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Interleukin-17 Cytokines Are Critical in Development of Fatal Lupus Glomerulonephritis

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SUMMARY

Systemic lupus erythematosus is a potentially fatal autoimmune disease. Although interleukin-17 (IL-17) has been linked to human lupus and mouse models of this disease, it has not been addressed whether this cytokine plays a critical role in fatal lupus pathology. Here we have demonstrated that increased production of IL-17 cytokines and their signaling via the adaptor protein CIKS (a.k.a. Traf3ip2, Act1) critically contributed to lethal pathology in an FcgammaR2b-deficient mouse model of lupus. Mice lacking IL-17 and especially those lacking CIKS showed greatly improved survival and were largely protected from development of glomerulonephritis. Importantly in this model, potential effects of IL-17 cytokines on antibody production could be distinguished from critical local contributions in kidneys, including recruitment of neutrophils and monocytes. These findings provide the proof of principle that signaling by IL-17 family cytokines mediated via CIKS presents promising therapeutic targets for the treatment of systemic lupus erythematosus, especially in cases with kidney involvement.

INTRODUCTION

Systemic lupus erythematosus (SLE) is one of the most common and devastating systemic autoimmune diseases. In health, various tolerance mechanisms prevent the generation of autoreactive B cells, but once these mechanisms are breached, autoantibodies may be produced, causing immune complex deposition and inflammation. Unless SLE patients receive effective therapies, chronic activation of the immune system and local inflammation may cause permanent organ damage, including glomerulonephritis, leading to renal failure and death.

To elucidate mechanisms underlying the pathogenesis of SLE, we investigated Fcgamma receptor II-b (*Fcgr2b*)-deficient mice (generated with 129 embryonic stem cells [ESCs], extensively backcrossed to C57BL/6), which develop a fatal lupus-like disease (Bolland and Ravetch, 2000; Daëron et al., 1995). *Fcgr2b* has been identified as a lupus susceptibility

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SUPPLEMENTAL INFORMATION

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gene in both humans and mice. This inhibitory receptor is thought to help maintain tolerance mechanisms that prevent formation of autoantibody-producing B cells and restrain inflammation in response to immune complex deposition (Baerenwaldt et al., 2011; Smith and Clatworthy, 2010). 129-derived gene variants immediately surrounding the deleted *Fcgr2b* locus in this mouse background are likely to also play a role in the breach in tolerance, consistent with the multigenic nature of lupus (Boross et al., 2011; Harley et al., 2008; Sato-Hayashizaki et al., 2011).

Interleukin-17 (IL-17, a.k.a. IL-17A) is the signature cytokine of T helper-17 cells, and these cells also produce the closely related IL-17F; both cytokines have been linked to the development of various autoimmune diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), and SLE. Patients with these diseases express increased amounts of IL-17A and IL-17F (Doreau et al., 2009; Matusevicius et al., 1999; Ziolkowska et al., 2000). Furthermore, IL-17A and IL-17F have been shown to be functionally relevant in the pathogenesis of collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) in mice; in addition, IL-17C is involved in psoriasis and may also contribute to other diseases, including EAE (Chang et al., 2011; Komiyama et al., 2006; Nakae et al., 2003; Ramirez-Carrozzi et al., 2011). IL-17A, IL-17F, and IL-17C are members of an extended family of IL-17 cytokines (A–F) that signal via heteromeric receptors composed of members of the IL-17 receptor family (RA–RE) (Gaffen, 2009). CIKS (a.k.a. Traf3ip2 or Act1) is an adaptor protein required for signaling by these cytokines (Chang et al., 2011; Gaffen, 2009). Consistent with a role for IL-17 cytokines in disease, the CIKS (Traf3ip2; Act1) adaptor is essential for development of CIA and EAE (Pisitkun et al., 2010; Qian et al., 2007).

Recombinant inbred BXD2 mice develop erosive arthritis and a lupus-like condition. Loss of *Il17ra*, a receptor chain required for signaling by IL-17 cytokines, abrogates spontaneous germinal center formation in this mouse model, whereas addition of IL-17 exacerbates this process (Hsu et al., 2008). IL-17 also synergizes with “B cell activating factor belonging to the TNF family” to promote the survival and/or proliferation of human B cells and their differentiation into antibody secreting cells in vitro (Doreau et al., 2009). These findings suggest possible roles for IL-17 in autoantibody production; however, it has not been addressed whether such contributions are actually relevant for the fatal outcome in the BXD2 mouse model. Other studies have provided evidence for increased amounts of IL-17 in kidneys of SLE patients and Mrl/*lpr* and BL6/*lpr* lupus-prone mice and for a functional role of IL-23 in the latter model (Crispín et al., 2008; Kyttaris et al., 2010). These findings suggest involvement of T helper 17 (Th17) cells and/or IL-17 cytokines in kidney pathology, but it has not been addressed whether IL-17 cytokines are in fact functionally relevant in development of fatal kidney disease in mouse models for lupus.

Here we demonstrate that CIKS (Traf3ip2) adaptor-mediated signaling from IL-17 cytokines, including IL-17A and likely also IL-17C, plays a major role in the development of fatal lupus pathology in *Fcgr2b*$^{-/-}$ mice. Loss of CIKS, which blocks signaling by all IL-17 cytokines, and to a lesser extent loss of IL-17A, significantly reduced mortality rates, specifically protecting mice from glomerulonephritis, by eliminating an important pathway for chemokine-mediated recruitment of inflammatory cells into kidneys, in particular neutrophils. Contributions of IL-17 cytokines to end-organ pathology were crucial for progression of the disease, and without these contributions autoantibodies alone were largely insufficient to cause severe kidney pathology. Consistent with this, signaling of these cytokines into B cells did not significantly contribute to the course of glomerulonephritis in *Fcgr2b*$^{-/-}$ mice. Importantly, we detected neutrophil extracellular traps (NETs) in kidneys of *Fcgr2b*$^{-/-}$ mice, but not in the absence of IL-17 cytokines signaling. We also demonstrated a requirement for CIKS-mediated signaling in a glomerular basement membrane (GBM)

antibody-induced model of glomerulonephritis, providing further evidence for a direct role of IL-17 cytokines "downstream" of autoantibodies. These findings identify IL-17 cytokines and the CIKS signaling adaptor as potential therapeutic targets in lupus nephritis.

RESULTS

Loss of the IL-17 Receptor Adaptor CIKS Improves Survival of *Fcgr2b*$^{-/-}$ Mice

We first examined IL-17 cytokine expression in the *Fcgr2b*$^{-/-}$ mice at or shortly after the onset of pathology at about 6 months of age. We detected an increased number of IL-17(A) and IL-17F producers among $CD3^+$ T cells, especially in lymph nodes, and to a lesser extent spleens (Figures 1A and 1B; see also Figure S1 available online). The fraction of $CD4^-CD8^-CD3^+$ (double-negative, DN-T) cells was specifically increased in lymph nodes, a population reportedly expanded in SLE patients and a lupus mouse model (Crispín et al., 2008; Kyttaris et al., 2010) (Figures 1C and 1D). We observed significant increases in IL-17A and/or IL-17F producing $CD4^+$ T cells (Th17 cells) in spleens and IL-17A and/or IL-17F producing DN-T cells in lymph nodes (Figures 1E and 1F; there was also a trend toward an increase in Th17 cells in lymph nodes). The IL-17-producing DN-T cell population in lymph nodes included $\gamma\delta^+$ and $\alpha\beta^+$ T cells, but not invariant natural killer T (iNKT) cells (Figure S1).

Next we asked whether IL-17 cytokines play a critical role in the fatal outcome in this lupus model. We crossed *Fcgr2b*$^{-/-}$ mice with *Traf3ip2*$^{-/-}$ C57BL/6 mice to generate doubly deficient mice (*Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$) and littermate controls deficient in *Fcgr2b* and heterozygous for *Traf3ip2* (*Traf3ip2* heterozygosity did not alter phenotypes of wild-type [WT] or *Fcgr2b*$^{-/-}$ mice). Mice were followed for up to 12 months of age, at which time just 7.7% (1 of 13) of *Fcgr2b*$^{-/-}$ were still alive, whereas 75% (9 of 12) of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice had survived (Figure 1G; an 89.7% improvement of survival rates). This significant difference in survival suggests that Traf3ip2 (CIKS) plays an important role in development of fatal lupus pathology in the *Fcgr2b*$^{-/-}$ mice.

CIKS Contributes to Germinal Center Formation in *Fcgr2b*$^{-/-}$ Mice

Unlike WT mice, *Fcgr2b*$^{-/-}$ mice readily formed spontaneous germinal centers and expanded their plasma cell numbers; however, in mice also lacking *Traf3ip2* these phenotypes were substantially, though not completely, reversed (Figures 2A–2E; Figure S2). We also assayed for preswitched (germline) and postswitched immunoglobulin transcripts with semi-quantitative RT-PCR to confirm these findings (Figure S2). We noted increased expression of MHC class II (H2-Ab1) on B cells in *Fcgr2b*$^{-/-}$ mice, and this expression was significantly reduced in mice also lacking *Traf3ip2* (Figures 2F and 2G). Together, these results suggest that IL-17 cytokines signaling via CIKS (Traf3ip2) contribute to spontaneous germinal center B cell formation, plasma cell development, and MHC class II expression on B cells in lupus-prone *Fcgr2b*$^{-/-}$ mice.

Loss of CIKS in B Cells Does Not Block Development of Glomerulonephritis

To determine whether IL-17 cytokines directly targeted B cells to promote formation of spontaneous germinal centers in *Fcgr2b*$^{-/-}$ mice and to cause disease, we generated mice in which *Traf3ip2* was conditionally deleted in B cells with Mb1-driven Cre recombinase (Figure S3; *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{fl/fl\text{-}Mb1}$). Although loss of *Traf3ip2* in B cells of *Fcgr2b*$^{-/-}$ mice clearly reduced splenomegaly and total number of B cells and GC B cells, it did not significantly reduce the percentage of GC B cells or plasma cells, although there was a trend toward lower numbers (Figures 3A and 3B; Figure S3). Expression of H2-Ab1 on B cells of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{fl/fl/Mb1}$ mice was reduced compared to *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{wt/fl}$ mice. Importantly, however, *Fcgr2b*$^{-/-}$ mice lacking *Traf3ip2* in B cells still developed

glomerulonephritis comparable to *Traf3ip2* sufficient *Fcgr2b*$^{-/-}$ mice (Figures 3D and 3E). Thus CIKS (Traf3ip2)-mediated signaling by IL-17 cytokines in B cells in *Fcgr2b*$^{-/-}$ mice promotes the overall increase in spleen size and total number of B cells but is not required for the increased percentage of GC B cells or for development of glomerulonephritis.

CIKS-Mediated Signaling Is Not Required for Autoantibody Production but Is Critical for End-Organ Pathology in *Fcgr2b*$^{-/-}$ Mice

Despite reduced numbers of spontaneous germinal centers and splenic plasma cells in *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ as compared to *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ littermates, the titers and staining patterns for anti-nuclear antibodies (ANA) did not differ (Figures 4A and 4B). Similarly, we did not observe any reduction in double-stranded DNA (dsDNA) IgG antibodies (regardless of isotype), nRNP antibodies, total serum IgG or IgG deposition in kidneys in the absence of *Traf3ip2 in Fcgr2b*$^{-/-}$ mice (Figures 4C and 4D; Figure S4). However, dsDNA antibody amounts do not always correlate with the clinical activities of lupus nephritis (Christensen et al., 2005; Mok, 2010). Indeed, mice deficient in *Traf3ip2* exhibited markedly reduced infiltration of inflammatory cells into kidneys (Figure 4E). Glomerular pathology was greatly ameliorated in *Traf3ip2* deficient *Fcgr2b*$^{-/-}$ mice as visualized in H&E and PAS stained sections (Figures 4F and 4G) and quantified as glomerular and interstitial scores (Figures 4H and 4I).

Our results suggest that although signaling of IL-17 cytokines via CIKS was able to promote formation of germinal centers in *Fcgr2b*$^{-/-}$ mice, this was not critical for autoantibody production or IgG deposition. Autoantibody production in this *Fcgr2b*$^{-/-}$ model is likely to be controlled by other factors. IFN-γ is reported to be critical for autoantibody production and IgG deposition in kidneys of lupus-prone (NZBxNZW)F1 mice as well as for development of lupus nephritis (Haas et al., 1998; Peng et al., 1997; Richards et al., 2001). We observed a significant expansion of IFN-γ producing cells, mainly T cells, in spleens of *Fcgr2b*$^{-/-}$ mice, irrespective of the presence of *Traf3ip2* (Figure 4J; Figure S4). Therefore IFN-γ may promote autoantibody production independent of IL-17 cytokines. These findings suggest that CIKS-mediated signaling by IL-17 cytokines contributes to the fatal outcome in this lupus model in a manner separate from autoantibody formation and IgG deposition.

We also observed increases in CD11b^{+}CD11c^{-} monocytic cells, CD11b^{+}CD11c^{+} myeloid DCs (mDCs), and CD11c^{+}B220^{+} plasmacytoid DCs (pDCs) in spleens from *Fcgr2b*$^{-/-}$ mice; notably, these increases were reversed in the absence of *Traf3ip2*. Furthermore the shift toward increased numbers of T effector-memory cells (CD4^{+}CD62L^{-}CD44^{+}) in this lupus model was partially reversed in the absence of CIKS (Figure S4). Therefore, CIKS-mediated signaling by IL-17 cytokines in *Fcgr2b*$^{-/-}$ mice promotes increases in numbers of monocytic cells, mDCs, and pDCs and contributes to activation and/or differentiation of T cells. These latter systemic changes could have had a role in initiating kidney pathology but could also have occurred as a consequence thereof.

Loss of IL-17A Does Not Reduce Formation of Spontaneous Germinal Centers but Does Protect against Fatal Glomerulonephritis in *Fcgr2b*$^{-/-}$ Mice

The CIKS adaptor is not only required for signaling by IL-17 but also other members of this cytokine family (Chang et al., 2011; Gaffen, 2009). We crossed *Fcgr2b*$^{-/-}$ mice with *Il17a*$^{-/-}$ mice to determine whether IL-17 and/or other members of this family might be critical for the pathogenesis in this lupus model. Although only 35.7% (5 of 14) of *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ mice were alive after 12 months, this number increased to 91.7% (11 of 12) of *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ littermates (Figure 5A; a 61.1% improvement of survival rates). This suggests that IL-17(A) plays an important, albeit not exclusive role in the CIKS-

dependent fatal outcome of *Fcgr2b*$^{-/-}$ mice. (*Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ mice were somewhat less susceptible to lupus pathology, most likely due to a subtly different BL6 background of *Il17a*$^{-/-}$ mice; there was no apparent difference between *Fcgr2b*$^{-/-}$.*Il17a*$^{+/+}$ and *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ mice). The protection afforded by loss of *Il17a* also failed to correlate with any reduction in ANA or dsDNA antibodies (Figures 5B and 5C). Unexpectedly, *Il17a* deficient mice failed to show any reduction in the spontaneous germinal centers that developed in *Fcgr2b*$^{-/-}$ mice (Figure 5D; Figure S5). Consistent with this, immunoglobulin isotype switching, numbers of plasma cells, and H2-Ab1 expression on B cells in the *Fcgr2b*$^{-/-}$ mice were not changed in the absence of *Il17*a (Figure S5). Because expression of IL-17B or IL-17C did not change in spleens of *Fcgr2b*$^{-/-}$ mice compared to WT mice (Figure S5), the increased amounts of IL-17F likely compensated for loss of IL-17A (Figures 1A and 1B; Figure S5), although this remains to be formally proven.

The fact that the survival of *Fcgr2b*$^{-/-}$ mice was significantly improved in the absence of IL-17, while spontaneous germinal center formation and autoantibody production continued unabated, strongly points to a critical and likely direct role for IL-17-mediated signaling in fatal end-organ pathologies. Consistent with this hypothesis, loss of *Il17a* significantly reduced inflammatory cell infiltration and glomerulonephritis in kidneys of *Fcgr2b*$^{-/-}$ mice (Figures 5E and 5F).

Loss of CIKS or IL-17A Prevents Recruitment of Inflammatory Cells into Kidneys of *Fcgr2b*$^{-/-}$ Mice

We investigated which, if any, of the potential IL-17-producing T cell subsets could be detected in perfused kidneys of *Fcgr2b*$^{-/-}$ mice. We noted significant increases in numbers of DN-T and CD4^{+} T cells and, to a lesser degree, CD8^{+} T cells in kidneys of *Fcgr2b*$^{-/-}$ mice (Figure 6A). Surprisingly, loss of *Traf3ip2* reversed the numbers of DN-T cells (Figure 6A). In contrast, the fraction of DN-T cells in lymph nodes did not change in the absence of *Traf3ip*2, suggesting that IL-17 cytokines might play a role in recruitment and/or maintenance of these cells in kidneys (Figure 6B). We also performed intracellular staining of cells isolated from kidneys and found that both DN-T cells and CD4^{+} T cells produced IL-17 (Figure S6). Next we tested for expression of IL-17 cytokines in kidneys of *Fcgr2b*$^{-/-}$ mice. We detected a significant increase in IL-17 as well as IL-17C, but not IL-17F or IL-17B messenger RNA (mRNA) expression in *Fcgr2b*$^{-/-}$ mice (Figure 6C). Preferentially increased expression of IL-17 is consistent with the fact that loss of this cytokine afforded significant protection (Figure 5A). Nevertheless, since loss of *Traf3ip2* improved survival rates of *Fcgr2b*$^{-/-}$ mice more than loss of *Il17a* (89.7% versus 61.1%), it is likely that IL-17C also contributed to kidney pathology (see Discussion).

We detected increased numbers of neutrophils (Ly6g^{+}Ly6c^{+}) and monocytic cells (Ly6c^{+}Ly6g^{-} CD11b^{+}F4/80^{lo}) in perfused kidneys of *Fcgr2b*$^{-/-}$ mice; by contrast, these increases were largely erased in the absence of *Traf3ip2* or *Il17a* (Figures 6D and 6E; Figure S6). However, although the absence of *Traf3ip2* in this lupus model largely prevented the increase in DN-T cells, this was not the case in mice lacking *Il17a* (Figure S6); therefore IL-17C could have had a role in recruitment and/or maintenance of these cells in kidneys. To determine the anatomical locations of infiltrating myeloid cells in kidneys of *Fcgr2b*$^{-/-}$ mice, and to confirm the absence of such cells in mice lacking *Traf3ip2* and *Il17a*, we stained tissue sections of kidneys for neutrophils (7/4^{+}Ly6g^{+}), monocytes and macrophages (7/4^{+}Ly6g^{-}) (Daley et al., 2008; Rosas et al., 2010), and for IgG deposition. We observed substantial foci of infiltrating neutrophils and monocytic cells in *Fcgr2b*$^{-/-}$ mice; infiltration of these cells extended into interstitial regions. Loss of either *Traf3ip2* or *Il17a* largely prevented infiltration of these cells, while IgG deposition remained (Figure 6F).

Next we examined whether CIKS-mediated signaling might contribute to increased expression of neutrophil- and monocyte-attracting chemokines in kidneys of *Fcgr2b*$^{-/-}$ mice. We observed CIKS (Traf3ip2)-dependent increases in *Cxcl1*, *Cxcl5*, *Ccl2*, and *TNF-α* and to a lesser extent *Ccl20* mRNA expression (Figure 6G); TNF-α is known to strongly synergize with IL-17 (Hartupee et al., 2007). We extended these analyses to kidney biopsies from lupus nephritis patients and found footprints of IL-17 cytokines and TNF-α in these samples, suggesting that our findings may be relevant for development of lupus nephritis in patients (Figure S6).

Increased ability to form pathogenic neutrophil extracellular traps (NETs) and impairment in their degradation have been associated with lupus nephritis in humans (Hakkim et al., 2010). To test whether neutrophils recruited into kidneys of *Fcgr2b*$^{-/-}$ mice might develop NETs in tissue, we performed immunofluorescence staining and detected NETs in kidneys of *Fcgr2b*$^{-/-}$ mice, but not in mice also deficient in *Traf3ip2* (Figure 6H).

These results indicate that CIKS-mediated signaling by IL-17 cytokines in kidneys of *Fcgr2b*$^{-/-}$ mice is involved in recruitment of DN-T cells, monocytic cells as well as neutrophils, with the latter cells able to form pathogenic NETs. These findings may explain the role of these cytokines in the development of fatal end-organ pathology.

CIKS Deficiency Protects Mice from the Development of Glomerular Basement Membrane Antibody-Induced Glomerulonephritis

The data suggest a critical role for CIKS-mediated signaling by IL-17 cytokines in development of glomerulonephritis, apparently acting downstream of autoantibodies. To directly test whether IL-17 cytokines can contribute to the pathogenic effects of autoantibodies, we challenged CIKS sufficient and deficient mice with glomerular basement membrane antibodies in an established model of accelerated nephrotoxic nephritis that is associated with glomerular damage (Vielhauer et al., 2005). Although IL-17 has been proposed to have a pathogenic role in similar models, it has also been suggested to have a protective role (Odobasic et al., 2011; Paust et al., 2012). We found an increase in expression of *Il17a*, but not *Il17c* or *Il17f* in kidneys of WT mice after treatment with CFA and GBM antibodies (Figure S7). The numbers of neutrophils, monocytes, and macrophages were also increased; by contrast, *Traf3ip2*-deficient mice showed significantly lower counts of neutrophils and monocytes and a trend toward lower counts of macrophages (Figures 7A–7D). These findings correlated well with decreased renal pathology scores (Figures 7E–7G) and inflammatory cell infiltrations (Figures 7H and 7I) in *Traf3ip2*-deficient mice. Of note, we only detected an increase in CD4^{+} but not DN-T cells in kidneys in this model (Figure S7). These data demonstrate that CIKS-mediated signaling is essential for disease in a model of anti-GBM mediated glomerulonephritis. This finding is consistent with a major role for IL-17 cytokines in renal injury at a step downstream of autoantibody production.

DISCUSSION

Increased amounts of IL-17 in serum of SLE patients have been correlated with disease activity; in addition, increased production of IL-17 is prominent in two mouse models of lupus (Hsu et al., 2008; Kozyrev et al., 2008; Nalbandian et al., 2009). As shown here, elevated expression of IL-17 was also observed in lupus-prone *Fcgr2b*$^{-/-}$ mice, in particular in lymph nodes and kidneys. Beyond these observations, however, the work presented establishes that IL-17 cytokines and their signaling via the adaptor Traf3ip2 (CIKS, Act1) play critical roles in the ultimately fatal outcome in a lupus disease model: loss of *Il17a* or *Traf3ip2* profoundly improved survival in *Fcgr2b*$^{-/-}$ mice, correlating with greatly ameliorated kidney pathology.

Lack of *Traf3ip2* in *Fcgr2b*$^{-/-}$ mice afforded greater protection than lack of *Il17a* (89.7% versus 61.1% improvement in survival rates, respectively). Although this could be due to slight genetic background differences, it is more likely that in addition to major contributions from IL-17, IL-17C too may have played a role, given that it also signals via CIKS (Chang et al., 2011; Gaffen, 2009) and given that its expression was significantly increased in kidneys of *Fcgr2b*$^{-/-}$ mice as well. Alternatively, it remains at least theoretically possible that CIKS could have made contributions independent of IL-17 cytokines (Valente et al., 2012).

Although absence of CIKS led to reduced spontaneous germinal center formation in this model, surprisingly, lack of this adaptor (or of IL-17) did not lead to a noticeable reduction in ANA, dsDNA antibodies, or immunoglobulin deposition in kidneys. Aside from germinal centers, autoantibodies could also have arisen from extrafollicular B cells (Sweet et al., 2010). Importantly, loss of *Il17a* or *Traf3ip2* did prevent the influx of inflammatory myeloid-derived cells into kidneys of *Fcgr2b*$^{-/-}$ mice. Therefore, the present model allowed us to separate potential inputs of IL-17 cytokines to autoantibody production from other, more direct contributions of these cytokines to kidney pathology. In further support of a critical local role of IL-17 cytokines in kidney disease, we demonstrated local production of IL-17 by infiltrating Th17 and DN-T cells, and of IL-17C, most likely by epithelial cells; in addition, we detected increased expression of TNF-α, known to synergize with IL-17 cytokines (Hartupee et al., 2007). The presence of these cytokines may represent a primary means for attracting inflammatory cells into kidneys via induced expression of chemoattractant genes such as *Cxcl1*, *Cxcl5*, *Ccl2*, and *Ccl20* (Ramirez-Carrozzi et al., 2011; Ruddy et al., 2004; Shahrara et al., 2010).

Only loss of *Traf3ip2*, but not *Il17a*, notably reduced spontaneous germinal center formation. Given that we observed elevated amounts of IL-17F, but not IL-17B or IL-17C in spleens of *Fcgr2b*$^{-/-}$ mice, it is reasonable to suggest that IL-17F could have compensated for lack of IL-17 to promote spontaneous germinal center formation. Loss of *Traf3ip2* in B cells largely reversed increases in spleen size, total GC B cells, and MHC Class II expression on B cells, but not the propensity of B cells to form germinal centers in spleens of *Fcgr2b*$^{-/-}$ mice, suggesting that IL-17 cytokines may also affect B cells indirectly.

Loss of IL-17RA in BXD2 mice caused definite reductions in autoantibodies and immunoglobulin deposition; in contrast, the absence of CIKS in *Fcgr2b*$^{-/-}$ mice did not, although spontaneous germinal center formation was reduced in both contexts. It is not clear why production of autoantibodies was more dependent on IL-17 cytokines in the BXD2 model than in the *Fcgr2b*$^{-/-}$ model. It has been reported that IFN-γ production is critical for autoantibody production in lupus-prone (NZBxNZW) F1 mice (Haas et al., 1998), and expression of this cytokine was elevated in *Fcgr2b*$^{-/-}$ mice, independent of IL-17 cytokine signaling; thus, IFN-γ may have been responsible for autoantibody production.

The local production of IL-17 in kidneys of *Fcgr2b*$^{-/-}$ mice from both CD4^{+} T cells and DN-T cells was likely instrumental in recruitment of neutrophils and monocytes, and thus the ensuing inflammation. The mechanisms leading to local IL-17 production remain to be determined. Interestingly, kidney infiltration of DN-T cells was dependent on CIKS-mediated signaling, but not IL-17, suggesting the possibility that IL-17C may have targeted these cells to exacerbate an already initiated, IL-17-dependent inflammatory process in *Fcgr2b*$^{-/-}$ mice. IL-17C has also been reported to enhance IL-17 production from infiltrating CD4^{+} T cells (Chang et al., 2011). Our data suggest that local production of IL-17 cytokines is critical for kidney pathology, consistent also with the observation that CIKS was required for glomerulonephritis and recruitment of neutrophils and monocytes into kidneys in the GBM antibody-induced model of glomerulonephritis. Of note, whereas CD4^{+} T cells

numbers and *Il17a* expression were elevated in kidneys of *Fcgr2b*$^{-/-}$ mice and GBM antibody-treated mice, DN-T cells and *Il17c* were only elevated in *Fcgr2b*$^{-/-}$ mice.

Prior reports have suggested that the presence of IL-17-producing Th17 and DN-T cells in kidneys of SLE patients and in the MRL/*lpr* and B6/*lpr* mouse models (Crispín et al., 2008; Zhang et al., 2009). Consistent with this, IL-23 receptor deficiency in B6/*lpr* mice mitigates glomerulonephritis in that model (Kyttaris et al., 2010). However, IL-23 is known to stabilize pathogenic Th17 cells and thus boost expression of not only IL-17 but also several other cytokines, including IFN-γ (Oppmann et al., 2000); furthermore, it can restrain regulatory T cell activity (Izcue et al., 2008). Indeed, loss of the IL-23 receptor in B6/*lpr* mice reduces autoantibody production and immune complex deposition in kidneys (Kyttaris et al., 2010). The present findings directly demonstrate the importance of specifically IL-17 cytokines in the development of fatal kidney pathology, likely including IL-17C, the expression of which is not known to be controlled by IL-23.

Our studies identify IL-17 cytokines and the CIKS-mediated signaling pathway as potential therapeutic targets in SLE, especially in lupus nephritis, and also in anti-GBM disease. Biologic therapies directed at B cells in mice and patients have met with limited success, especially in more severe cases of lupus nephritis (Navarra et al., 2011; Sanz and Lee, 2010); thus, there is great need for new treatment options. The present findings validate IL-17 cytokines and CIKS as promising targets. Because these cytokines have also been implicated in other local inflammatory conditions, including RA, MS, and psoriasis, therapies targeting them or their signaling pathways may have wide-ranging benefits.

EXPERIMENTAL PROCEDURES

Mice

Traf3ip2$^{-/-}$, *Fcgr2b*$^{-/-}$, *Il17a*$^{-/-}$, and Mb1-cre mice have been described (Claudio et al., 2009; Hobeika et al., 2006; Nakae et al., 2002; Takai et al., 1996). Mice were intercrossed to generate animals with compound deficiency of *Fcgr2b* and *Traf3ip2*, *Fcgr2b* and *Il17a*, and control littermates. To generate *Traf3ip2* floxed mice, exon 2 was flanked by loxP sites (Ozgenes, Australia) (Figure S3). WT/Flox mice were crossed with Mb1-cre mice to generate mice with B cells specific *Traf3ip2* deletion. The experimental mice were used at the age of 6–8 months (*Fcgr2b*$^{-/-}$.*Traf3ip2* strain) and 10–12 months (*Fcgr2b*$^{-/-}$.*Il17a* strain). Mice were bred and housed in a facility at the National Institute of Allergy and Infectious Diseases (NIAID), and all experiments were performed with the approval of the NIAID Animal Care and Use Committee and in accordance with all relevant institutional guidelines.

Glomerular Basement Membrane Antibody Induced Glomerulonephritis

The protocol for GBM Ab-induced glomerulonephritis has been described (Rosenkranz et al., 1999; Vielhauer et al., 2005). Briefly, mixed rabbit IgG at a final concentration of 0.1 mg/ml in CFA (2.5 mg/ml) was injected subcutaneously on day −3, and then heat inactivated anti-GBM serum was intravenously injected (100 μl) via tail vein on day 0. The 8- to 10-week-old mice were followed up and sacrificed to collect tissues for analysis on day 14.

Cellular Analysis

Splenocytes were isolated as previously described (Pisitkun et al., 2010) and stained with the following antibodies: B220(RA3-6B2), GL7(Ly-77), FAS(Jo2), CD138(281-2), IgM(II/41), IAb(AF6-120.1), CD4(L3T4), CD44(IM7), CD11b(M1/70), Ly6c(AL-21), CD3e(145-2C11), CD11c(HL3), and CD62L(Ly-22) (BD Biosciences); CD19(eBio1D3),

ICOS(7E.17G9), F4/80(BM8), CD8a (53-6.7), and IL17A(eBio17B7) (eBioscience); Ly6g (IA8) (Biolegend), CD1dtetramer(PBS57) (NIH tetramer facility), and Aqua (Invitrogen). Cells were stimulated with PMA (5 ng/ml), Ionomycin (500 ng/ml), and Golgi stop (BD) for 4 hr before intracellular staining. Data were collected with a FACSCanto instrument (BD Biosciences).

In Vivo Immunoglobulin Isotype Switching

B cells were isolated from splenocytes using anti-CD19 beads (Miltenyi). RNA isolation from B cells and complimentary DNA (cDNA) synthesis were performed as described (Pisitkun et al., 2010). RT-PCR for immunoglobulin transcription was performed by using primers and condition as described (Muramatsu et al., 2000).

Autoantibody Analysis

Anti-nuclear antibodies (ANA) were assayed with Hep-2 cells (BION). Slides were mounted with Vectashield (Vector Labs) and visualized using a fluorescence microscope. Mouse dsDNA IgG was detected by ELISA using the dsDNA IgG kit (5120) (Alpha Diagnostic International).

Histological Analysis

Kidneys were fixed in 4% paraformaldehyde. Tissue sections were stained with H&E or Periodic acid–Schiff (PAS) and visualized by Olympus BX50. Histological scores were evaluated as described (Chan et al., 1997). We analyzed kidney pathology from these mice compared to their control littermates at between 6–8 months (*Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$) and 10–12 months (*Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$) of age. In GBM induced glomerunlonephritis, the histology scores were determined as described (Odobasic et al., 2011).

Immunofluorescence

Frozen sections (5 μm thickness) were fixed in acetone, and blocked with 1% BSA in PBS. Diluted antibodies were incubated for 1 hr. Endogenous biotin was blocked using Streptavidin-Biotin blocking kit (Vector Labs). The following antibodies were used: IgD(11-26c.2a) and CD35(8C12) (BD Biosciences), IgG(A11029, Invitrogen), 7/4(Cedarlane), Ly6g(IA8, Biolegend), MPO(2D4, Abcam), and LL37(LS-B2687 CAMP, LS Biosciences). Slides were mounted with Vectashield with or without DAPI (Vector Labs) and visualized using a Leica AF6000LX fluorescence microscope.

Isolation of Single Cells from the Kidney for FACS Analysis

Isolation of single cells from the kidneys was modified from a described procedure (Gunaratne et al., 2010). In brief, perfusion of the kidneys was performed with 20 ml of prewarmed HBSS while the mice were anesthesized with Avertin (0.4–0.75 mg/g of body weight). The perfused kidneys were digested with collagenase B (0.230 U/ml) (Roche Diagnostics) in 10% FCS in IMEM for 20 min (37°C). Single cells of kidneys were isolated the same way as splenocytes (see above) and cell suspensions were subjected to density separation to eliminate the epithelial or tubular cells of the kidneys using Lympholyte-M (Cedarlane).

Quantitative Real-Time PCR Analysis

RNA was purified using TRIzol (Invitrogen), cDNA synthesis, and quantitative real-time PCR was performed as described (Pisitkun et al., 2010). Control RNA of human kidneys was purchased from Ambion, Agilent, and Origene (CR560857, CR560624). The mouse or human primers (Taqman) for *actin*, *IL-17*, *IL-17F*, *IL-17C*, *IL-17B*, *TNF-α*, *CXCL1*, *CXCL5*, *CCL2*, and *CCL20* were obtained from Applied Biosystems.

Statistical Analysis

Gehan-Breslow-Wilcoxon test was performed with GraphPad Prism 5 to determine statistical significance of survival curves. Student's t test (one-tailed) was used for other comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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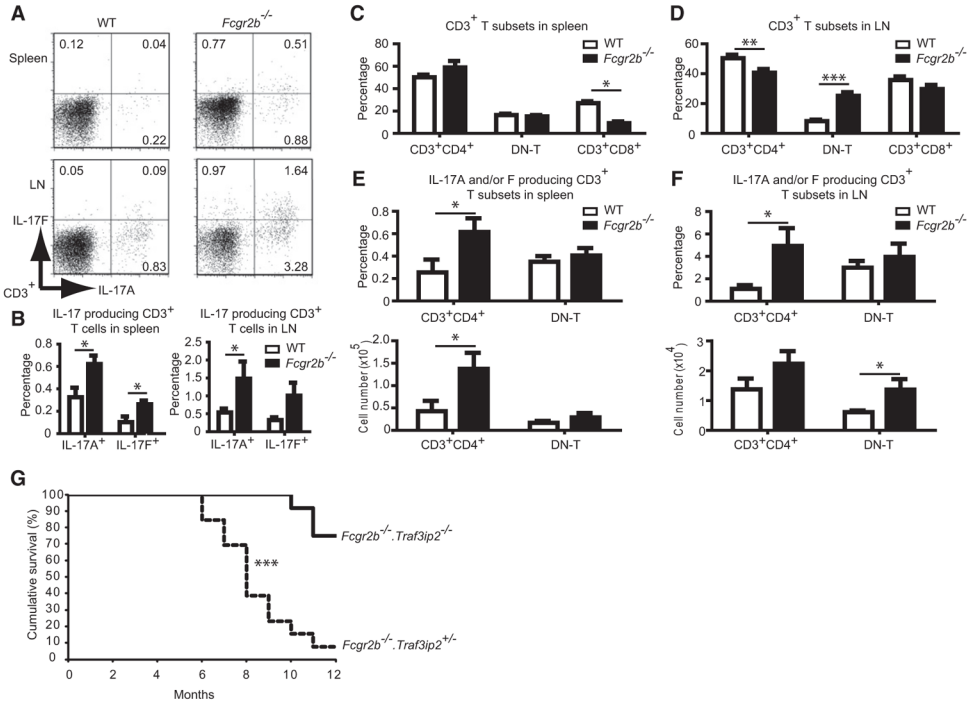

Figure 1. Loss of the IL-17 Signaling Adaptor CIKS Improves Survival of *Fcgr2b*$^{-/-}$ Mice

(A) Cells from spleen and lymph node (LN) of WT and *Fcgr2b*$^{-/-}$ mice (6–8 months old) were gated on CD3$^+$ and stained for intracellular expression of IL-17A and IL-17F. A profile representative of five mice per group is shown.

(B) Percentage of IL-17A and F producing T cells in spleen and LN (n = 5–7).

(C and D) Percentage of CD3$^+$ T subsets in spleen (n = 5–9) and LN (n = 10–16). DN-T cells are CD3$^+$CD4$^-$CD8$^-$.

(E and F) Percentage and numbers of IL-17 producing CD3$^+$ T subsets in spleen (n = 4–7) and LN (n = 7–10). (*p < 0.05, **p < 0.01, ***p < 0.001; mean ± SEM).

(G) Cumulative survival of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ mice (n = 13) and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice (n = 12); (***p < 0.001). Percent survival rate change = ([% survival of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ − % survival of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$] /% survival of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$) × 100 = ([75 − 7.7]/75) × 100 = 89.7%.

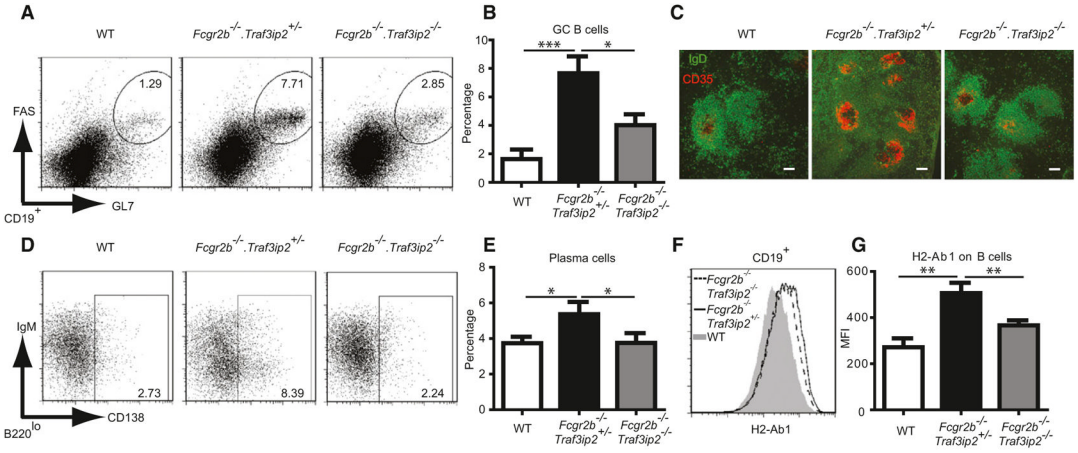

Figure 2. CIKS Contributes to Germinal Center Formation in *Fcgr2b*$^{-/-}$ Mice

(A and B and D and E) Flow cytometric analysis of spleen cells of WT, *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$, and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice. Representative staining profiles and percentages of (A and B) germinal center B cells (CD19^{+}GL7^{hi}FAS^{hi}) and (D and E) plasma cells (B220^{lo}CD138^{+}) are shown.

(C) Immunofluorescence staining of spleen sections for IgD (green) and CD35 (red) expression. Scale bar = 100 μm. Data are representative of five mice per group.

(F and G) Representative histogram (F) and mean fluorescence intensity (MFI) (G) for H2-Ab1 on CD19^{+} cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; mean ± SEM; n = 7–9).

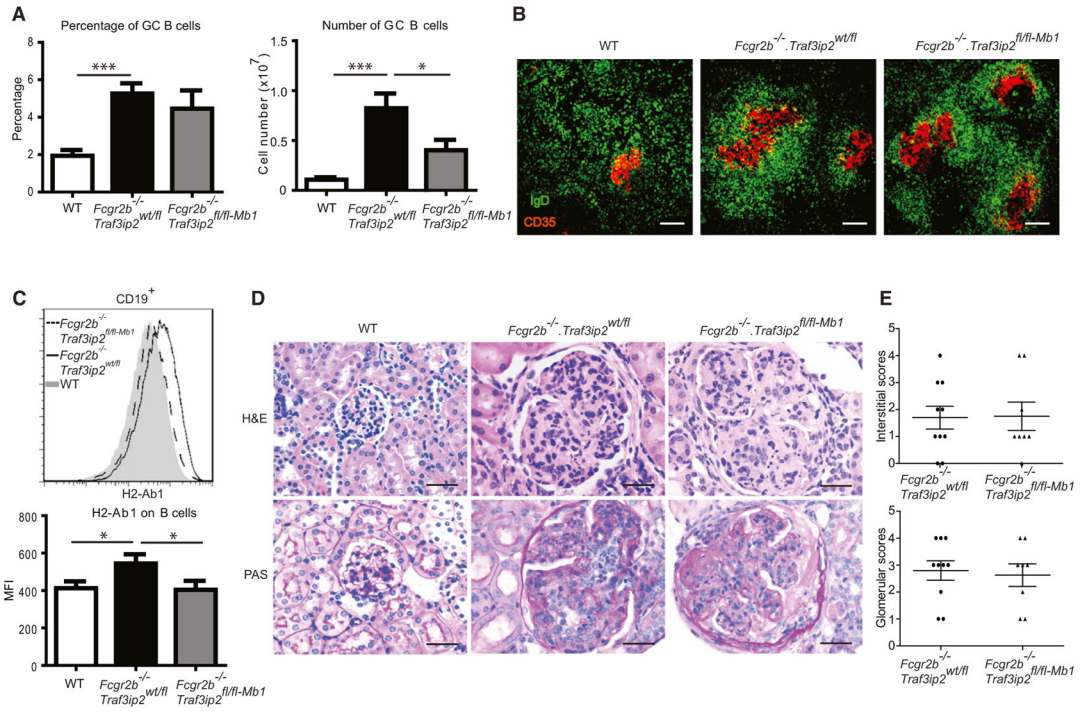

Figure 3. Loss of IL-17 Cytokine Signaling in B Cells Does Not Block Development of Glomerulonephritis

(A) Flow cytometric analysis of splenocytes of WT, *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{wt/fl}$, and *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{fl/fl\text{-}Mb1}$ mice, showing percentage and total numbers of CD19^{+}GL7^{hi}FAS^{hi} (GC B cells). (*$p < 0.05$, ***$p < 0.001$; mean ± SEM; n = 9–11).

(B) Immunofluorescence staining of spleen sections for IgD (green) and CD35 (red) expression. Scale bar = 100 μm. Data representative of five mice per group.

(C) Representative histogram and mean fluorescence intensity (MFI) for H2-Ab1 on CD19^{+} cells (*$p < 0.05$; mean ± SEM; n = 9–11).

(D) Upper panels show H&E staining (40×) showing increased glomerular hypercellularity in kidney sections of *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{wt/fl}$ and *Fcgr2b*$^{-/-}$*. Traf3ip2*$^{fl/fl\text{-}Mb1}$ mice (6–8 months old). Lower panels show PAS staining (40X) showing increased numbers of intracapillary leukocytes and double contours of the glomerular basement membrane in kidney sections of *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{wt/fl}$ and *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{fl/fl\text{-}Mb1}$ mice. Data are representative of 8–10 mice per group. Scale bar = 50 μm.

(E) Glomerular scores and interstitial scores of *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{wt/fl}$ and *Fcgr2b*$^{-/-}$*.Traf3ip2*$^{fl/fl\text{-}Mb1}$ mice (mean ± SEM; n = 8–10).

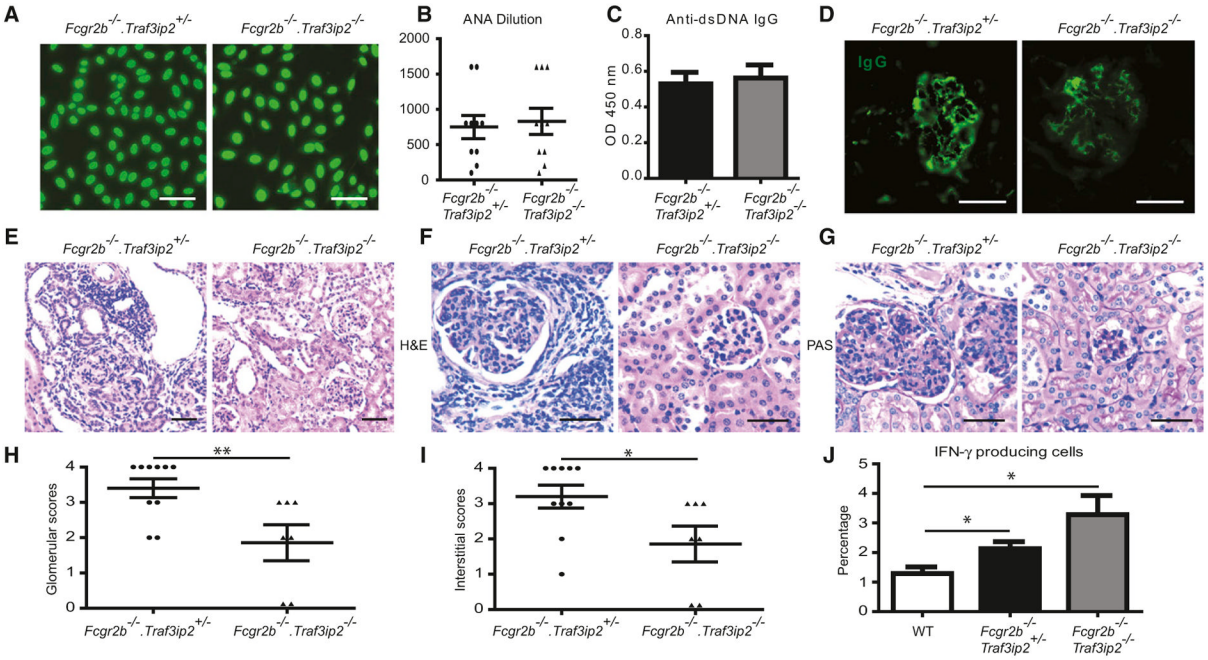

Figure 4. CIKS-Mediated Signaling Is Not Required for Autoantibody Production but Is Critical for End-Organ Pathology in *Fcgr2b*$^{-/-}$ Mice

(A) Anti-nuclear antibody (ANA) staining of Hep-2 cell coated slides incubated with diluted serum (1:100) from *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice. Data are representative of ten mice per group. Scale bar = 50 μm.

(B) ANA titers; shown are final dilution factors of serum still ANA positive (mean ± SEM; n = 10).

(C) ELISA assays for dsDNA IgG (mean ± SEM; n = 7–10).

(D) Immunofluorescence staining for IgG deposition in kidney sections from *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice. Data are representative of 7 or 8 mice per group. Scale bar = 50 μm.

(E) H&E staining of kidney sections (20X) showing substantial leukocyte infiltration in the interstitium of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ but not *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice. Scale bar = 100 μm.

(F) H&E staining (40X) showing glomerular hypercellularity with intracapillary proliferation, and periglomerular leukocyte infiltration in kidney sections of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$, but not *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice (6–8 months old).

(G) PAS staining (40X) showing increased numbers of intracapillary leukocytes and double contours of the glomerular basement membrane in *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ but not *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice. Data are representative of 7–10 mice per group. Scale bar = 50 μm.

(H and I) Glomerular scores and interstitial scores of *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice (*p < 0.05, **p < 0.01; mean ± SEM; n = 7–10).

(J) Percentage of IFN-γ producing cells from splenocytes evaluated by flow cytometry (*p < 0.05; mean ± SEM; n = 5–7).

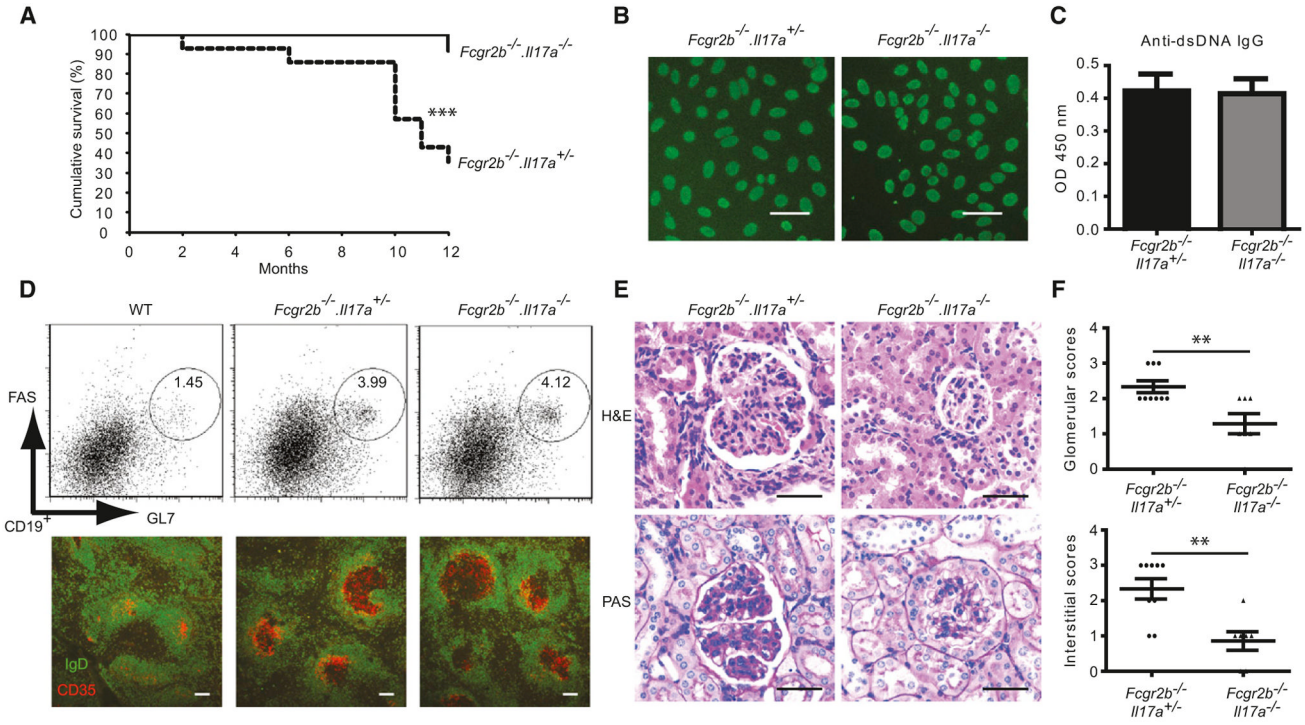

Figure 5. Loss of IL-17A Does Not Reduce Formation of Spontaneous Germinal Centers but Does Protect against Fatal Glomerulonephritis in *Fcgr2b*$^{-/-}$ Mice

(A) Cumulative survival of *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ mice (n = 14) and *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ mice (n = 12); (***p < 0.001). Percent survival rate change = ([% survival of *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ − % survival of *Fcgr2b*$^{-/-}$.*Il17*$^{+/-}$] /% survival of *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$) × 100 = ([91.7 − 35.7]/91.7) × 100 = 61.1%.

(B) ANA staining of Hep-2 cell coated slides incubated with diluted serum (1:100) from *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$, and *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ mice. Data are representative of 10 mice per group. Scale bar = 50 μm.

(C) ELISA assays for dsDNA IgG (mean ± SEM; n = 11).

(D) Representative flow cytometric staining profile for germinal center B cells ($CD19^{+}GL7^{hi}FAS^{hi}$) from mice as shown; data representative of ten mice per group. Immunofluorescence staining of spleen sections for IgD (green) and CD35 (red) expression. Scale bar = 100 μm. Data are representative of five mice per group.

(E) Upper panels show H&E staining (40X) showing glomerular hypercellularity with intracapillary proliferation in kidney sections of *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ but not *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ mice (10–12 months old). Lower panels show PAS staining (40X) showing increased numbers of intracapillary leukocytes and double contours of the glomerular basement membrane in kidney sections of *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ but not *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ mice. Data are representative of 7–9 mice per group. Scale bar = 50 μm.

(F) Glomerular and interstitial scores of *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ mice (**p < 0.01; mean ± SEM; n = 7–9).

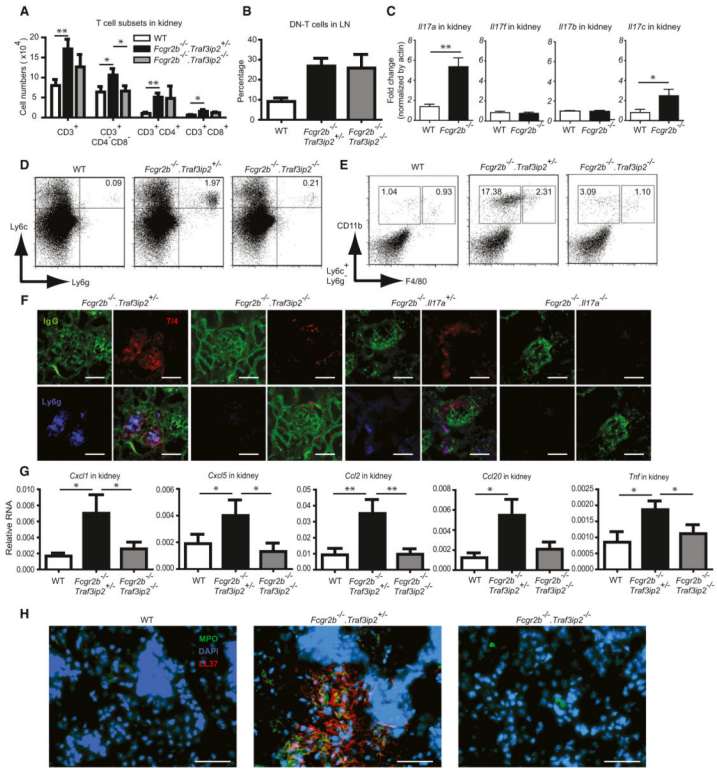

Figure 6. Loss of CIKS or IL-17A Prevents Recruitment of Inflammatory Cells into Kidneys of *Fcgr2b*$^{-/-}$ Mice

(A) Flow cytometric analysis of cells isolated from kidneys of WT, *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice, showing numbers of CD3^{+}, CD3^{+} CD4^{-}CD8^{-} (DN-T), CD3^{+}CD4^{+}, and CD3^{+}CD8^{+} cells (*p < 0.05, **p < 0.01; mean ± SEM; n = 13).

(B) Percentage of DN-T (CD3^{+}CD4^{-}CD8^{-}) in LNs of mice as shown (mean ± SEM; n = 5–8).

(C) Fold change in relative mRNA expression of *Il17a*, *Il17f*, *Il17b*, and *Il17c* in kidneys of WT and *Fcgr2b*$^{-/-}$ mice (*p < 0.05, **p < 0.01; mean ± SEM; n = 4–5).

(D and E) Representative staining profiles of cells isolated from kidneys of WT, *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$, and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice showing (D) neutrophils (Ly6g^{+}Ly6c^{+}) and (E) inflammatory monocytes (CD11b^{+}F4/80^{lo}) and macrophages (CD11b^{+}F4/80^{hi}) after gating on Ly6c^{+}Ly6g^{-} cells. Data are representative of 13 mice per group.

(F) Immunofluorescence of kidney sections from *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice (6–8 months old) or *Fcgr2b*$^{-/-}$.*Il17a*$^{+/-}$ and *Fcgr2b*$^{-/-}$.*Il17a*$^{-/-}$ mice (10–12 months old) stained with anti-IgG (green), anti-7/4 (red), and anti-Ly6g (blue) to identify immune complexes, inflammatory monocytes and macrophages (7/4^{+}Ly6g^{-}), and neutrophils (7/4^{+}Ly6g^{+}).

(G) Relative mRNA expression (normalized by actin) of *Cxcl1*, *Cxcl5*, *Ccl2*, *Ccl20*, and *TNF-α* in kidney tissues of WT, *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{+/-}$, and *Fcgr2b*$^{-/-}$.*Traf3ip2*$^{-/-}$ mice (*p < 0.05, **p < 0.01; mean ± SEM; n = 6–8).

(H) Immunofluorescence of kidney sections (genotypes as indicated), stained with anti-MPO (green), anti-LL37 (red), and DAPI (blue). Scale bar = 50 μm. Data are representative of 4–6 mice per group.

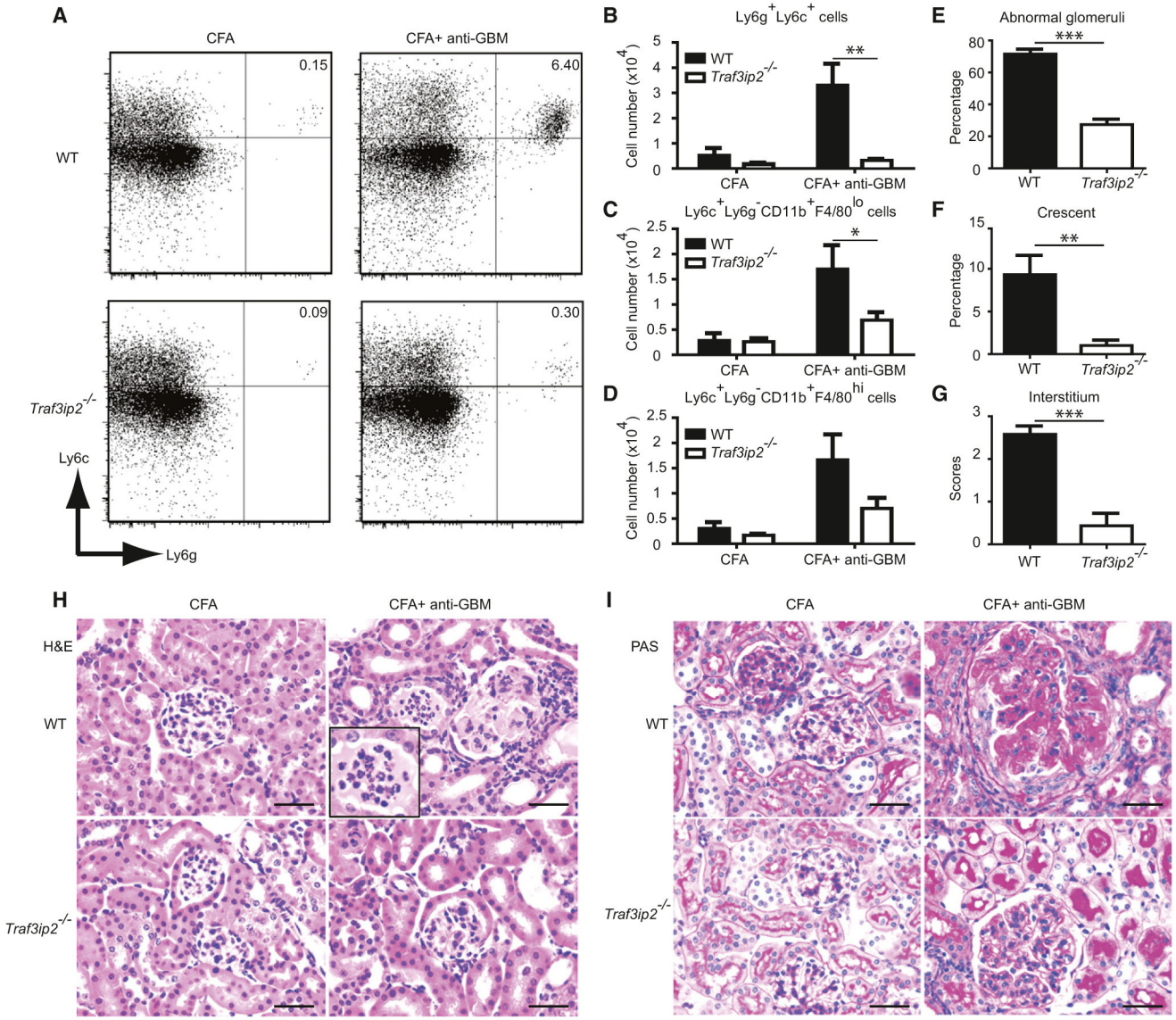

Figure 7. CIKS Deficiency Protects Mice from the Development of Antibody-Induced Glomerulonephritis

(A) Representative staining profiles of cells isolated from kidneys of WT and *Traf3ip2*$^{-/-}$ mice showing neutrophils (Ly6g^{+}Ly6c^{+}). Data are representative of ten mice per group.

(B–D) Flow cytometric analysis of infiltrating cells in kidneys of WT and *Traf3ip2*$^{-/-}$ mice showing (B) neutrophils (Ly6g^{+}Ly6c^{+}), (C) inflammatory monocytes (Ly6c^{+}Ly6g^{-} CD11b^{+}F4/80^{lo}) and (D) macrophages (Ly6c^{+}Ly6g^{-} CD11b^{+}F4/80^{hi}). (*p < 0.05, **p < 0.01; mean ± SEM; n = 10).

(E–G) Histology scores showing percentage of (E) abnormal glomeruli, (F) crescent, and (G) interstitial scores of WT and *Tra3ip2*$^{-/-}$ mice (**p < 0.01, ***p < 0.001; mean ± SEM; n = 10).

(H and I) H&E staining (H) showing inflammatory cell infiltration in kidney sections of WT but not *Traf3ip2*$^{-/-}$ mice. PAS staining (I) showing glomerular hypercellularity, glomerular deposition of PAS stained material, and crescentric glomerunlonephritis of WT but not *Traf3ip2*$^{-/-}$ mice. Data are representative of ten mice per group. Scale bar = 50 μm.