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Fcγ Receptor III and Fcγ Receptor IV on Macrophages Drive Autoimmune Valvular Carditis in Mice

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Objective. **Arthritis and valvular carditis coexist in several human rheumatic diseases, including systemic lupus erythematosus, rheumatic fever, and rheumatoid arthritis. T cell receptor–transgenic K/BxN mice develop spontaneous autoantibody-associated arthritis and valvular carditis. The common Fc receptor γ (FcRγ) signaling chain is required for carditis to develop in K/BxN mice. FcRγ pairs with numerous receptors in a variety of cells. The aim of this study was to identify the FcRγ-associated receptors and Fcγ receptor (FcγR)–expressing cells that mediate valvular carditis in this model.**

Methods. **We bred K/BxN mice lacking the genes that encode activating Fcγ receptors (FcγRI, FcγRIII, and FcγRIV), and we assessed these mice for valvular carditis. We similarly evaluated complement component C3–deficient K/BxN mice. Immunohistochemistry, bone marrow transplantation, and macrophage depletion were used to define the key FcRγ-expressing cell type.**

Results. **Genetic deficiency of only one of the activating Fcγ receptors did not prevent carditis, whereas deficiency of all 3 activating Fcγ receptors did. Further analysis demonstrated that FcγRIII and FcγRIV were the key drivers of valve inflammation; FcγRI was dispensable. C3 was not required. FcRγ expression by radioresistant cells was critical for valvular carditis to develop, and further analysis indicated that macrophages were the key candidate FcγR-expressing effectors of carditis.**

Conclusion. **FcγRIII and FcγRIV act redundantly to promote valvular carditis in K/BxN mice with systemic autoantibody-associated arthritis. Macrophage depletion reduced the severity of valve inflammation. These findings suggest that pathogenic autoantibodies engage Fcγ receptors on macrophages to drive valvular carditis. Our study provides new insight into the pathogenesis of cardiovascular inflammation in the setting of autoantibody-associated chronic inflammatory diseases.**

Several systemic autoimmune diseases characterized by autoantibody production, including systemic lupus erythematosus (SLE), antiphospholipid syndrome, acute rheumatic fever, and rheumatoid arthritis (RA), affect both the synovial joints and the heart (1–3). Most notable is the increased cardiovascular morbidity and mortality due to atherosclerotic coronary artery disease among patients with SLE and RA. This increased risk is not fully explained by traditional risk factors, strongly suggesting that chronic inflammatory diseases themselves contribute to poor cardiovascular outcomes (4–6). Cardiac manifestations of these diseases also include valvular carditis (2). The immunologic mechanisms by which these diseases provoke inflammation of the joints and cardiovascular system remain poorly understood.

T cell receptor (TCR)–transgenic K/BxN mice develop spontaneous, fully penetrant, autoantibody-associated arthritis and valvular carditis (7,8). The valve inflammation in these mice shares several pathologic features with the valvular carditis found in patients with rheumatic conditions. Specifically, it affects left-sided

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valves and is characterized by IgG and complement C3 binding to the valves and a cellular infiltrate comprising predominantly T cells and mononuclear myeloid cells (2,7,9,10). In these mice, neutrophils are not found in the inflamed valves (7). This mouse model is therefore well-suited for research into how systemic autoimmune inflammatory diseases drive cardiac pathology.

Autoimmunity in K/BxN mice is initiated by a breach of immunologic self-tolerance, when T lymphocytes bearing the KRN TCR transgene recognize peptides derived from the ubiquitously expressed antigen glucose-6-phosphate isomerase (GPI), which is presented by the class II major histocompatibility complex (MHC) molecule I-A^{g7} (8,11). This ultimately leads to the sustained production of anti-GPI IgG autoantibodies. Transfer of anti-GPI autoantibodies causes arthritis in recipient mice (12). Interruption of any of the events leading to the production of autoantibodies abrogates the development of both arthritis and valvular carditis (7). However, the downstream immune effector mechanisms responsible for arthritis and carditis in this model differ. Specifically, arthritis requires complement component C5 but not the common Fc receptor γ signaling chain (FcRγ). Conversely, valvular carditis requires FcRγ but not C5 (7).

FcRγ is an immunoreceptor tyrosine–based activation motif (ITAM)–containing signaling molecule that pairs with activating Fcγ receptors and several other types of receptors; it is required for cell surface expression of the receptors and for signal transduction (13–15). Fcγ receptors bind the Fc portion of IgG (13,15). There are 2 general categories of Fcγ receptors: activating and inhibitory. In mice, the activating Fcγ receptors are FcγRI, FcγRIII, and FcγRIV. These receptors have unique IgG-binding α-chains, but share the common γ signaling chain, FcRγ. The inhibitory receptor, FcγRII, does not associate with FcRγ. Activating Fcγ receptors have distinct cellular expression patterns, predominantly on myeloid cells. They bind the different IgG subtypes with varying affinities: FcγRI and FcγRIV predominantly bind IgG2a/c and IgG2b, whereas FcγRIII binds IgG1 more strongly than it binds IgG2a/c and IgG2b (13,15). The knowledge that the common γ signaling chain FcRγ is required for valvular carditis in K/BxN mice led to 2 hypotheses: 1) that one or more of the activating Fcγ receptors is required or 2) that a different FcRγ-associated receptor is involved. Herein we used a genetic approach to examine these possibilities, and we also studied the FcRγ-expressing cells that are key drivers of valvular carditis in K/BxN mice.

MATERIALS AND METHODS

Mice. KRN TCR–transgenic mice on a C57BL/6 (B6) background and B6 mice congenic for H-2^{g7} (B6.g7) (7,8) were gifts from Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA and Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). C5-deficient B6 mice congenic for the NOD-derived *Hc* allele (encoding nonfunctional C5) (7,16) were also a gift from Drs. Mathis and Benoist. C3-deficient mice on a B6 background (17) were a gift from Michael Carroll (Harvard Medical School, Boston, MA). Mice on a B6 background that were deficient in the gene for FcRγ (*Fcer1g*) (18) were purchased from Taconic. Mice with targeted deletion of *Fcgr1* have been previously described (19). Mice with targeted deletion of *Fcgr4* were a gift from Jeffrey Ravetch (The Rockefeller University, New York, NY) and have been previously described (19,20). Generation and characterization of FcγRII/FcγRIII/FcγRIV-deficient mice and FcγRI/FcγRII/FcγRIII/FcγRIV-deficient mice will be described elsewhere (Verbeek JS: unpublished observations). Notably, *Fcgr2*, *Fcgr3*, and *Fcgr4* are in close proximity on mouse chromosome 1, and this entire region was targeted; *Fcgr1* is on mouse chromosome 3. *Fcgr3*-deficient mice on a B6 background (*Fcgr3*^{tm1Sjv}; stock no. 003171), B6 mice congenic for CD90.1 (B6.PL-Thy1^{a}/Cy3; stock no. 000406), and *Rag1*-deficient B6 mice (*Rag1*^{tm1Mom}; stock no. 002216) (21,22) were purchased from The Jackson Laboratory.

Mice with the described targeted alleles of the genes that encode the various Fcγ receptors and complement components were interbred with KRN mice and B6.g7-congenic mice to generate mice for this study. For ease of nomenclature, we refer to KRN^{+}H-$2^{b/g7}$ mice as "K/BxN mice" (7). Genotype was determined by polymerase chain reaction for all mice and confirmed by flow cytometry for the FcγR-deficient mice. Mice were bred in specific pathogen–free colonies and maintained at the University of Minnesota as per Institutional Animal Care and Use Committee–approved protocols (0909A72086 and 1207A17481).

Antibodies. Antibodies used for flow cytometry included anti-CD3 (145-2C11) and anti-CD4 (RM4-5) (BioLegend), anti-CD4 (RM4-5), anti-CD90.1 (HIS51), anti-CD44 (IM7), anti–interferon-γ (anti-IFNγ) (XMG1.2), anti–interleukin-17 (anti–IL-17) (eBioTc11-18H10.1), anti-CD62L (MEL-14), anti-CD16/32 (clone 93), anti–Gr-1 (RB6-8C5), anti-F4/80 (BM8), and anti-B220 (RA3-6B2) (eBioscience), anti-$V_{\beta}6$ (RR4-7), anti-CD11b (M1/70), and anti-CD64 (X54-5/7.1) (BD PharMingen), and anti–Armenian hamster IgG (Jackson ImmunoResearch). The anti-FcγRIV–specific antibody (9E9) was generously provided by Jeffrey Ravetch and has been previously described (23).

Additional antibodies used for immunohistochemical analysis included anti-CD64 (N-19) (Santa Cruz Biotechnology), anti-CD16/32 (clone 93) and biotinylated anti-F4/80 (BM8) (eBioscience), anti-CD11c (N418) and anti-CD31 (clone 390) (BioLegend), and anti-Langerin (929F3.098) (Imgenex). Biotin-conjugated antibodies recognizing IgG1, IgG2b, and IgG2c were from Jackson ImmunoResearch. Secondary antibodies included bovine anti-goat DyLight 594 and streptavidin Alexa Fluor 488 (Jackson ImmunoResearch), goat anti-

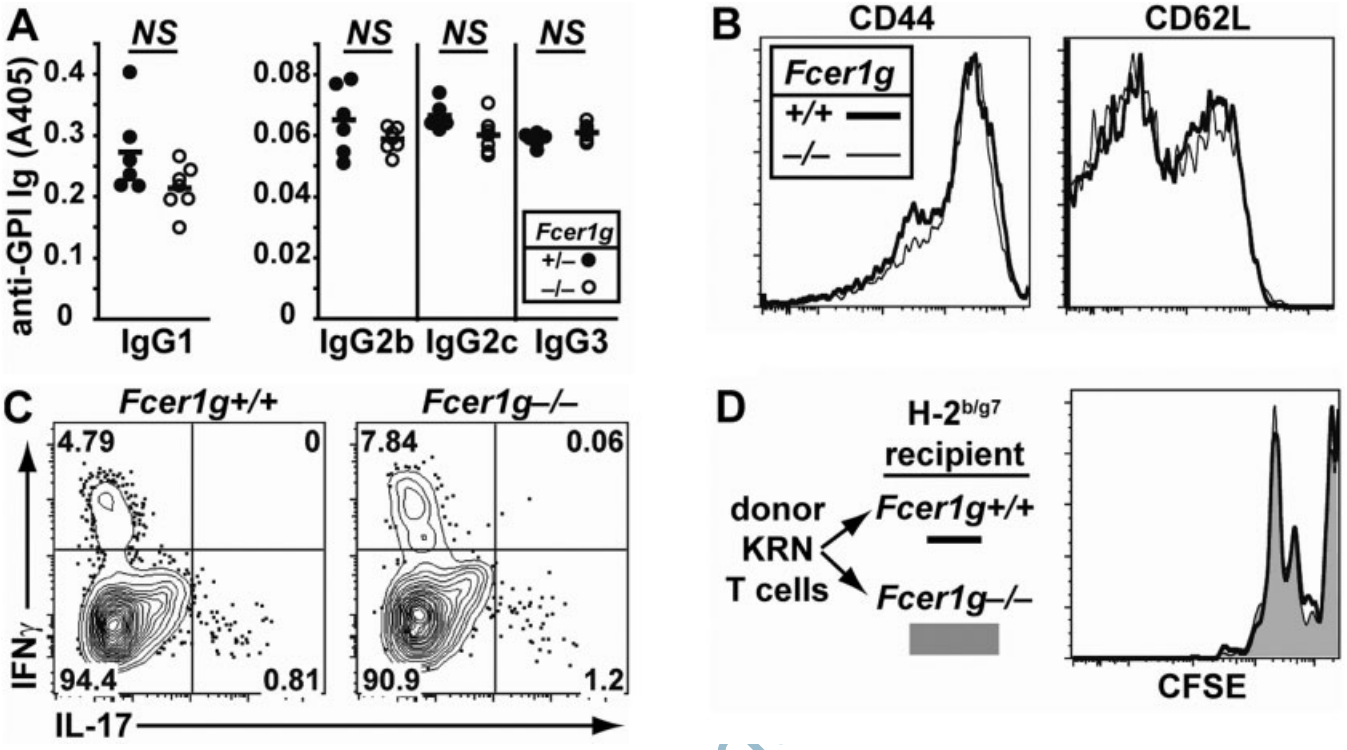

Figure 1. Normal adaptive immunity in Fc receptor γ (FcRγ)–deficient K/BxN mice. **A,** Anti–glucose-6-phosphate isomerase (anti-GPI) IgG1, IgG2b, IgG2c, and IgG3 antibody titers in serum from FcRγ-sufficient (n = 6) and FcRγ-deficient (n = 7) K/BxN mice. Each data point represents a single mouse; horizontal lines show the mean. *P* values were calculated using Student's *t*-test and were not significant (NS). **B,** Flow cytometric assessment of CD44 and CD62L expression on CD4+V_β6+ splenocytes and lymph node cells from FcRγ-sufficient K/BxN control mice and FcRγ-deficient K/BxN mice. Data are representative of 1 experiment with a total of 2 control mice and 3 FcRγ-deficient mice. V_β6 is the KRN transgene–encoded T cell receptor β-chain. Activated T cells are $CD44^{high}$ and $CD62L^{low}$. **C,** Representative flow cytometric assessment of the percentage of CD4+V_β6+ splenocytes from the FcRγ-sufficient K/BxN control mice and FcRγ-deficient K/BxN mice expressing intracellular interferon-γ (IFNγ) and interleukin-17 (IL-17). Data are representative of 2 experiments with a total of 3 control mice and 5 FcRγ-deficient mice. **D,** Proliferation of congenically marked carboxyfluorescein succinimidyl ester (CFSE)–labeled lymphocytes from KRN mice transferred into FcRγ-sufficient control mice or FcRγ-deficient recipient mice expressing $H\text{-}2^{g7}$. Cells were gated on CD3+CD4+90.1+V_β6+ cells. Data are representative of 2 experiments with a total of 2 control mice and 3 FcRγ-deficient mice.

rabbit DyLight 594 (Poly4054) and goat anti–Armenian hamster DyLight 594 (Poly4055) (BioLegend), and streptavidin DyLight 550 (Thermo Scientific).

Assessment of arthritis, anti-GPI titers, and histology. Arthritis was assessed as previously described (24,25). Serum anti-GPI total IgG titers and IgG subtype (IgG1, IgG2b, IgG2c, and IgG3) titers were measured as described (25). Except where indicated, cardiac valves of 8–10-week-old mice were assessed histopathologically.

Immunohistochemistry and toluidine blue staining. Frozen sections were first blocked for Fcγ receptors and with avidin/biotin (Invitrogen), when necessary. Sections were incubated with the unconjugated primary antibody recognizing the Fcγ receptor of interest (anti-CD64, anti-CD16/32, or FcγRIV-specific antibody) or with appropriate isotype controls, and fluorescence-conjugated secondary antibodies were used for detection of Fcγ receptors. For the detection of CD11c, CD31, and Langerin, frozen sections were stained with the Alexa Fluor 488–conjugated antibodies described above or with appropriate isotype controls. For the detection of F4/80, frozen sections were stained with biotinylated anti-F4/80 followed by fluorescent strepavidin. Nuclei were counterstained with DAPI. Immunohistochemical analysis of IgG1, IgG2b, and IgG2c was performed using biotinylated primary antibodies (Jackson ImmunoResearch) or the appropriate isotype controls. The primary antibodies were detected by application of ImmPACT 3,3′-diaminobenzidine peroxidase substrate with an avidin–biotin–peroxidase kit (Vector). Mast cells were detected in frozen sections by staining with toluidine blue according to the toluidine blue–staining protocol (IHC World). Slides were viewed on an Olympus BX51 microscope equipped with a digital camera and DP-BSW software.

Flow cytometry. Intracellular cytokine staining for IL-17 was performed according to the instructions of the manufacturer (eBioscience). Flow cytometry was performed using a FACSCalibur and an LSRII (BD Biosciences), and cells were analyzed using FlowJo version 8.8.7 software (Tree Star).

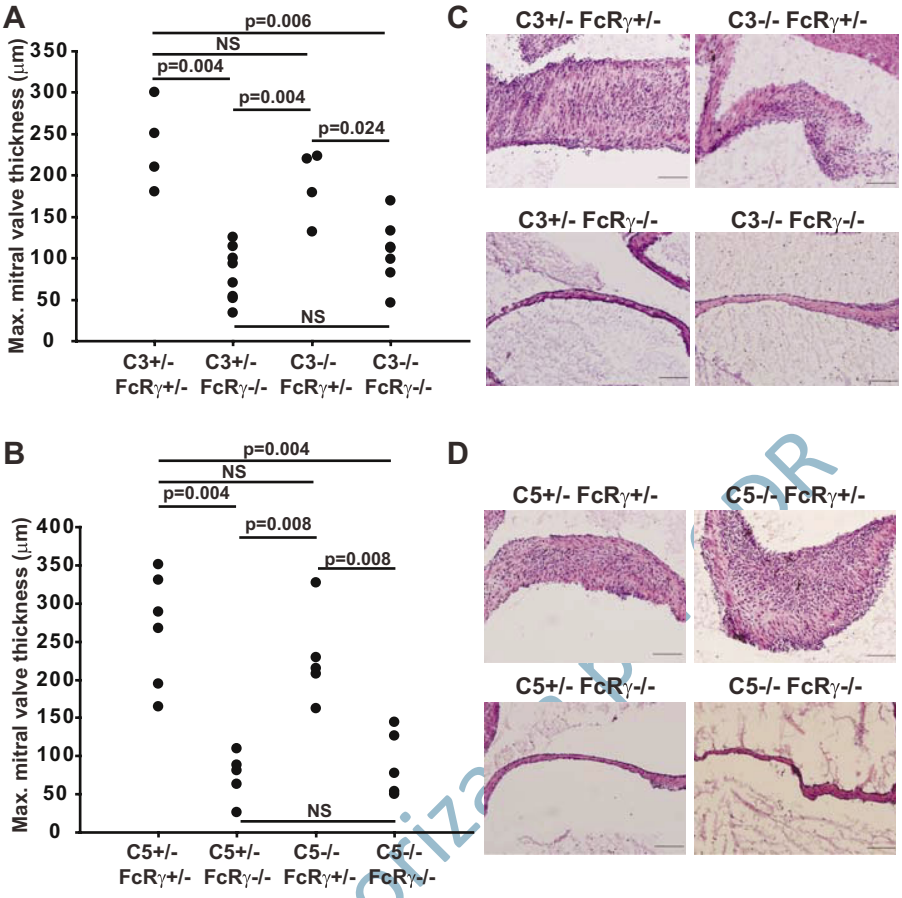

Figure 2. Complement components C3 and C5 are not necessary for valvular carditis. **A** and **B**, Maximum (max.) mitral valve thickness in 8-week-old K/BxN mice of the indicated genotypes for the genes encoding C3 and Fc receptor γ (FcRγ) (**A**) and C5 and FcRγ (**B**). Each data point represents a single mouse. *P* values were calculated using the Mann-Whitney U test. NS = not significant. **C** and **D**, Representative photomicrographs of mitral valves from K/BxN mice of the indicated genotypes for the genes encoding C3 and FcRγ (**C**) and C5 and FcRγ (**D**). Hematoxylin and eosin stained. Bars = 100 μm; original magnification × 20.

Bone marrow transplantation. Nine–15-week-old *Rag1*-deficient recipient mice were irradiated with 300 rads. Four hours following irradiation, 3×10^6 donor bone marrow cells were injected intravenously. Hearts were harvested 8 weeks after bone marrow transplantation.

Adoptive transfer. After labeling with carboxyfluorescein succinimidyl ester (CFSE), 5×10^6 donor cells were injected intravenously into H-2^{g7}–expressing recipient mice. Lymphocytes were harvested from recipient mice 40 hours later and analyzed by flow cytometry.

Macrophage depletion. Three-week-old K/BxN mice received weekly intraperitoneal injections (from weeks 3 to 7) of clodronate liposomes or control phosphate buffered saline (PBS)–containing liposomes (100 μl/10 gm of body weight) (ClodronateLiposomes.com). Mice underwent weekly assessment for arthritis. Hearts were harvested at 8 weeks and assessed for carditis. The presence of splenic macrophages was analyzed by flow cytometry.

Serum-transfer arthritis. Pooled serum (150 μl/dose) from K/BxN mice was injected intraperitoneally into recipient mice on days 0 and 2. The mice were monitored for 10 days for the development of arthritis.

Statistical analysis. Repeated-measures analysis of variance was used to compare arthritis severity scores. Tukey's multiple comparison post hoc test was used when >2 groups were compared. Data on mitral valve thickness were compared by Mann-Whitney 2-tailed U test. All other statistical differences between the mean values were calculated using Student's unpaired 2-tailed *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Antibody production, T cell activation, and antigen presentation occur normally in FcRγ-deficient K/BxN mice. Protection against valvular carditis in K/BxN mice lacking FcRγ (*Fcer1g*) despite normal production of anti-GPI IgG antibodies has previously been demonstrated (7). We found no difference in the pro-

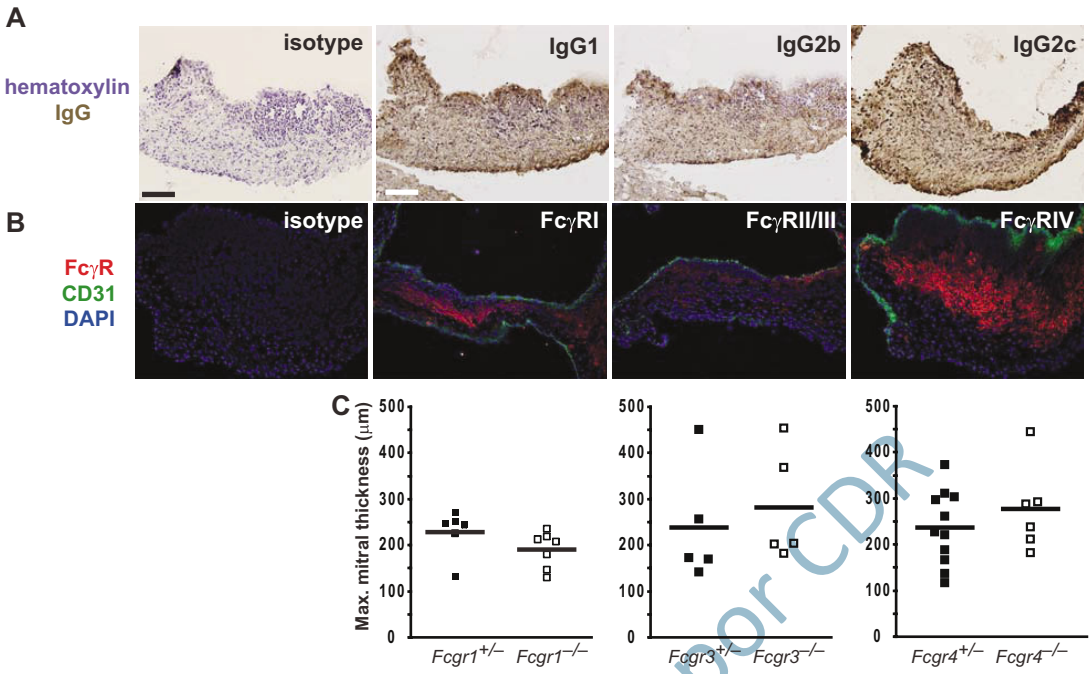

Figure 3. Activating Fcγ receptor (FcγR) expression in inflamed mitral valves of K/BxN mice. Sections of the inflamed mitral valve of an arthritic K/BxN mouse were probed via immunohistochemistry analysis for the indicated antigens. **A,** Brown staining indicates the presence of IgG1, IgG2b, or IgG2c. Sections were counterstained with hematoxylin (blue). **B,** Red staining indicates the presence of FcγRI, FcγRII/FcγRIII, or FcγRIV. The endothelial marker CD31 is shown in green. Nuclei were counterstained with DAPI (blue). No staining of isotype control antibodies was observed. Bars = 100 μm; original magnification × 20. **C,** K/BxN mice expressing null alleles of the genes encoding FcγRI, FcγRIII, or FcγRIV were bred and assessed for valvular carditis. The development of valvular carditis was not affected by the deficiency of any one activating FcγR as measured by maximum (max.) mitral valve thickness. Each data point represents a single mouse; horizontal lines show the mean. *P* values were calculated using the Mann-Whitney U test and were not significant.

duction of anti-GPI IgG subtypes (i.e., no difference in titers of IgG1, IgG2b, IgG2c, and IgG3) in FcRγ-deficient K/BxN mice as compared with the production of anti-GPI IgG subtypes in wild-type K/BxN mice (Figure 1A). Because CD4+ T cells are key contributors to the valve pathology in this model (26), we questioned whether these cells were activated normally in FcRγ-deficient K/BxN mice. Indeed, we found no difference in T cell expression of CD44 and CD62L or in intracellular cytokine production (IFNγ and IL-17) (Figures 1B and C). KRN T cells that were transferred into FcRγ-deficient or FcRγ-sufficient B6.g7 mice proliferated equivalently (Figure 1D). We therefore concluded that the protection against valvular carditis afforded by FcRγ deficiency in K/BxN mice is not due to impaired T cell or B cell activation or autoantibody synthesis, but rather to later effector events.

Complement is not required for the development of valvular carditis. Because autoantibodies appear critical for the effector phase of valvular carditis, we investigated whether antibody-mediated activation of the complement system was pathogenically important. Previously it was shown that complement component C5 is not required for valvular carditis in this model (7). It remained possible, however, that the upstream complement component C3 could play a role, for instance, by binding to receptors for C3 split products, particularly since C3 is bound to the inflamed valves (7). We therefore bred K/BxN mice carrying null alleles of the genes encoding FcRγ and/or complement components C3 or C5. We found that C3, like C5, was not necessary for the development of valvular carditis (Figure 2). These findings confirm that the autoantibody-associated valve inflammation in this model depends on FcRγ and not the complement system.

Presence of IgG- and FcγR-expressing cells in the inflamed K/BxN mouse mitral valve. Given the requirement for FcRγ in the development of valvular carditis, we used immunohistochemistry to probe inflamed mitral valves for the presence of individual activating Fcγ receptors and for the various IgG subtypes. Although IgG1 is the predominant autoantibody

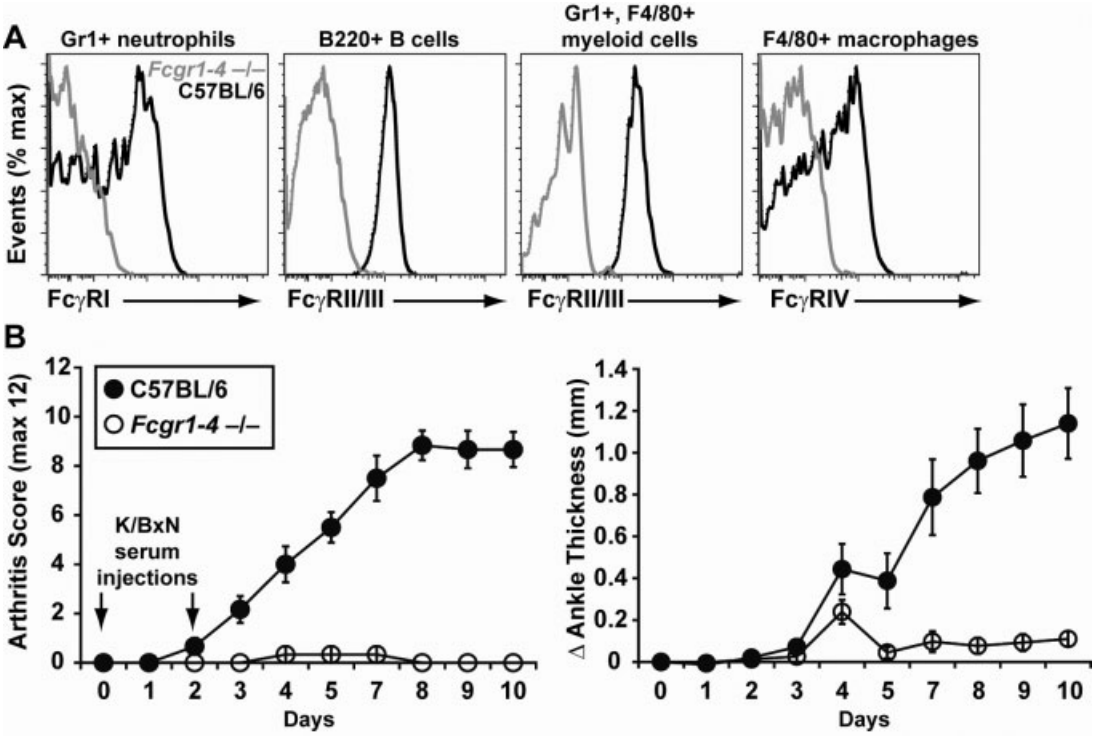

Figure 4. Characterization of *Fcgr1–4*–deficient mice. **A,** Fcγ receptor (FcγR) expression on cells from mice on a C57BL/6 background lacking all of the Fcγ receptors (*Fcgr1–4*$^{-/-}$) and cells from C57BL/6 control mice was assessed by flow cytometry. **B,** Pooled serum from arthritic K/BxN mice was injected intraperitoneally (150 μl/dose on days 0 and 2) into control mice and 6-week-old *Fcgr1–4*–deficient mice on a C57BL/6 background (n = 6 mice per group). The mice were monitored for 10 days for the development of arthritis and assessed for arthritis severity (maximum [max] possible score 12) and change in ankle thickness. Values are the mean ± SEM.

subtype produced in K/BxN mice, we found that IgG1, IgG2b, and IgG2c were all bound to the inflamed mitral valve (8) (Figure 3A). We also detected expression of each of the activating Fcγ receptors in the inflamed cardiac valve (Figure 3B). These findings supported the notion that any or all of the activating Fcγ receptors could be the key driver(s) of valvular carditis in K/BxN mice.

No protection against valvular carditis in K/BxN mice lacking single activating Fcγ receptors. We next investigated if the absence of any one of the activating Fcγ receptors protected against valve inflammation. Mice with null alleles of the genes encoding the α-chains of each of the activating Fcγ receptors (*Fcgr1*$^{-/-}$, *Fcgr3*$^{-/-}$, and *Fcgr4*$^{-/-}$) were bred with K/BxN mice and assessed for the development of valvular carditis. We found that K/BxN mice lacking expression of only one of the activating Fcγ receptors developed valvular carditis equivalent in severity to the valvular carditis seen in controls (Figure 3C). As expected, these mice also developed arthritis comparable in severity to the arthritis seen in controls (data not shown), since absence of the gene encoding FcRγ did not reduce the severity of spontaneous arthritis in K/BxN-transgenic mice (7).

Because no single activating FcγR was solely responsible for the development of valvular carditis, we considered the possibilities that more than one activating FcγR was contributing (i.e., redundancy) or that an alternative FcRγ-associated receptor, not in the FcγR family, was responsible. To discriminate between these hypotheses, we used recently developed mice that lacked all of the FcγR α-chain genes (*Fcgr1, Fcgr2, Fcgr3,* and *Fcgr4*) (Figure 4A). Although the common signaling chain FcRγ is not required for spontaneous arthritis in K/BxN-transgenic mice (7), it is required for arthritis that is induced by passive transfer of serum from K/BxN mice into naive recipients (27,28). Similarly, complete protection against serum-transfer arthritis in these new mice lacking all of the FcγR α-chain genes (Figure 4B) confirmed the expected functional defect that the absence of activating Fcγ receptors engenders in the serum transfer model.

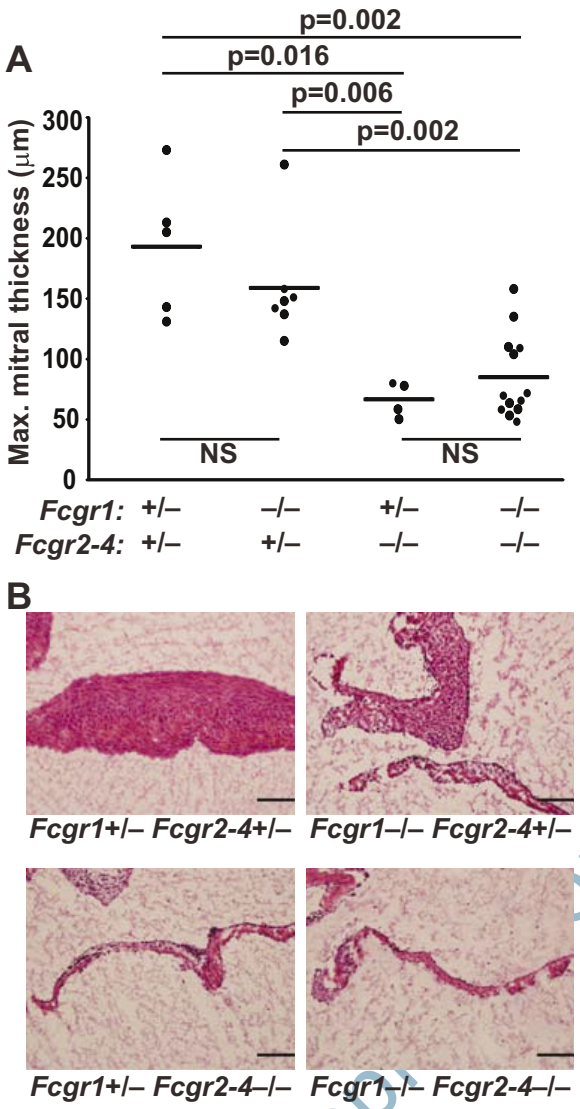

Figure 5. Protection against valvular carditis in K/BxN mice lacking Fcγ receptor III (FcγRIII) and FcγRIV. Eight-week old K/BxN mice expressing null alleles of heterozygous or homozygous *Fcgr1* and/or *Fcgr2–4* were evaluated for valvular carditis. **A,** Maximum (max.) mitral valve thickness in K/BxN mice of the indicated genotypes. Each data point represents a single mouse; horizontal lines show the mean. *P* values were calculated using the Mann-Whitney U test. NS = not significant. **B,** Representative photomicrographs of hematoxylin and eosin–stained mitral valves from the same mice. Bars = 100 μm; original magnification × 20.

FcγRIII and FcγRIV are key mediators of valvular carditis. We bred K/BxN mice lacking FcγRI, FcγRII, FcγRIII, and FcγRIV and found that protection against valvular carditis was evident (Figure 5). Furthermore K/BxN mice lacking only FcγRII/FcγRIII/FcγRIV were also protected, whereas FcγRI deficiency had no effect (Figure 5). Arthritis severity scores were not different between these various groups, with one exception: the *Fcgr1*$^{+/-}$,*Fcgr2-4* $^{-/-}$ group had slightly lower scores than the *Fcgr1*$^{+/-}$,*Fcgr2-4*$^{+/-}$ group (P = 0.044) (data not shown). K/BxN mice deficient in *Fcgr2–4* also had slightly lower levels of total anti-GPI IgG than mice sufficient for *Fcgr2–4* (data not shown), a somewhat unexpected finding since absence of the inhibitory receptor FcγRII often, but not always, leads to increased autoantibody titers (29,30). These decreases in arthritis severity and antibody titers were slight compared with the dramatic protection against valvular carditis. These findings demonstrate that the FcRγ-associated activating receptors FcγRIII and FcγRIV are the key drivers of cardiac valve inflammation in this model—there is no need to invoke roles for other FcRγ-associated receptors.

FcRγ expression on radioresistant cells promotes valvular carditis. To explore which FcRγ-expressing cells are required for the development of valvular carditis, we performed a reciprocal bone marrow transplantation experiment. We transplanted bone marrow from FcRγ-sufficient or FcRγ-deficient K/BxN mice into sublethally irradiated FcRγ-sufficient or FcRγ-deficient *Rag1*-deficient host mice. We found that recipient mice lacking FcRγ developed less severe valvular carditis as compared to controls, whereas the FcRγ status of the donor did not influence carditis severity (Figure 6A). Therefore, FcR γ-chain expression of radioresistant host cells, rather than of radiosensitive bone marrow–derived cells, contributed to the development of valvular carditis in K/BxN mice. Candidate cells included mast cells, Langerhans' cells, dendritic cells (DCs), macrophages, and endothelial cells, based on the described radioresistance of subpopulations of these cell types (31–36).

FcγR-expressing cells in inflamed mitral valves of K/BxN mice. We next explored which of the candidate cell types were present in the inflamed K/BxN mouse mitral valves and colocalized with one or more of the activating Fcγ receptors. We found that FcγRI (CD64), FcγRII/FcγRIII (CD16/32), and FcγRIV all colocalized with F4/80, a macrophage marker, on the inflamed mitral valve (Figure 6B). We could not detect Langerhans' cells or mast cells in the inflamed mitral valves (Figure 6B). None of the Fcγ receptors colocalized with CD31, an endothelial cell marker, as shown in Figure 3. CD11c, a marker of activated macrophages and DCs, colocalized with FcγRII/FcγRIII and also with F4/80 (Figure 6C), but not with FcγRI or FcγRIV (results not shown). These data indicated that the most likely FcγR-

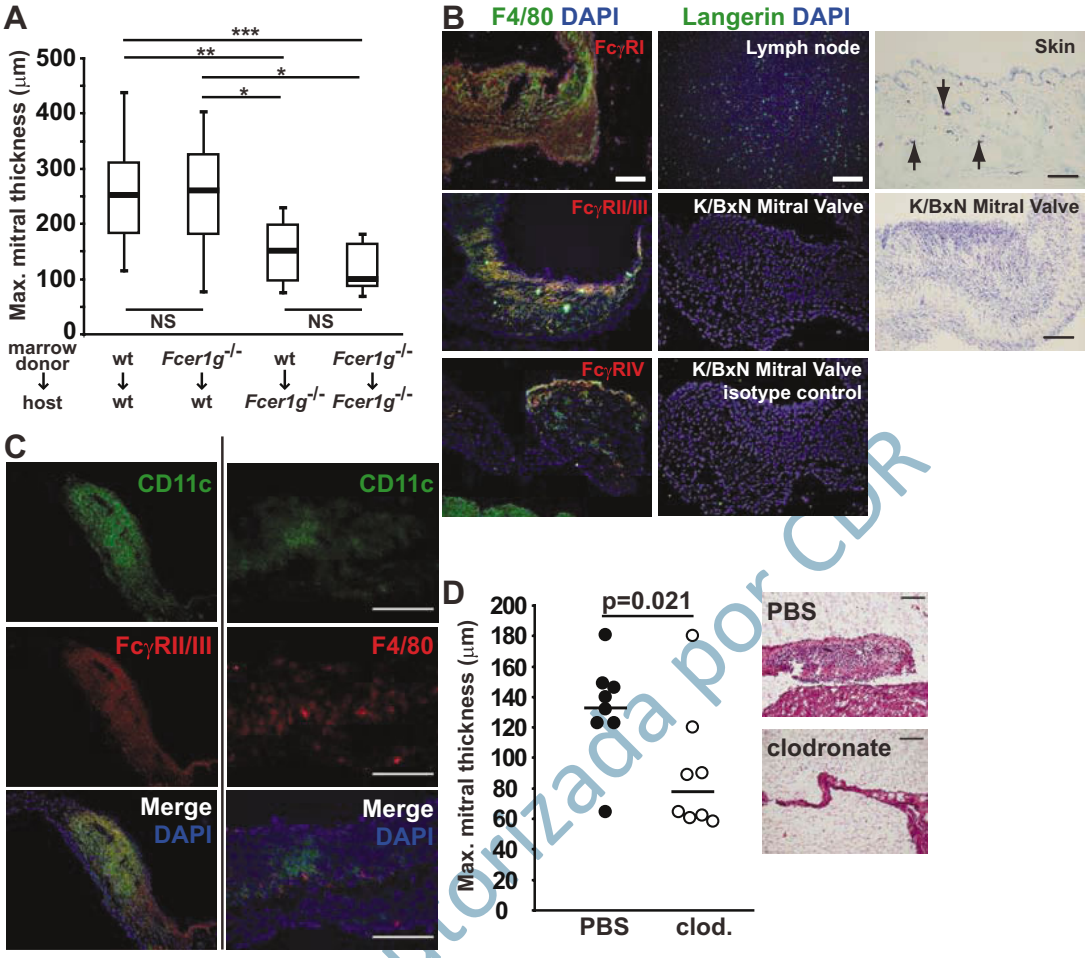

Figure 6. Macrophages are the critical Fc receptor γ (FcRγ)–expressing cell type mediating valvular carditis. **A,** Bone marrow was transplanted from donor mice to host mice in the manner indicated. Eight weeks later, the hearts were analyzed and maximum (max.) mitral valve thickness was determined. Data are shown as box plots, where the boxes represent the first and third quartiles, the lines inside the boxes represent the median, and the whiskers represent the minimum and maximum values. Results are representative of 2 experiments (n = 8–11 mice per group). $* = P < 0.05$; $* * = P = 0.01$; $* * * = P = 0.001$, by Mann-Whitney U test. NS = not significant; WT = wild type. **B,** The mitral valves of K/BxN mice were probed for the indicated activating Fcγ receptors (red) and the macrophage marker F4/80 (green) (left). Lymph node and K/BxN mouse mitral valve sections were stained with anti-Langerin antibody (green) (middle). Nuclei were stained with DAPI (blue). Toluidine blue–positive mast cells (**arrows**) were detected in skin (positive control); no mast cells were identified in the mitral valves of K/BxN mice (right). Bars = 100 μm; original magnification × 20. **C,** Staining of inflamed K/BxN mouse mitral valves for CD11c plus anti-FcγRII/FcγRIII (left) and CD11c plus F4/80 (right) demonstrates colocalization in the merged panels. Bars = 100 μm; original magnification × 40. **D,** Mitral valves of 8-week-old K/BxN mice treated with clodronate (clod.) liposomes or control phosphate buffered saline (PBS)–containing liposomes were assessed. Each data point represents a single mouse; horizontal lines show the mean. The *P* value was calculated using the Mann-Whitney U test. Representative hematoxylin and eosin–stained sections are shown. Bars = 100 μm; original magnification × 20.

expressing cells mediating valvular carditis are radioresistant macrophages and/or closely related "macrophage-like" inflammatory DCs. Initial activation of KRN T cells depends on conventional DCs and other professional antigen-presenting cells (APCs) (12). Our finding that KRN T cells were activated normally in the absence of FcRγ (Figure 1) therefore suggests that FcγR expression by conventional DCs is not required for KRN T cell activation. This indicates that FcγR-expressing macrophages or similar cells have a key role in effecting carditis.

Clodronate liposome administration reduces valvular carditis severity. We used clodronate liposomes to deplete macrophages from K/BxN mice, starting at 3 weeks of age, and assessed the mice for the development of valvular carditis at 8 weeks of age. K/BxN mice

treated with clodronate liposomes developed less severe valvular carditis compared with mice treated with control PBS-containing liposomes (Figure 6D). We confirmed the efficiency of macrophage depletion by flow cytometric enumeration of CD11b+F4/80+ splenic macrophages, which averaged 4.55×10^5 in mice treated with clodronate liposomes and 2.75×10^6 in mice treated with PBS-containing liposomes ($P < 0.0001$). Clodronate liposome treatment had no effect on arthritis severity or anti-GPI antibody titers (data not shown). These data suggest that macrophages or similar clodronate-sensitive cells are important cellular effectors of valve inflammation. Collectively, our findings are consistent with a model in which pathogenic autoantibodies engage FcγRIII and FcγRIV on macrophages to drive the development of valvular carditis in K/BxN mice.

DISCUSSION

Studies in both humans and rodents on the pathogenesis of valvular carditis in rheumatic heart disease, antiphospholipid antibody syndrome, and Libman-Sacks endocarditis have demonstrated the deposition of IgG and complement bound in the subendothelial connective tissue of the valve, as well as the presence of CD4+ T cells and macrophages (2,9,10,37,38). The K/BxN mouse model of inflammatory arthritis and valvular carditis recapitulates these characteristics. We have demonstrated the power of the K/BxN mouse model in the investigation of the immunologic mechanisms that mediate cardiovascular pathology in the setting of systemic autoantibody-associated inflammatory disease. Despite the presence of complement C3 bound to the inflamed valves in K/BxN mice, we found that C3 was not necessary for the development of valvular carditis. Rather, our results suggest that pathogenic antibodies produced by the adaptive immune system engage activating Fcγ receptors on innate immune effector cells to drive the development of valvular carditis.

In this study, we found that no activating FcγR (FcγRI, FcγRIII, or FcγRIV) was solely responsible for the development of valvular carditis in K/BxN mice. This is not surprising given the diversity of FcγR-expressing cells and the presence of multiple IgG subtypes in the inflamed mitral valves. Our findings are consistent with other examples of autoimmune disease that require the contribution of more than one activating FcγR. For example, in a passive mouse model of autoimmune hemolytic anemia induced by IgG2a and IbG2b subclasses of the anti-erythrocyte antibody, FcγRI, FcγRIII, and FcγRIV all contributed to a severe anemia phenotype induced by IgG2a antibodies, while FcγRIII and FcγRIV, but not FcγRI, were required for both the mild and severe anemia phenotypes induced by IgG2b antibodies (39). Similarly, in a murine model of acute glomerular inflammation induced by switch variant monoclonal antibodies, FcγRIII and FcγRIV were required, whereas FcγRI was dispensable (40). Our results show that FcγRIII and FcγRIV drive the development of valvular carditis in K/BxN mice and that FcγRI is not necessary. Because FcγRIV does not bind to IgG1 (23), these findings suggest that although IgG1 is the predominant IgG subtype produced in K/BxN mice, other IgG subtypes (specifically IgG2b and/or IgG2c) are also pathogenically important mediators of carditis.

Our conclusion that macrophages are the critical FcγR-expressing cell type driving the development of valvular carditis is based on our findings that a radioresistant FcγR-expressing cell type is involved, that clodronate liposomes deplete this key cell type, and that macrophage markers colocalize with the activating Fcγ receptors in the inflamed mitral valves. Could DCs also be involved? This question is complicated by the fact that the cell surface markers CD11b, F4/80, and CD11c can be expressed both by activated macrophages and by monocyte-derived DCs; our histologic studies cannot distinguish these cell types (7) (Figure 6). Furthermore, clodronate liposomes can deplete some DC populations (41–43). We consider it unlikely that conventional DCs play a role, based on our finding that professional APC–mediated activation of KRN T cells occurred normally in the absence of FcRγ (Figure 1). The absence of inflammatory cells in the native mitral valve strongly suggests that the CD11b+F4/80+CD11c+ cells present in the inflamed valve are recruited from the circulating blood. Circulating monocytes give rise not only to macrophages but also to inflammatory DCs or tumor necrosis factor/inducible nitric oxide synthase–producing DCs, with phenotypic and functional characteristics that overlap considerably with those of activated macrophages (44), leading some investigators to assert that the distinction between these subsets is artificial (45).

Our data support the conclusion that the key FcγR-expressing cell types driving the development of valvular carditis in K/BxN mice are macrophages and/or phenotypically and functionally similar monocyte-derived DCs. We therefore postulate that autoantibodies engage FcγRIII and FcγRIV on these cells to drive the development of valvular carditis in K/BxN mice. We are currently investigating how activating FcγR engage-

ment influences macrophage function, with particular attention to defining which of the proinflammatory products are critical mediators of carditis.

We have previously shown that CD4+ T cells are key effectors of valvular carditis in this model (26). Our new findings support the notion that CD4+ T cells and macrophages cooperate to provoke cardiac inflammation. Future investigations will focus on evaluating how these cell types are recruited into the valve tissue and on characterizing their molecular interactions.

Our findings provide important insight that can be used to guide the rational choice of agents to treat cardiovascular inflammation in the setting of systemic autoantibody-associated diseases. Interventions that modulate Fc receptor function, such as intravenous immunoglobulin, may be attractive (46). Alternatively, the FcγR-expressing effector cells could be eliminated. Additionally, the key Fcγ receptors themselves, or their downstream intracellular signaling molecules, could be targeted. Because the activating Fcγ receptors rely on spleen tyrosine kinase (Syk) for signal transduction, Syk inhibitors, such as fostamatinib (R788) or R406, might be good candidates for the treatment of autoantibody-associated cardiovascular inflammation (47,48). Future investigations to determine which proinflammatory pathways are activated by FcγR engagement on macrophages (or other cell types) to drive the development of valvular carditis in these mice are expected to reveal additional potential therapeutic targets.

In summary, our findings delineate several previously unreported key features in the pathogenesis of autoimmune valvular carditis in K/BxN mice. FcRγ deficiency does not affect the T cell–dependent and B cell–dependent initiation phase in the development of valvular carditis, but rather it affects the downstream effector phase. The activating receptors FcγRIII and FcγRIV drive valvular carditis, while FcγRI is dispensable. Moreover, macrophages contribute critically to the development of carditis in this model. Our findings provide new insight into the pathogenesis of cardiovascular inflammation in the setting of autoantibody-associated chronic inflammatory diseases and offer new directions from which to pursue research on basic pathogenic mechanisms and rational therapeutic approaches.

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Binstadt had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Asanuma Y, Oeser A, Shintani AK, Turner E, Olsen N, Fazio S, et al. Premature coronary-artery atherosclerosis in systemic lupus erythematosus. N Engl J Med 2003;349:2407–15.
2. Blank M, Aron-Maor A, Shoenfeld Y. From rheumatic fever to Libman-Sacks endocarditis: is there any possible pathogenetic link? Lupus 2005;14:697–701.
3. Chung CP, Oeser A, Raggi P, Gebretsadik T, Shintani AK, Sokka T, et al. Increased coronary-artery atherosclerosis in rheumatoid arthritis: relationship to disease duration and cardiovascular risk factors. Arthritis Rheum 2005;52:3045–53.
4. Avina-Zubieta JA, Choi HK, Sadatsafavi M, Etminan M, Esdaile JM, Lacaille D. Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies. Arthritis Rheum 2008;59:1690–7.
5. Symmons DP, Gabriel SE. Epidemiology of CVD in rheumatic disease, with a focus on RA and SLE. Nat Rev Rheumatol 2011;7:399–408.
6. Gustafsson JT, Simard JF, Gunnarsson I, Elvin K, Lundberg IE, Hansson LO, et al. Risk factors for cardiovascular mortality in patients with systemic lupus erythematosus, a prospective cohort study. Arthritis Res Ther 2012;14:R46.
7. Binstadt BA, Hebert JL, Ortiz-Lopez A, Bronson R, Benoist C, Mathis D. The same systemic autoimmune disease provokes arthritis and endocarditis via distinct mechanisms. Proc Natl Acad Sci U S A 2009;106:16758–63.
8. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. Cell 1996;87:811–22.
9. Kaplan MH, Bolande R, Rakita L, Blair J. Presence of bound immunoglobulins and complement in the myocardium in acute rheumatic fever: association with cardiac failure. N Engl J Med 1964;271:637–45.
10. Tenedios F, Erkan D, Lockshin MD. Cardiac involvement in the antiphospholipid syndrome. Lupus 2005;14:691–6.
11. Matsumoto I, Staub A, Benoist C, Mathis D. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. Science 1999;286:1732–5.
12. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 1999;10:451–61.
13. Boross P, Verbeek JS. The complex role of Fcγ receptors in the pathology of arthritis. Springer Semin Immunopathol 2006;28:339–50.
14. Humphrey MB, Lanier LL, Nakamura MC. Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. Immunol Rev 2005;208:50–65.
15. Nimmerjahn F, Ravetch JV. Fcγ receptors as regulators of immune responses. Nat Rev Immunol 2008;8:34–47.
16. Wetsel RA, Fleischer DT, Haviland DL. Deficiency of the murine fifth complement component (C5): a 2-base pair gene deletion in a 5'-exon. J Biol Chem 1990;265:2435–40.
17. Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC.

Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proc Natl Acad Sci U S A 1995;92:11490–4.
18. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR γ chain deletion results in pleiotrophic effector cell defects. Cell 1994;76: 519–29.
19. Ioan-Facsinay A, de Kimpe SJ, Hellwig SM, van Lent PL, Hofhuis FM, van Ojik HH, et al. FcγRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. Immunity 2002;16:391–402.
20. Nimmerjahn F, Lux A, Albert H, Woigk M, Lehmann C, Dudziak D, et al. FcγRIV deletion reveals its central role for IgG2a and IgG2b activity in vivo. Proc Natl Acad Sci U S A 2010;107: 19396–401.
21. Hazenbos WL, Gessner JE, Hofhuis FM, Kuipers H, Meyer D, Heijnen IA, et al. Impaired IgG-dependent anaphylaxis and Arthus reaction in FcγRIII (CD16) deficient mice. Immunity 1996;5:181–8.
22. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 1992;68:869–77.
23. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcγRIV: a novel FcR with distinct IgG subclass specificity. Immunity 2005; 23:41–51.
24. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science 2002;297:1689–92.
25. Nguyen LT, Jacobs J, Mathis D, Benoist C. Where FoxP3-dependent regulatory T cells impinge on the development of inflammatory arthritis. Arthritis Rheum 2007;56:509–20.
26. Haasken S, Auger JL, Binstadt BA. Absence of β_2 integrins impairs regulatory T cells and exacerbates $CD4^+$ T cell-dependent autoimmune carditis. J Immunol 2011;187:2702-10.
27. Corr M, Crain B. The role of FcγR signaling in the K/B × N serum transfer model of arthritis. J Immunol 2002;169:6604–9.
28. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, et al. Arthritis critically dependent on innate immune system players. Immunity 2002;16:157–68.
29. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. Nature 1996;379:346–9.
30. Sharp PE, Martin-Ramirez J, Mangsbo SM, Boross P, Pusey CD, Touw IP, et al. FcγRIIb on myeloid cells and intrinsic renal cells rather than B cells protects from nephrotoxic nephritis. J Immunol 2013;190:340–8.
31. Fukuzumi T, Waki N, Kanakura Y, Nagoshi J, Hirota S, Yoshikawa K, et al. Differences in irradiation susceptibility and turnover between mucosal and connective tissue-type mast cells of mice. Exp Hematol 1990;18:843–7.
32. Allan RS, Smith CM, Belz GT, van Lint AL, Wakim LM, Heath WR, et al. Epidermal viral immunity induced by $CD8\alpha^+$ dendritic cells but not by Langerhans cells. Science 2003;301:1925–8.
33. Merad M, Manz MG, Karsunky H, Wagers A, Peters W, Charo I, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol 2002;3:1135–41.
34. Bogunovic M, Ginhoux F, Wagers A, Loubeau M, Isola LM, Lubrano L, et al. Identification of a radio-resistant and cycling dermal dendritic cell population in mice and men. J Exp Med 2006;203:2627–38.
35. Oghiso Y, Yamada Y. Heterogeneity of the radiosensitivity and origins of tissue macrophage colony-forming cells. J Radiat Res 1992;33:334–41.
36. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity 2013;38:792–804.
37. Roberts S, Kosanke S, Dunn ST, Jankelow D, Duran CM, Cunningham MW. Pathogenic mechanisms in rheumatic carditis: focus on valvular endothelium. J Infect Dis 2001;183:507–11.
38. Guilherme L, Kalil J, Cunningham M. Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. Autoimmunity 2006;39:31–9.
39. Baudino L, Nimmerjahn F, Azeredo da Silveira S, Martinez-Soria E, Saito T, Carroll M, et al. Differential contribution of three activating IgG Fc receptors (FcγRI, FcγRIII, and FcγRIV) to IgG2a- and IgG2b-induced autoimmune hemolytic anemia in mice. J Immunol 2008;180:1948–53.
40. Giorgini A, Brown HJ, Lock HR, Nimmerjahn F, Ravetch JV, Verbeek JS, et al. FcγRIII and FcγRIV are indispensable for acute glomerular inflammation induced by switch variant monoclonal antibodies. J Immunol 2008;181:8745–52.
41. Atibalentja DF, Murphy KM, Unanue ER. Functional redundancy between thymic $CD8\alpha^+$ and $Sirp\alpha^+$ conventional dendritic cells in presentation of blood-derived lysozyme by MHC class II proteins. J Immunol 2011;186:1421–31.
42. Weisser SB, van Rooijen N, Sly LM. Depletion and reconstitution of macrophages in mice. J Vis Exp 2012;4105.
43. Ward NL, Loyd CM, Wolfram JA, Diaconu D, Michaels CM, McCormick TS. Depletion of antigen-presenting cells by clodronate liposomes reverses the psoriatic skin phenotype in KC-Tie2 mice. Br J Dermatol 2011;164:750–8.
44. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol 2011;11:762–74.
45. Hume DA. Macrophages as APC and the dendritic cell myth. J Immunol 2008;181:5829–35.
46. Durandy A, Kaveri SV, Kuijpers TW, Basta M, Miescher S, Ravetch JV, et al. Intravenous immunoglobulins–understanding properties and mechanisms. Clin Exp Immunol 2009;158 Suppl 1:2–13.
47. Braselmann S, Taylor V, Zhao H, Wang S, Sylvain C, Baluom M, et al. R406, an orally available spleen tyrosine kinase inhibitor blocks Fc receptor signaling and reduces immune complex-mediated inflammation. J Pharmacol Exp Ther 2006;319:998–1008.
48. Bahjat FR, Pine PR, Reitsma A, Cassafer G, Baluom M, Grillo S, et al. An orally bioavailable spleen tyrosine kinase inhibitor delays disease progression and prolongs survival in murine lupus. Arthritis Rheum 2008;58:1433–44.