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Renal pathology in systemic lupus erythematosus involves both autoantibody deposition and a cellular inflammatory response, both of which are mediated by effector CD4 T cells. MRL<sup>lpr</sup> mice spontaneously develop massive perivascular infiltrates, but the pathways that regulate the development, trafficking, and effector functions of kidney-infiltrating T cells are poorly defined. To address these questions, we first surveyed inflammatory chemokine protein levels in nephritic kidneys from lupus-prone MRL<sup>lpr</sup> mice. After identifying highly elevated levels of the CXCR3 ligand CXCL9, we found that kidney-infiltrating effectors are enriched for expression of CXCR3, as well as P-selectin ligand and ICOS. Using genetic ablation, we demonstrate that ICOS plays an essential role in the establishment of renal perivascular infiltrates, although a small number of infiltrating cells remain around the blood vessels. Interestingly, though IgG autoantibody production is substantially reduced in Icos<sup>−/−</sup> MRL<sup>lpr</sup> mice, the progression of immune complex glomerulonephritis is only modestly diminished and the production of inflammatory chemokines, such as CXCL9, remains high in the kidney. We find that Icos<sup>−/−</sup> effector cell numbers are only slightly reduced and these have normal expression of CXCR3 and P-selectin ligand with intact migration to CXCL9. However, they have impaired production of inflammatory cytokines and fail to show evidence of efficient proliferation in the kidney. Thus, while dispensable for acquisition of renal trafficking receptor expression, ICOS is strictly required for local inflammatory functions of autoreactive CD4 T cells in murine lupus.


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ystemic lupus erythematosus is a common autoimmune syndrome in both humans and inbred and mutant mouse strains, typified by high-titer anti-nuclear Ab (ANA)<sup>5</sup> production and inflammation of multiple organ systems (1). ANA cause tissue damage by deposition and fixation of complement in the microvasculature, the most pathologically important site being the kidney glomerulus (2). CD4 T cells are essential for renal injury in systemic lupus erythematosus (3, 4), not only via their role in autoreactive B cell help and subsequent generation of immune complexes but also through Ab-independent direct effects (5). In the MRL/MpJ-Fas<sup>−/−</sup> (MRL<sup>lpr</sup>) murine lupus model, mononuclear renal infiltrates containing CD4 T cells occur in periglomerular and tubulointerstitial spaces, with the most massive infiltrates occurring around large blood vessels (6). Similar infiltrates also arise in other lupus models, including the (NZB × NZW)<sub>F</sub><sub>1</sub> (7) and NZM2388 strains (8). Cellular inflammation in the kidney, although not necessarily in other organs, is mediated by Th1 effector cells and is rescued by both IFN-γ and T-bet deficiency (9–14). Th1 effector cells also appear to play a critical role in renal inflammation in human lupus (15). CD4 T cells in nephritic kidneys from lupus patients bear an activated phenotype (16) and undergo local expansion (17). The presence of these inflammatory infiltrates indicates a poor prognosis (18).

T cell homing to inflamed tissues involves the inducible expression of selectin ligands and chemokine receptors, which are sequentially involved in rolling, integrin activation and extravasation across the endothelium (19). Although the molecules that control T cell trafficking in lupus nephritis are not completely defined, some pathways have been implicated. For instance, CCL2 deletion reduces cellular infiltration around glomeruli and tubules, but does not prevent the more prominent perivascular lesions (20, 21). Recent genomic analysis of MRL<sup>lpr</sup> spleens has also shown elevations of the inflammatory chemokines CXCL9 and CXCL10 (22). Expression of CXCR3, the receptor for these chemokines, has been associated with Th1 differentiation (23), and thus may contribute to the trafficking of kidney-infiltrating T cells. Th1, but not Th2, differentiation also leads to acquisition of the ligand for P-selectin (P-selectin-L), through expression of glycosyltransferases such as FucT-VII (24). Inflammatory signals induce widespread P-selectin expression on endothelium, and this is thought to play a central role in leukocyte rolling in the vasculature of multiple tissues (25). P-selectin is expressed on endothelial cells in humans with proliferative glomerulonephritis (26, 27) as well as in kidneys...
of mice with experimental Ab-induced nephritis, although the site of its renal expression in the latter is uncertain (28, 29). Although P-selectin-L− effector cells have been shown to mediate an inflammatory response in the skin (30), whether kidney-infiltrating effector cells in lupus express P-selectin-L is unknown.

The generation and maintenance of effector T cells is regulated in part by costimulatory receptors, which function generally to signal the presence of non-self. CD28 is essential for the initiation of T cell responses, and MRL−/− mice doubly deficient in B7.1 and B7.2 have dramatic reductions in kidney-infiltrating leukocytes (31). ICOS is a costimulatory molecule closely related to CD28 that is important for both type 1 and type 2 CD4 T cell-mediated inflammation, being most critical during the effector phase of the response (32, 33). We therefore hypothesized that ICOS signaling would be important for generation of renal-infiltrating CD4 T cells in lupus and that such cells could be identified in secondary lymphoid organs by their pattern of chemokine receptor expression.

In this study, we report that CXCL9 protein levels are dramatically elevated in MRL−/− kidneys and that kidney-infiltrating CD4 T cells are enriched for expression of CXCR3, P-selectin-L, and ICOS. By analyzing Icos−/− MRL−/− mice, we find that ICOS expression is only partially required for autoantibody-mediated glomerulonephritis, but plays an essential role in perivascular renal infiltration. Without ICOS, CD4 effector T cells are able to acquire CXCR3 and P-selectin-L expression and migrate to CXCL9, but have impaired production of inflammatory cytokines and fail to form perivascular lesions. Thus, in a systemic autoimmune response, ICOS is selectively required for effector functions while being dispensable for expression of P-selectin-L and CXCR3, which contribute to the kidney-homing phenotype.

Materials and Methods

Mice

MRL−/− animals were obtained from The Jackson Laboratory and maintained in specific pathogen-free conditions at the Yale School of Medicine. The Institutional Animal Care and Use Committee of Yale University approved all procedures. The disrupted Icos allele was generated as described previously (34) and backcrossed to the MRL−/− background for six generations. Animals so derived were fixed for the MRL genome at all MRL susceptibility intervals (35, 36) except the centromeric region of Lmb1 (37). Since MRL−/− mice carrying the B6 allele of Lmb1 have slightly increased splenic lymphoproliferation, but autoantibody production is unaffected, Lmb1 has a minimal impact on the development of autoimmunity. Moreover, if anything, the B6 allele contributes to an increase in lymphocyte activation and therefore would not invalidate our conclusions; thus, the impact of Icos deficiency may actually be slightly stronger than we describe. Animals were then intercrossed and MRL−/− Icos−/− mice were subsequently maintained as homozygotes; to avoid any confounding effects of sex bias, precisely sex-matched groups were analyzed in all experiments. Control animals included Fas-intact MRL.AND mice bearing rear- ranged TCR transgenes (38) and C57BL/6 (B6) mice, which were both maintained in our colony.

Flow cytometry

Spleens were extracted and homogenized by pressing through a 40-μm nylon filter. RBC were lysed by hypotonic shock by brief exposure to a hypotonic buffer followed by immediate isotonic restoration with 10× PBS. Lymphocytes from homogenized kidneys were isolated by centrifugation over Ficoll. Surface staining was conducted in ice-cold PBS with 1% FCS in the presence of FcR blocking Ab clone 2.4G2. For experiments involving chemokine receptor staining, cells were incubated with Ab at 37°C for 30 min. Cells were resuspended in PBS and analyzed on an LSRII 4 laser flow cytometer (BD Biosciences). Samples were analyzed and unfixed and dead cells were excluded based on staining with Hoescht 33342 (Sigma-Aldrich), added immediately before acquisition. Abs used were CD4 (GK1.5; BD Biosciences), B220 (RA3-6B2; BD Biosciences), CD44 (IM7; eBioscience), CD62L (MEL-14; BD Biosciences), CXCR3 (220803; R&D Systems), CCR3 (83101; R&D Systems), CCR5 (C34-3448; BD Biosciences), PSGL-1 (2PH-1; BD Biosciences), Ki-67 (B56; BD Biosciences), and ICOS (C398-4A; eBioscience). P-selectin-L was detected using P-selectin-Ig fusion protein (BD Biosciences), followed by anti-human Ig (Southern Biotechnology Associates). Fluorochromes used in each channel were: FITC or Alexa Fluor (AF) 488, PE, PE-Texas Red, allopacyocyanin or AF647, allopacyocyanin-AF750, Hoechst 33342, and Pacific Blue. In all analyses, autofluorescent events were excluded based on their fluorescence in the PE-Texas Red (B220) channel.

Chemokine and cytokine measurements

Renal chemokine measurements were made directly ex vivo on whole kidney extracts. Kidneys were ground in a rotor-stator homogenizer in 800 μl of PBS with 1% Triton X-100 and the homogenate was cleared by centrifugation. Chemokines in the supernatants were measured by a LumineX assay using Beadlyte anti-mouse chemokine beads (Upstate Biotechnol- ogy), except CXCL10 and CXCL11, which were measured by a Quan- tikine ELISA kit (R&D Systems) according to the manufacturer’s instruc- tions. For effector T cell cytokine production, cells were sorted as indicated and cultured with 50 ng/ml PMA and 1 μM ionomycin for 24 h in complete Click’s medium. IL-2, IFN-γ, TNF-α IL-17, IL-5, and IL-10 from culture supernatants were measured by LumineX.

Immunofluorescence microscopy

Kidneys were snap frozen in OCT, cut into 6-μm sections, and fixed with ice-cold acetone. PBS rehydrated sections were blocked with 10% rat serum and stained with CD19-FITC, CD11c-biotin, and CD4-FA647, or ICOS-FITC and CD4-FA647. Secondary stains included anti-IFITC-FA488 and streptavidin-Cy3, as appropriate. Images were collected on a Zeiss 510 META laser-scanning confocal microscope.

Serology

Total serum lgs were measured by sandwich ELISA using anti-IgM or anti-IgG capture Abs and isotype-specific detection Abs conjugated to HRP (Southern Biotechnological Associates). For anti-DNA Abs, plates were coated with dsDNA and also developed using isotype-specific detection Abs. Four 5-fold serial dilutions of sera were plated and OD values con- forming to the linear portion of the standard curve were used to generate histograms. Fluorescent ANA (FANA) assays were performed on HEP-2 slides (Bio-Rad) and detected with anti-mouse IgM-FITC (BD Biosciences) or anti- rat IgG-FITC (BD Biosciences). Fluorescent micrographs were taken of each slide and the photographs were randomized and coded for blind analysis. Scores represent intensity of staining: +++, strong; +, moderate; +/−, close to background; nil, no staining.

Pathology

Slides of formalin-fixed kidneys were cut and stained by H&E or periodic acid-Schiff by the Department of Pathology at the Yale School of Medi- cine. Slides were randomized, coded, and blindly scored for glomerulonephritis, tubulointerstitial nephritis, perivascular mononuclear infiltration by M.J.K. Proteinuria was measured by Yale-New Haven Hospital Clinical Laboratories. Dermatitis was subjectively assessed based on the presence of mild to moderate lesions (score 1), moderate dorsal lesions (score 2), or severe dorsal lesions and/or ear degradation (score 3).

Statistical analysis

All data analysis was performed using Prism 4.0a (GraphPad Software). In most cases, two-tailed p values were calculated by an unpaired t test. For populations that were unlikely to approximate a Gaussian distribution, two- tailed p values were calculated using the Mann-Whitney U test. Categorical results were subjected to χ2 analysis and binary outcomes were analyzed by Fisher’s exact test.
Results

Kidney-infiltrating CD4 T cells express CXCR3 and P-selectin-L

Because the mechanisms of autoreactive T cell trafficking to the kidney are incompletely defined, we screened for elevations in a panel of chemokines known to recruit T cells to inflamed tissues: CXCL1, CXCL2, CXCL9, CCL2, CCL3, CCL4, and CCL5. We proceeded to examine kidney-infiltrating CD4 T cells for expression of the receptors for the two most elevated chemokines, CXCL9 and CCL5. CCL5 can be bound by CCR1, CCR3, or CCR5, while CXCL9 is recognized by CXCR3 (41), and satisfactory staining reagents were available for CCR5 and CXCR3. After multicolor staining of single-cell kidney suspensions, we gated CD4 T cells and then on CD44high or CD44low, as well as CD44highCD62Llow effector cells, as well as CD44lowCD62Lhigh, likely blood-borne, naive cells as an internal control. In contrast to

fold) (Fig. 1A). A previous study has described increases in Ccl2 and Ccl5 mRNAs in the MRLpr kidney (39) and two more recent studies have also identified elevated Cxcl9, Cxcl10, and Cxcl11 transcripts (22, 40). Strikingly, our data indicated that CXCL9 was by far the most abundant chemokine at the protein level, at a concentration two or more orders of magnitude greater than the others. Subsequently, we also identified high levels of CXCL10, but CXCL11 levels were not above background (Fig. 5; data not shown).

We proceeded to examine kidney-infiltrating CD4 T cells for expression of the receptors for the two most elevated chemokines, CXCL9 and CCL5. CCL5 can be bound by CCR1, CCR3, or CCR5, while CXCL9 is recognized by CXCR3 (41), and satisfactory staining reagents were available for CCR5 and CXCR3. After multicolor staining of single-cell kidney suspensions, we gated first on Hoescht “CD4+” B220+ T cells and then on CD44highCD62Llow effector cells, as well as CD44lowCD62Lhigh, likely blood-borne, naive cells as an internal control. In contrast to
CXCR3, for which only a few cells expressed detectable levels, a significant fraction of kidney-infiltrating cells expressed CXCR3, correlating with robust expression of its ligand (Fig. 1B). By contrast, very few CXCR3+ cells were detected in the peripheral blood of MRL/lpr mice or kidneys of Foxn-intact MRL AND Tcr-transgenic mice (data not shown). Analysis of the spleen also revealed a population of CXCR3+ cells (Fig. 1B), likely representing effector cells with tissue-homing potential.

P-selectin-L expression occurs on Th1 effectors and has been associated with inflammatory potential (24, 30); therefore, we proceeded to measure the expression of this adhesion molecule on kidney-infiltrating effector CD4 T cells. Since P-selectin-L is formed by inducible carbohydrate modifications of PSGL-1 (25), we stained for the PSGL-1 scaffold as well. Using a P-selectin-Ig fusion protein, we found an enrichment of P-selectin-L+ cells in the kidneys of MRL/lpr mice as compared with the spleen (Fig. 1B). Together, these data suggest that P-selectin-mediated rolling and CXCL9-mediated transmigration may contribute to kidney trafficking, and by inference that CXCR3 and P-selectin-L expression in secondary lymphoid organs identifies effector cells with inflammatory potential in lupus.

**ICOS is elevated on CD4 T cells from spleen and kidney**

Immunofluorescence staining of MRL/lpr kidneys confirmed that perivascular infiltrates contain a proportionally large number of CD4+ cells, with a smaller number of B cells (Fig. 2A). However, costaining with CD11c indicates that a fair number of these CD4+ cells are dendritic cells (DC) (Fig. 2, B and C). We suspected that the ICOS-B7RP-1 costimulatory pair was involved in T cell-APC interactions in inflammatory nephritis, given its essential role in the collaboration of CD4 T cells with B cells and DC and the subsequent development of inflammatory effector functions (32–34, 42–44). Importantly, the interaction of CD4 T cells with B cells is essential for nephritis, not only via the production of autoantibodies but also for autoantibody-independent inflammation (5). Tissue staining indicated that ICOS is expressed on kidney-infiltrating T cells (Fig. 2, D–F), and this finding was confirmed by flow cytometric analysis (Fig. 2G). ICOS was also highly expressed in the spleen (Fig. 2G) and thus may potentially regulate effector cell functions at either or both the priming and effector phases of the autoimmune response.

**Autoantibody levels and glomerulonephritis are partially dependent on ICOS**

To define the contribution of ICOS to lupus pathogenesis, we analyzed MRL/lpr mice with a disruption in the Icos gene (Icos+/−) compared with wild-type Icos+/+ controls. Upon measuring total spontaneous Ab levels in the serum, we found that although IgM and IgG3 levels were unchanged, Icos+/− animals showed a marked decrease in IgG1, IgG2a, and, most strikingly, IgG2b Abs.
and shown as total mass per single kidney, in age-matched
compared with expressed as mean

of ICOS (56.5% in total incidence of glomerulonephritis did not change in the absence
by a lessening of the severity of glomerulonephritis. Although the
tions indicated that the reduction in serum IgG was accompanied
pus is the kidney glomerulus. Pathological analysis of kidney sec-
neous nuclear staining.

C

This decrease paralleled a reduction, albeit not an

A

B

C

D

FIGURE 5. Mononuclear infiltration is severely diminished in kidneys of Icos−/− MRLnpr mice despite elevated levels of inflammatory chemo-
kines. A and B, Representative H&E micrographs of perivascular region of
kidneys from 18-wk-old Icos+/+ (A) and Icos−/− (B) mice; arrows point to
residual infiltrates in the latter. C, H&E-stained renal tissue sections were
randomized, coded, and blindly scored for perivascular mononuclear infil-
tration by a pathologist (M.J.K.). Scores of 1–3 indicate frank infiltrates of
residual infiltrates in the latter.

K

Icos

Icos

Icos

Icos

FIGURE 6. T cell activation in MRLnpr mice is partially dependent on ICOS.
A, Absolute numbers of splenic CD4 T cells (TCRβ−CD4+ B220−), CD8 T cells
(TCRβ−CD8+ B220−), and B cells (CD19+ B220+ TCRβ+) in the spleens of 16-
to 18-wk-old Icos+/+ and Icos−/− animals; bar indicates median value; n = 12–21
spleens were examined. B, Expression of CD44, CD62L, and CD25 on CD4 T
cells from the spleen; gate frequencies indicate mean values. C and D, Frequency
of naive (CD44hiCD62Llo) and activated (CD44hiCD62Lhi, CD44hi
CD62Lhi) subsets among CD4 and CD8 T cells as indicated. Frequency of CD25+ cells, including effector and regulatory T cells, is shown in C. Bars indicate
mean values; n = 5–6 spleens were examined. Asterisk (*) indicates significant
difference from Icos+/+ (p < 0.05, t test).

absence, of IgG deposition in glomeruli (data not shown). Mea-
surement of urinary protein, an indicator of kidney injury, did not
reveal a significant reduction in Icos−/− animals (Fig. 4C),

(3A). Thus, both type 1 and type 2 isotypes are ICOS depend-
ent in this model. Assessment of serum anti-dsDNA Abs revealed
a similar pattern of IgG isotype reductions in the Icos−/− group,
with autoantigen-specific IgM and IgG3 Abs remaining at levels
comparable to those of wild-type controls (Fig. 3B). In a comple-
mentary analysis, we used the FANA assay to measure total nu-
clear Ag-reactive Abs. In agreement with the ELISA data, this
analysis showed a substantial reduction in IgG staining of nuclei,
while IgM ANA levels were unchanged by the

A

B

C

D

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indicating that substantial glomerular injury still occurred. This finding is consistent with the fact that IgG2a and IgG3, which were least affected by the ICOS mutation, are thought to be most pathogenic in the kidney (12). Similarly, apart from two outliers, Icos−/− animals did not have significantly elevated BUN levels compared with the Icos+/+ cohort (Fig. 4D). The development of dermatis was unaffected by Icos disruption (Fig. 4B).

**ICOS controls effector cytokines but not homing receptor expression**

At 18 wk of age, 100% of Icos+/+ MRL+/+ animals displayed frank perivascular infiltrates in the kidney, while essentially all Icos−/− animals displayed negligible numbers of infiltrating cells in the interstitium (Fig. 5, A–C). Importantly, although the numbers of infiltrating cells in Icos−/− kidneys were remarkably low, they were not completely absent (Fig. 5B, arrows), suggesting that ICOS is not absolutely required for cell recruitment to the kidney. Glomerular injury by autoantibodies is thought to incite inflammatory chemokine expression and secondary infiltration of the interstitium (39). We hypothesized, therefore, that a defect in production of one or more of the IgG isotypes in Icos−/− mice would result in impaired chemokine induction. Surprisingly, Icos−/− kidneys contained high levels of all of the chemokines measured, including the CXCR3 ligands CXCL9 and CXCL10 (Fig. 5D). Only CCL5 was reduced in comparison to Icos+/+ mice (p < 0.05, t test), but this chemokine was still significantly elevated over that found in control B6 mice. The lack of infiltrating T cells likely accounts for the reduction in CCL5 in Icos−/− kidneys, since infiltrating T cells themselves are likely a major source of this chemokine (45). These results show that the loss of infiltrates in Icos−/− kidneys is not likely due to inadequate levels of inflammatory chemokines. We further infer from these data that induction of chemokines is causally prior to cellular infiltration of the nephritic kidney.

Because Icos+/+ MRL+/+ kidneys contained abundant CXCL9 levels, but had severely reduced infiltrates, we thought it likely that ICOS signaling was required for T cell (and/or B cell) expansion or activation in the spleen. To address this issue, we determined T cell and B cell counts, revealing that ICOS deficiency did not reduce splenic T cell numbers (Fig. 6A). By analysis of CD44, CD62L, and CD25 expression, we also showed that although CD4 T cell activation was somewhat impaired in these mice, with the frequency of CD44highCD62Llow effector CD4 T cells in Icos−/− mice 70% of that in Icos+/+ controls, a substantial number of activated splenic T cells developed in the absence of ICOS (Fig. 6, B–D).

Since it seemed that the stark reduction in renal infiltrates in Icos−/− animals could not be accounted for by these differences, we next asked whether ICOS signaling was required during T cell differentiation for expression of CXCR3, P-selectin-L, or both. In this study, we analyzed Icos−/− splenic effector cells; however, surprisingly, both of these tissue-homing molecules were expressed in an ICOS-independent manner (Fig. 7, A and C). To confirm that the CXCR3 detected on Icos−/− cells was functional, we measured their migration in vitro. Indeed, ICOS deficiency did not impair the cells’ ability to migrate to CXCL9 (Fig. 7B), indicating that the inflammatory defect did not lie in the inability of effector T cells to respond this chemokine.
The normal expression of trafficking molecules was consistent with the fact that infiltrating cells, upon close inspection of kidney sections, are not completely absent in 

Icos \(^{-/-}\) MRL \(^{pr}\) mice, but rather are drastically reduced in number. Considered together, these data were consistent with a failure of local effector cell expansion in the absence of ICOS. To address this possibility directly, we were able to collect enough effector CD4 T cells from 

Icos \(^{-/-}\) kidneys to measure expression of Ki-67, a surface marker of proliferation. These data indicated that, indeed, a significantly smaller fraction of presumably kidney-infiltrating effector cells were actively proliferating in the absence of ICOS (Fig. 8, B and C).

Cytokines such as IFN-\(\gamma\) play an essential role in tissue inflammation, including nephritis. For instance, renal infiltrates are almost completely abolished in IFN-\(\gamma\)- or IFN-\(\gamma\)-receptor-deficient MRL \(^{pr}\) or (NZB \(\times\) NZW)F\(_1\) mice (9–14), similar to the phenotype of 

Icos \(^{-/-}\) mice described here. As local effector cell proliferation is almost certainly dependent on MHc class II (MHCIi), it is important to note that IFN-\(\gamma\) is absolutely required for MHCIi expression in the kidney (12). Therefore, we addressed whether defective cytokine production could account for the failure of inflammatory lesions to develop. We sorted B220 \(^{+}\) CD4 \(^{+}\)CD44\(^{high}\) CD62L\(^{low}\) effector cells from spleens of both 

Icos \(^{+/+}\) and 

Icos \(^{-/-}\) MRL \(^{pr}\) mice and compared their cytokine production to sorted naive T cells following ex vivo restimulation. Effector cells made IFN-\(\gamma\), TNF-\(\alpha\), IL-10, IL-5, and an insignificant amount of IL-17, and ICOS deficiency reduced secretion of all of these effector cytokines except the latter (Fig. 8A). Therefore, the data suggest that ICOS signaling is not absolutely required to generate effector cells capable of infiltrating the kidney but rather promotes the ability of those cells to execute their effector functions and expand in the tissue.

**Discussion**

In a study of chemokine expression in lupus nephritis, an RNase protection assay was used to screen for transcripts of nine inflammatory chemokines and 

Ccl2 and Ccl5 were identified as the two most abundant (39). Expression of Ccl2 and its receptor, Ccr5, occurs primarily around glomeruli (39, 46). Deletion of Ccl2 ablates cellular infiltration in and around glomeruli, but does not affect perivascular infiltration (20). A more recent microarray analysis has identified elevated transcripts of two more chemokines, 

Cxc9 and Cxc10 (22). We show here, at the protein level, that the level of CXCL9 in MRL \(^{pr}\) kidneys far outweighs those of CCL2 and CCL5, and this elevated level of expression is accompanied by massive perivascular infiltration of effector CD4 T cells expressing the CXCL9 receptor CXCR3. Although CCR5 \(^{+}\) T cells have been previously identified around glomeruli (39), the fact that we observed relatively few of them in the kidney likely reflects that cells in glomeruli, although clinically important, are rare in comparison to those in the perivascular area. Although it remains unclear whether CXCL9 and CXCR3 are required for perivascular inflammation in MRL \(^{pr}\) mice, mice lacking either gene have reductions in both glomerular and tubulo-interstitial infiltration in a model of nephrotoxic serum nephritis (40).

In addition to CXCR3, we also found that kidney-infiltrating T cells were enriched for expression of P-selectin-L. The expression level of P-selectin correlates with the progression of nephritis in MRL \(^{pr}\) mice (47), deficiencies in P-selectin or PSGL-1 paradoxically exacerbate glomerular inflammation through enhanced expression of CCL2 (48). However, as indicated by 

Ccl2 \(^{-/-}\) mice (20), trafficking to glomeruli and perivascular tubulo-interstitial regions are controlled by distinct mechanisms, suggesting that P-selectin interactions may still be operative in the latter.

Renal-infiltrating T cells also expressed ICOS and deletion of the Icos gene led to a dramatic reduction in perivascular infiltration. Production of IgG autoantibodies was substantially reduced, owing to the failure to generate helper T cells in the extrafollicular Ab response (37), the principal site for generation of class-switched and somatically mutated autoantibodies in this mouse model of lupus (49). Ab blockade of ICOS interactions also down-modulates pathogenic autoantibody production and immune complex glomerulonephritis that arises in (NZB \(\times\) NZW)F\(_1\) lupus-prone mice (50). In concert with these observations, Kelley and colleagues (51) found that 

Icos \(^{-/-}\) MRL \(^{pr}\) mice had reductions in class-switched and anti-dsDNA Ab levels, also without substantial alterations in glomerulonephritis or renal function. However, in distinct contrast to our observations, these investigators did not observe any effect of Icos deficiency on perivascular infiltrates (51). The reasons for this stark difference between our studies is not clear, but may reflect the impact of B6/129-derived genetic modifiers of interstitial nephritis, as our cohort was backcrossed to the MRL background six generations, whereas Zeller et al. (51) analyzed an N5 group; our animals also were genotyped and contained defined MRL susceptibility loci or, in the case of the 

Lmnb locus, a B6 allele that may actually potentiate disease (see Materials and Methods). Importantly, these data are consistent with other studies indicating a requirement for ICOS in cell-mediated inflammation (32, 33, 52–57). Alternatively, differences in the original knockout strains (34, 42) and/or variance in the MRL \(^{pr}\) renal phenotype in different mouse colonies may contribute to the disparity.

Despite the reduction in IgG levels, we did not find a substantial effect on chemokine induction in the kidney. This finding was somewhat surprising in light of the current model that immune complex-induced inflammation leads to secondary cellular infiltration via engagement of Fc receptors on renal parenchymal and hematopoietic cells with subsequent inflammatory chemokine release (7, 20, 39, 58, 59). Our data indicate either that the lower levels of autoantibodies present in 

Icos \(^{-/-}\) MRL \(^{pr}\) mice are sufficient for inflammatory chemokine induction or that Ab-independent inflammatory processes are more significant than generally appreciated, possibilities that are not mutually exclusive. In support of the latter view, renal infiltrates can occur in the absence of secreted Abs (5). Thus, it appears most likely that both Ab-dependent and -independent pathways contribute to organ inflammation, suggesting that T cells and other locally inflammatory cells are critical to disease initiation and/or maintenance, a view supported by the observations that T cells are a major contributor to proinflammatory cytokine production in murine lupus (60) and have the capacity to initiate renal parenchymal injury (61).

In addition to having its minimal impact on renal chemokine levels, ICOS was also unexpectedly dispensable for the expression of the trafficking molecules CXCR3 and P-selectin-L on effector CD4 cells as well as their migration to CXCL9. Of course, while this suggested an intact potential for migrating to the kidney, we are unable to directly analyze the rate of renal trafficking to the kidney. Staining with Ki-67 suggested that kidney-infiltrating cells proliferated poorly in the kidney. In light of these data, we believe 

Icos \(^{-/-}\) T cells that reach the kidney neither efficiently expand in situ nor recruit other cells through the production of inflammatory cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\). Since 

Icos \(^{-/-}\) effector T cells in the spleen show defects in cytokine production after recall with PMA and ionomycin, the lack of ICOS signaling during differentiation appears to cause an intrinsic defect in the acquisition of these effector functions. These data indicate that CXCR3 and P-selectin-L expression can be uncoupled from production of IFN-\(\gamma\), even though all of these genes are under control of the Th1-specific
transcription factor T-bet (62). Thus, in systemic autoimmunity, ICOS signaling is necessary for only part of the Th1 program, though how these disparate sets of genes are controlled is not clear.

In addition to developmental defects in effector T cells, another, although not exclusive, possibility is that local ICOS-B7RP1 interactions in the kidney are required for effector cell expansion and function. Indeed, cognate interactions are likely to be important in renal injury, as MHCIIC expression is seen on several kidney resident cell types in MRL/lpr mice, including tubular epithelia, endothelium, and mesangial cells (3, 12). However, neither B7.1 nor B7.2 are expressed on intrinsic kidney cells, even under inflammatory conditions (31). On the other hand, B7RP-1, the sole ligand for ICOS (63), is expressed in multiple renal tissues (64), indicating that ligation of the latter on kidney-infiltrating T cells has a high likelihood of occurring, and that this interaction may be critical for proliferation and cytokine secretion. In vitro, ICOS signaling strongly activates the Akt pathway through PI3K (65) and through this pathway likely affects cell survival and cytokine production (66). In vivo, ICOS blockade during the effenter inflammatory response is sufficient to prevent tissue infiltration in such disparate tissues as the lung, brain, and heart (32, 33, 57). Taken together, these studies and our data support a dual role for ICOS in both the differentiation of armed effector Th1 cells as well as a local activation signal in lupus nephritis.

In addition to direct effects on effector T cells, a number of extrinsic factors may contribute to the reduction of renal infiltrates in Icos−/− mice. First, since the production of isotype-switched, and likely affinity-matured, Ab production is impaired in these mice, there may be changes in inflammatory chemokines, cytokines, or endothelial adhesion molecules that were not assessed. Although we argue that it is primarily an effect, rather than a cause, the partial reduction in CCR5 (Fig. 5D) may also contribute to some extent to reduced renal infiltration. The probable reduction in high-affinity B cells may also impact the efficiency of T cell activation, particularly in light of the Ag-presenting role B cells play in the development of nongerminate T cells (5), which could in part contribute to poor cytokine secretion by Icos−/− effectors.

Pathological analysis of human lupus nephritis suggests that pathogenic mechanisms similar to murine models may be operative. Inflammatory infiltrates are comprised of CD45RO+ (activated/memory) T cells, macrophages, and B cells (67). CD4 T cells from nephritic kidneys, such as those occurring in the tubulointerstitial regions of patients with World Health Organization class IV involvement and from the peripheral blood of more severely ill patients, have a proinflammatory Th1 phenotype with IFN-γ production (15). These renal-infiltrating T cells also express CXCR3, presumably responding to locally produced CXCL9 or CXCL10 (68, 69). The finding that infiltrating T cells in lupus patients express ICOS (16) suggests that ICOS-B7RP1 interactions may also be important in the pathogenic response in human lupus nephritis. Data from Ab-blocking experiments in (NZB × NZW)F1 mice (50) and our genetic analysis of Icos−/− MRL/lpr mice provide direct data that ICOS-B7RP1 interactions contribute to the inflammatory process in lupus nephritis.

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Disclosures

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References

ICOS CONTROLS EFFECTOR T CELL FUNCTION IN LUPUS


