Msr1−/− K/BxN mice (thick line) or Msr1−/− K/BxN mice (thin line) were adoptively transferred into H-2b+/+–expressing hosts and harvested 3 d later for flow cytometric analysis. Data represent two experiments with a total of seven recipient mice. No CFSE dilution was seen in H-2b hosts (data not shown). (G) Expression of the T cell anergy markers CD73 and FR4 was evaluated among CD4+ splenocytes and lymph node cells in the indicated mice. Data represent two experiments with a total of three mice per group.

Msr1 regulates serum GPI concentration

Although normally found in the cytoplasm, low levels of soluble GPI can also be detected in the serum of mice and humans (30–32). Because Msr1 functions as a scavenger receptor, we hypothesized that Msr1 might bind and clear excess Ag (GPI) from the circulation. To address this, we incubated peritoneal macrophages from Msr1+/+ and Msr1−/− with fluorescently labeled GPI or an irrelevant protein (bovine albumin), using fluorescence as a measure of uptake. Macrophages lacking Msr1 took up both GPI and albumin less efficiently than did wild-type macrophages, and this defect...
appeared more pronounced for GPI (Fig. 6A). Correspondingly, we found elevated levels of GPI in the serum of Msr1-deficient mice, which ranged between 2 and 20 μg/ml by Western blot analysis (Fig. 6B). Although the GPI standards detected as little as 23 ng/ml, the serum concentration of GPI in Msr1+/− mice was below the level of detection for the assay, indicating that the serum concentration of GPI in Msr1+/− mice was a log or more higher than in Msr1+/+ mice. Deficiency of Msr1 did not impact the serum concentration of mouse albumin (42.9 ± 16 mg/ml in Msr1+/+ versus 45.9 ± 14 mg/ml in Msr1+/− [mean ± S.D., p = 0.76, n = 5 mice/group]). We speculate that although Msr1 may be able to mediate uptake of albumin (as in Fig. 6A), this effect does not measurably affect the concentration of a protein in high abundance in the serum. In contrast, the absence of Msr1-mediated uptake of a low abundance protein such as GPI can cause a detectable and immunologically significant increase in its serum concentration. These results demonstrate that Msr1 normally acts to maintain low levels of soluble GPI in the circulation.

We hypothesized that adding Msr1-expressing APCs to capture and “normalize” the concentration of GPI should reverse the defect in B cell activation seen in Msr1−/− K/BxN mice. To test this, we created bone marrow chimeric mice in which bone marrow from nonarthritic Msr1+/+ K/BxN or arthritic Msr1+/+ K/BxN mice was mixed with bone marrow from Rag1-deficient mice (as an additional source of Msr1-expressing APCs) 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 equivalently to control mice (Fig. 6C). Furthermore, the incidence of arthritis among the mice transplanted with Msr1+/− K/BxN marrow was significantly higher than the incidence among the Msr1+/− K/BxN TCR transgenic animals (90 versus 52.5%; p = 0.0033). In subsequent experiments in which the additional donor Rag1-deficient bone marrow was omitted, all Rag1-deficient recipients of either Msr1−/− K/BxN or Msr1+/− K/BxN bone marrow developed arthritis, al-
transplantation experiments confirm our earlier impression that CD4+ KRN T cells from Msr1+/− K/BxN mice can provide productive B cell help and that intrinsic B cell Msr1 deficiency per se is not responsible for the reduced arthritis severity in Msr1+/− K/BxN mice. Rather, these experiments suggest that Msr1 regulates the serum concentration of the soluble self-Ag GPI.

Thus, in the presence of Msr1 on macrophages, GPI levels remain low, and KRN*CD4+ autoreactive T cells are able to locate and activate GPI-specific B cells. In the absence of Msr1, GPI levels are higher; T cells are still activated, but are inefficient at activating GPI-specific B cells. Indeed, we found that Msr1+/−/K/BxN mice had significantly fewer follicular Th (Tfh) cells than did their wild-type counterparts (Fig. 7A, 7B). Furthermore, immunohistologic examination of lymph nodes revealed T cells located in close proximity to B cells within germinal centers in Msr1+/− K/BxN mice. In contrast, in Msr1+/− K/BxN mice, the T cells were situated in the paracortical area of the lymph node and B cells remained in primary lymphoid follicles, similar to naïve B6 mice and Msr1−/− mice lacking the KRN TCR transgene (Fig. 7C). Below we discuss potential mechanisms by which a relatively autoantigen-rich environment might paradoxically lead to less efficient T-B cell collaboration and decreased differentiation to helper Th cells.

**Discussion**

In this paper, we report that Msr1 deficiency ameliorates systemic autoimmune disease 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. Despite normal activation of autoreactive CD4+ T cells, GPI-specific B cells remained relatively naïve, resulting in decreased autoantibody production and protection against arthritis development.

The increased circulating concentration of GPI in Msr1-deficient mice could theoretically impact the fate of GPI-specific B cells in a number of ways. For example, high concentrations of GPI could lead to more efficient clonal deletion of high-affinity, GPI-specific B cells or induce them to become anergic. Notably, naïve B cells may be rendered anergic, or hyporesponsive to antigenic stimuli, if they encounter their cognate Ag (signal 1) in the absence of TLR ligation or appropriate costimulation (signal 2) (6, 35). However, in Msr1−/− mice lacking autoreactive T cells, the number of GPI-specific B cells was not reduced (see Fig. 4B), suggesting that clonal deletion was not more efficient. It is possible that the GPI-specific B cells in the Msr1−/− mice bind GPI with lower affinity or are relatively anergic compared with their counterparts in Msr1+/+ mice. However, our finding that GPI-reactive B cells from Msr1−/− proliferated equivalently to those derived from Msr1+/+ mice following adoptive transfer suggests that they were neither anergic nor of substantially lower affinity.

We favor a model in which the higher concentration of circulating GPI in Msr1-deficient mice impairs B cell activation indirectly by altering the cognate interactions between autoreactive CD4+ T cells and B cells. In the Msr1−/− environment of relative GPI excess, T cells are more likely to encounter professional APCs presenting GPI peptide/MHCII complexes, resulting in T cell “stop” signals (36). Widespread, sustained arrest of the autoreactive T cells would in turn reduce the likelihood that an activated autoreactive T cell would encounter a rare GPI-reactive B cell, leading to the reduced number of Tfh cells that we observed. The net effect of this disruption of cognate T-B cell collaborations would be that GPI-reactive B cells would paradoxically appear to be in a clonally ignorant, naïve state despite the high concentration of soluble self Ag. As a corollary, because the precursor frequency of autoreactive...
The authors have no financial conflicts of interest.
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