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ICOS Controls Effector Function but Not Trafficking Receptor Expression of Kidney-Infiltrating Effector T Cells in Murine Lupus[1]

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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^{lpr} 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^{lpr} 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*$^{-/-}$ MRL^{lpr} 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*$^{-/-}$ 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. *The Journal of Immunology,* **2009, 182: 4076–4084.**

Systemic lupus erythematosus is a common autoimmune syndrome in both humans and inbred and mutant mouse strains, typified by high-titer anti-nuclear Ab (ANA)[5] 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*^{lpr} (MRL^{lpr}) 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)F$_1$ (7) and NZM2338 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^{lpr} 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

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[5] Abbreviations used in this paper: ANA, anti-nuclear Ab; BUN, blood urea nitrogen; PSGL-1, P-selectin glycoprotein receptor 1; P-selectin-L, ligand for P-selectin; AF, Alexa Fluor; FANA, fluorescent ANA; BUN, blood urea nitrogen; DC, dendritic cell; MHCII, MHC class II.

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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^{lpr} 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^{lpr} kidneys and that kidney-infiltrating CD4 T cells are enriched for expression of CXCR3, P-selectin-L, and ICOS. By analyzing *Icos*$^{-/-}$ MRL^{lpr} 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^{lpr} 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^{lpr} 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^{lpr} 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^{lpr} *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 rearranged 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 distilled water 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, allophycocyanin or AF647, allophycocyanin-AF750, Hoescht 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 Biotechnology), except CXCL10 and CXCL11, which were measured by a Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions. 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.

Chemotaxis assay

Splenocytes were isolated in prewarmed serum-free Click's medium with 0.5% BSA and 2 mM HEPES, and 1×10^6 cells in 100 μl were added to the upper chamber of 5 μM Transwell plates (Millipore) containing 500 μl of medium in the lower chamber. Cells were preincubated for 1 h at 37°C in 5% CO_2, then 100 μl of medium containing varying concentrations of CXCL9 was added to the lower chamber and cells were allowed to migrate for 3 h. Percent migration was calculated by dividing the number recovered in the lower chamber by the number of input for each population. Specific migration was determined by subtracting the percent migrated without chemokine. Samples were run in duplicate at four dilutions of CXCL9: 1000, 500, 250, and 125 μg/ml.

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-AF647, or ICOS-FITC and CD4-AF647. Secondary stains included anti-FITC-AF488 and streptavidin-Cy3, as appropriate. Images were collected on a Zeiss 510 META laser-scanning confocal microscope.

Serology

Total serum Igs were measured by sandwich ELISA using anti-IgM or anti-IgG capture Abs and isotype-specific detection Abs conjugated to HRP (Southern Biotechnology 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 conforming 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 Medicine. Slides were randomized, coded, and blindly scored for glomerulonephritis and perivascular mononuclear infiltration by M.J.K. Proteinuria was assessed at 18 wk of age using Bayer Multistix. Blood urea nitrogen (BUN) levels were measured by Yale-New Haven Hospital Clinical Laboratories. Dermatitis was subjectively assessed based on the presence of mild dorsal 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 χ^2 analysis and binary outcomes were analyzed by Fisher's exact test.

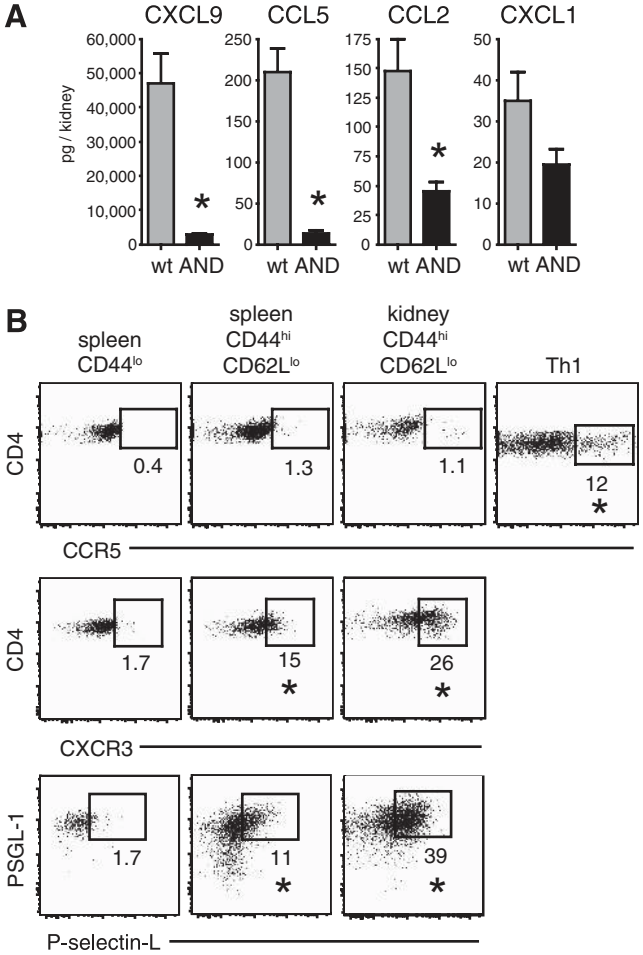

FIGURE 1. CXCL9 is elevated in nephritic kidneys and renal-infiltrating CD4 T cells express CXCR3 and P-selectin-L. *A*, Protein levels of a panel of inflammatory chemokines were measured in fresh kidney homogenates from 16-wk-old nephritic MRL^{lpr} (wild type (wt), $n = 9$) and 12-wk-old MRL$^{+/+}$ AND TCR-transgenic (AND, $n = 3$) mice as controls. Chemokines are ordered by descending expression level, from *left* to *right*. CXCL2, CCL3, and CCL4 were below background (<20 pg) and are not shown. Data shown are mean picograms per kidney ± SEM. *B*, Expression of CCR5, CXCR3, PSGL-1, and P-selectin-L were measured on naive (CD44^{low}) and effector (CD44^{high}CD62L^{low}) B220^{-}CD4^{+} T cells from spleen and kidney as indicated. In vitro-differentiated Th1 cells served as a positive control for CCR5 staining. Gate frequencies indicate mean values; for spleen and kidney gates, $n \geq 5$ and for Th1 gate, $n = 3$. Significant difference from control (AND in *A*, CD44^{low} cells in *B*) is indicated by an asterisk (*) ($p < 0.05$, *t* test).

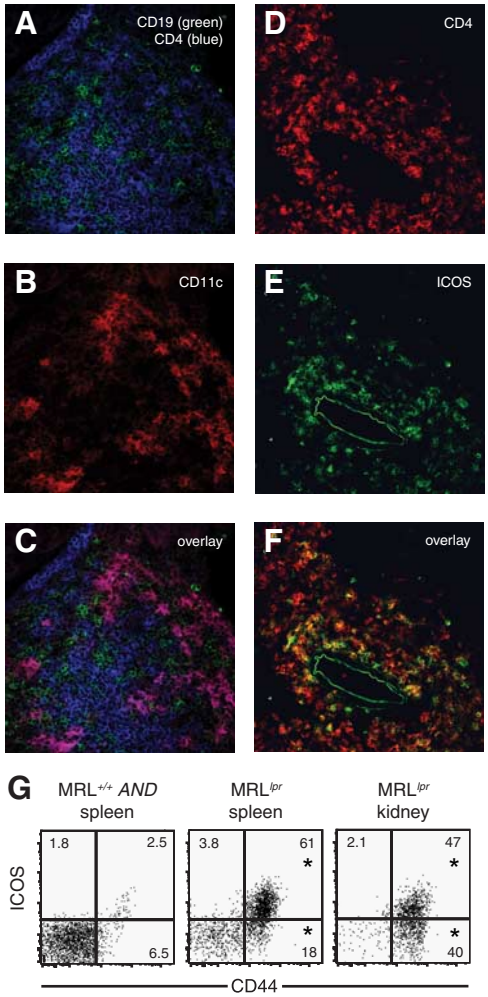

FIGURE 2. Renal-infiltrating cells include B cells, DC, and ICOS^{+} CD4 T cells. *A–F*, Confocal micrographs of perivascular renal infiltrates from 16- to 18-wk-old MRL^{lpr} animals. *A–C,* Representative of 12 mice analyzed. *C*, Merge of CD19 (blue)/CD4 (green) image shown in *A* with CD11c (red) image shown in *B*, and colocalization of blue and red signals is indicated by magenta. *F*, Merge of CD4 (red) image shown in *D* with ICOS (green) image shown in *E*, with colocalization indicated by yellow. Two slides each from four mice were analyzed and a typical image is shown. *G*, CD44 and ICOS expression on B220^{-}CD4^{+} T cells from indicated mice and organs is shown. Gate frequencies indicate mean values; for MRL$^{+/+}$.AND, $n = 2$ and for MRL^{lpr}, $n = 5$. Significant difference from *AND* control is indicated by an asterisk (*) ($p < 0.05$, *t* 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 measured the protein levels of these chemokines in fresh, unmanipulated homogenates of kidneys from nephritic MRL^{lpr} mice using as negative controls Fas-intact MRL mice bearing the foreign Ag-specific *AND Tcr* transgenes that we have previously shown to rescue renal pathology (38). CCL3, CCL4, and CXCL2 levels fell below the linear detection limits of the assay and were excluded. The remaining four chemokines measured were all found at elevated levels in nephritic MRL^{lpr} kidneys: CXCL9 (15.6-fold), CCL5 (15.2-fold), CCL2 (3.2-fold), and CXCL1 (1.8-fold) (Fig. 1*A*). A previous study has described increases in *Ccl2* and *Ccl5* mRNAs in the MRL^{lpr} 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 (see 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 CD44^{high} CD62L^{low} effector cells, as well as CD44^{low}CD62L^{high}, likely blood-borne, naive cells as an internal control. In contrast to

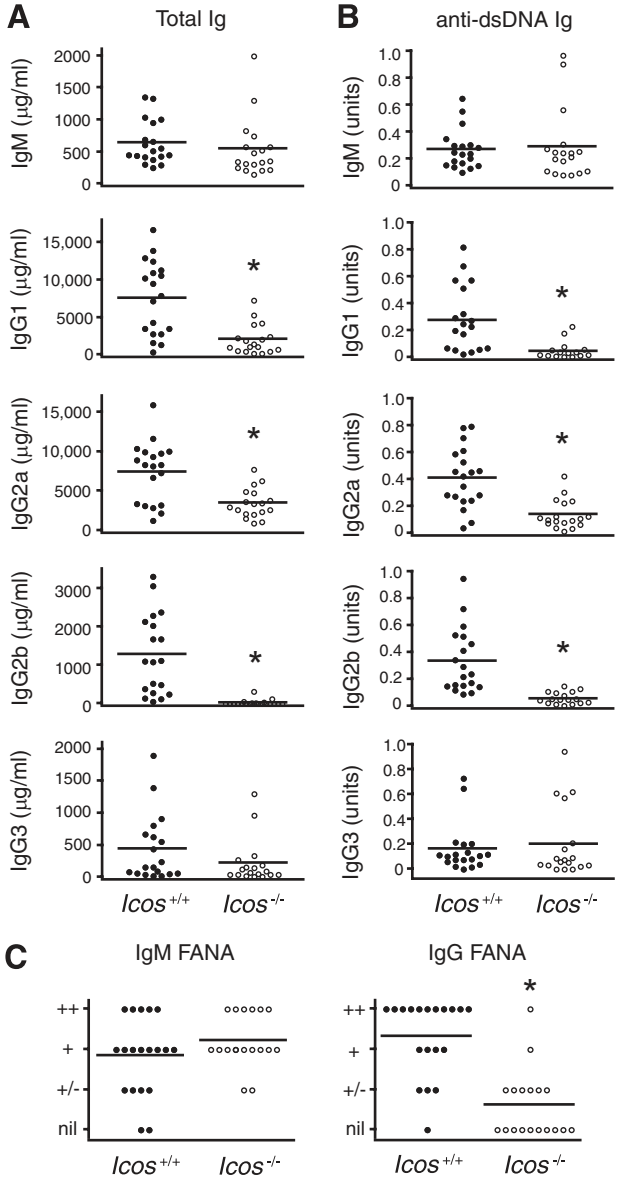

FIGURE 3. IgG autoantibody production is ICOS dependent. Abs were measured in sera from 18-wk-old $Icos^{+/+}$ ($n = 19$, 47.4% female) and $Icos^{-/-}$ ($n = 23$, 47.8% female) MRL^{lpr} animals. *A*, ELISA for total Ig of each isotype is indicated, with results expressed in μg/ml; $Icos^{+/+}$, ●; $Icos^{-/-}$, ○. *B*, ELISA for anti-dsDNA Ig of each isotype is indicated in arbitrary units. *C*, FANA assays were performed using HEp-2 cells as substrate and detected with anti-mouse IgM or IgG, as indicated. Bars represent mean values. Asterisk (*) indicates $p < 0.05$ (*A* and *B*, *t* test; *C*, χ^2).

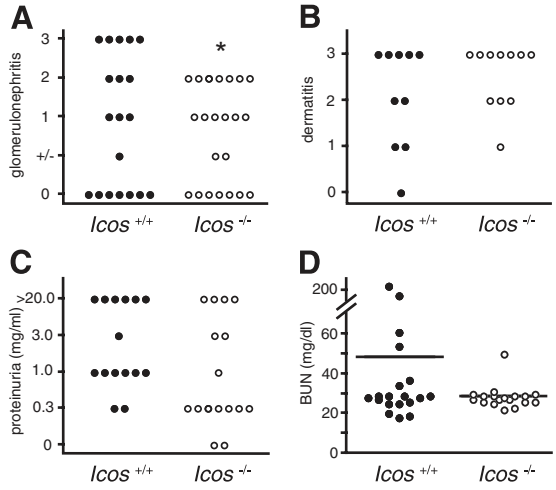

FIGURE 4. *Icos* deficiency does not protect mice from renal failure or dermatitis. *A*, Glomerulonephritis scores assigned by a pathologist (M.J.K.) in a double-blind assessment of both H&E and periodic acid-Schiff-stained slides, with appreciable disease scored on a scale of 1–3. 0 indicates normal histology and +/− indicates negligible changes. Asterisk (*) denotes significant reduction in the incidence of severe glomerulonephritis (score 3; $p < 0.05$, Fisher's exact test). *B*, Dermatitis was scored on a scale of 0–3: 0, normal; 1, mild; 2, moderate; and 3, severe. *C*, Protein levels were measured in fresh urine using Bayer Multistix, with results expressed in mg/ml. *D,* BUN levels were measured in sera and expressed in mg/dl. Bars represent mean values. $Icos^{+/+}$, $n = 19$; $Icos^{-/-}$, $n = 23$.

CCR5, 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. 1*B*). By contrast, very few $CXCR3^+$ cells were detected in the peripheral blood of MRL^{lpr} mice or kidneys of Fas-intact MRL *AND Tcr*-transgenic mice (data not shown). Analysis of the spleen also revealed a population of $CXCR3^+$ cells (Fig. 1*B*), 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. 1*B*). 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. 2*A*). 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. 2*G*). ICOS was also highly expressed in the spleen (Fig. 2*G*) 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

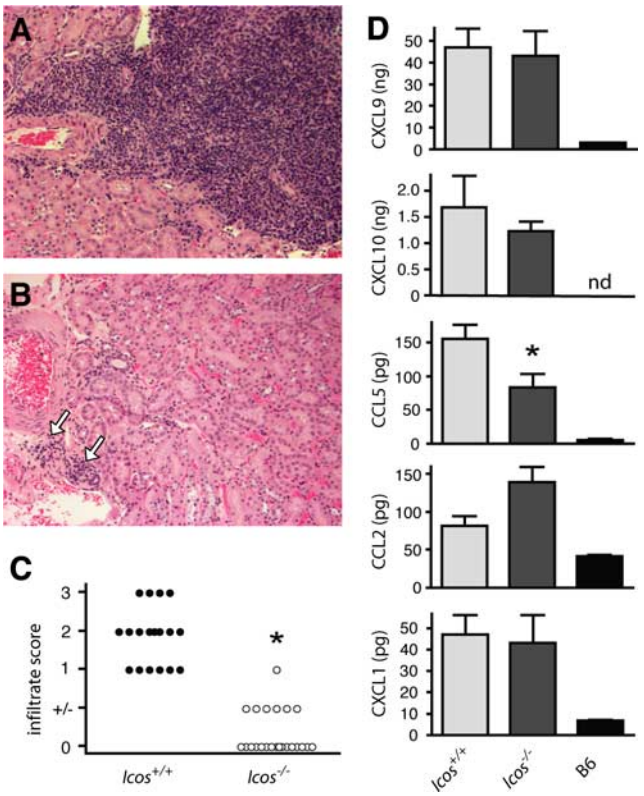

FIGURE 5. Mononuclear infiltration is severely diminished in kidneys of $Icos^{-/-}$ MRL^{lpr} mice despite elevated levels of inflammatory chemokines. *A* and *B*, Representative H&E micrographs of perivascular region of kidneys from 18-wk-old $Icos^{+/+}$ (*A*) and $Icos^{-/-}$ (*B*) mice; arrows point to residual infiltrates in the latter. *C*, H&E-stained renal tissue sections were randomized, coded, and blindly scored for perivascular mononuclear infiltration by a pathologist (M.J.K.). Scores of 1–3 indicate frank infiltrates of increasing size, +/− indicates very minor infiltrate (~1 cell thick) and 0 indicates no apparent infiltrate. Bars represent mean values. $Icos^{+/+}$, n = 19; $Icos^{-/-}$, n = 23. *D*, Renal chemokine levels, as measured by Luminex and shown as total mass per single kidney, in age-matched $Icos^{+/+}$ (n = 5) and $Icos^{-/-}$ (n = 4) MRL^{lpr} mice are shown along with values from kidneys of B6 (n = 3) healthy controls. Chemokines are ordered by descending expression level, from *top* to *bottom* (note units for CXCL9). Data are expressed as mean ± SEM. Asterisk (*) indicates significant reduction compared with $Icos^{+/+}$ (*C*, χ^2; *D*, *t* test); nd, Not determined.

(Fig. 3*A*). Thus, both type 1 and type 2 isotypes are ICOS dependent 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. 3*B*). In a complementary analysis, we used the FANA assay to measure total nuclear 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 *Icos* mutation (Fig. 3*C*). We did not detect a shift in the pattern of autoantibody reactivity, with both groups most commonly displaying homogeneous nuclear staining.

One of the major targets of autoantibody-mediated injury in lupus is the kidney glomerulus. Pathological analysis of kidney sections indicated that the reduction in serum IgG was accompanied by a lessening of the severity of glomerulonephritis. Although the total incidence of glomerulonephritis did not change in the absence of ICOS (56.5% in $Icos^{-/-}$, 57.9% in $Icos^{+/+}$ animals), the incidence of severe (score 3) glomerular injury was significantly reduced (Fig. 4*A*). This decrease paralleled a reduction, albeit not an

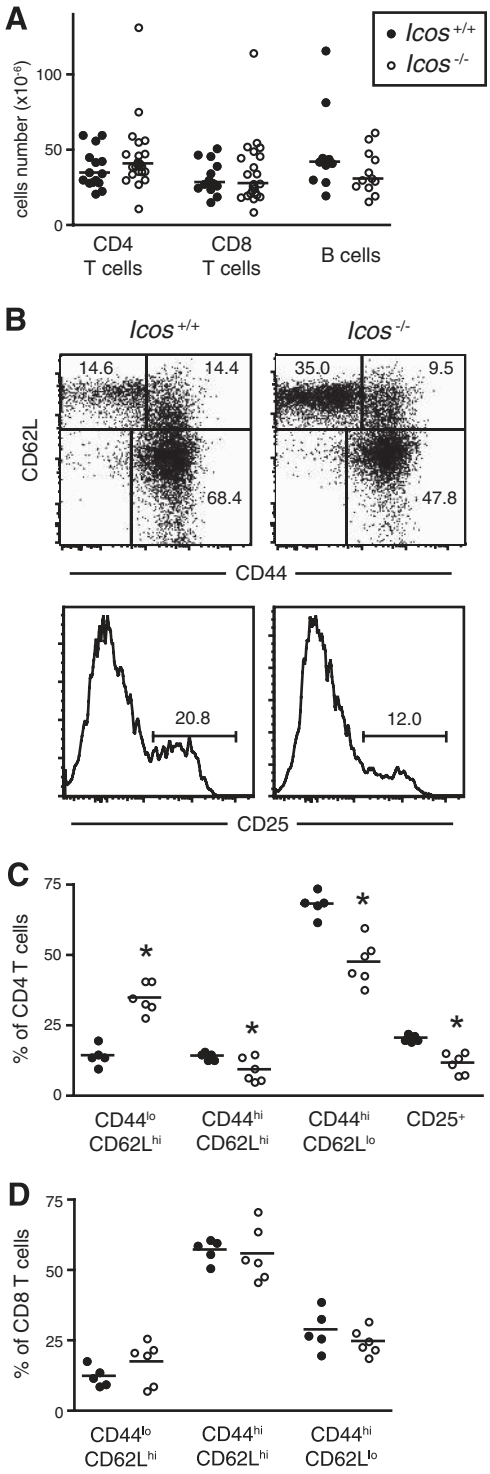

FIGURE 6. T cell activation in MRL^{lpr} mice is partially dependent on ICOS. *A*, Absolute numbers of splenic CD4 T cells (TCRβ^+CD4$^+$B220$^-$), CD8 T cells (TCRβ^+CD8$^+$B220$^-$), and B cells (CD19$^+$B220$^+$TCRβ^-) in the spleens of 16- to 18-wk-old $Icos^{+/+}$ and $Icos^{-/-}$ animals; bar indicates 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 (CD44^{low}CD62L^{high}) and activated (CD44^{high}CD62L^{high}, CD44^{high} CD62L^{low}) 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). Measurement of urinary protein, an indicator of kidney injury, did not reveal a significant reduction in $Icos^{-/-}$ animals (Fig. 4*C*),

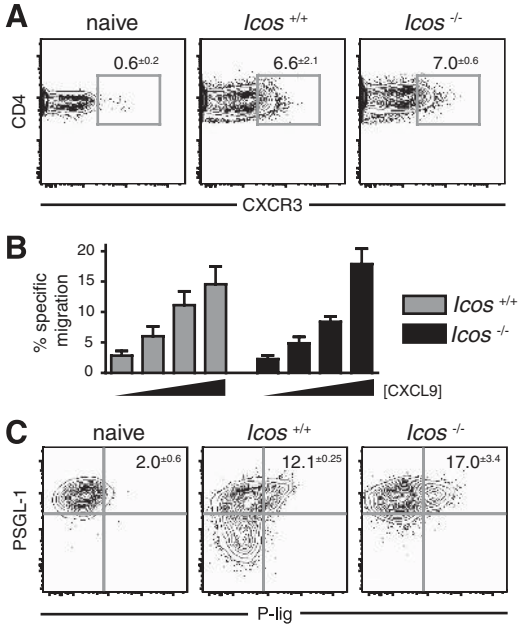

FIGURE 7. ICOS is not required for CXCR3 and P-selectin-L expression in effector CD4 T cells from MRL^{lpr} mice. *A* and *C*, Expression of CXCR3 and P-selectin-L was measured by flow cytometry on B220$^-$CD4$^+$ CD44^{high}CD62L^{low} effector CD4 T cells from the spleens of 16- to 18-wk-old *Icos*$^{+/+}$ and *Icos*$^{-/-}$ animals, with *Icos* $^{+/+}$ CD44^{low}CD62L^{high} naive CD4 T cells gated as a negative control. *B*, Migration of effector CD4 T cells to CXCL9 was measured in a Transwell chemotaxis assay. The percentage of migrated CD44^{high}CD62L^{low} effector cells minus background is displayed.

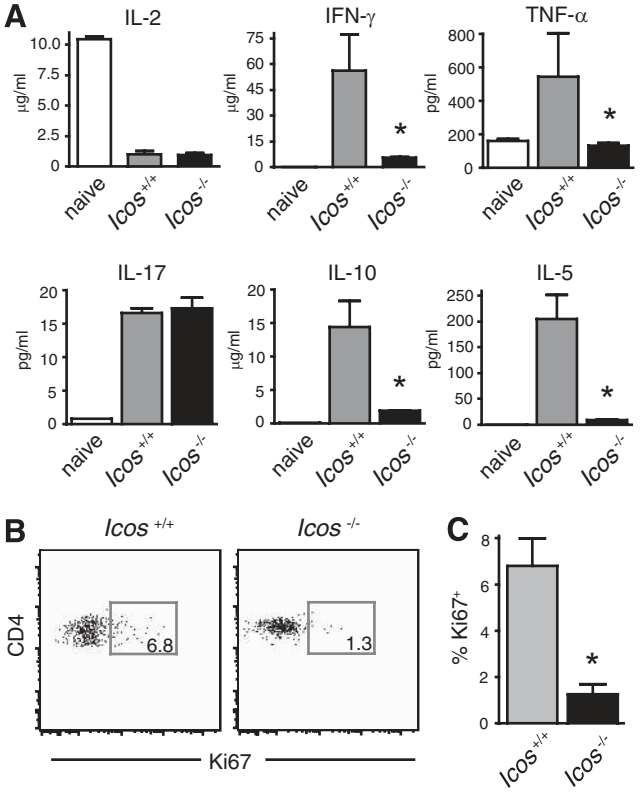

FIGURE 8. ICOS is required for T cell effector function and local proliferation in the kidney. *A*, *Icos*$^{+/+}$ and *Icos*$^{-/-}$ CD44^{high}CD62L^{low} effectors and control naive cells were sorted from three to four pooled donors, 5×10^5 cells stimulated in vitro, and secreted cytokines were measured by Luminex. Data are expressed as mean ± SEM and are representative of two experiments. *B*, Representative Ki-67 staining of CD4 effector T cells from the kidneys of *Icos*$^{+/+}$ ($n = 4$) and *Icos*$^{-/-}$ ($n = 4$) mice. Mice were all female and between 18 and 20 wk of age. *C*, Mean expression of Ki-67 ± SEM, $n = 4$. Asterisk (*) indicates significant reduction compared with *Icos*$^{+/+}$ ($p < 0.05$, *t* test).

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. 4*D*). The development of dermatitis was unaffected by *Icos* disruption (Fig. 4*B*).

ICOS controls effector cytokines but not homing receptor expression

At 18 wk of age, 100% of *Icos*$^{+/+}$ MRL^{lpr} 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. 5*B*, 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. 5*D*). 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^{lpr} 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. 6*A*). 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 CD44^{high}CD62L^{low} 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. 7*B*), 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^{lpr} 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-γ play an essential role in tissue inflammation, including nephritis. For instance, renal infiltrates are almost completely abolished in IFN-γ- or IFN-γ receptor-deficient MRL^{lpr} or (NZB × 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-γ 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^{lpr} mice and compared their cytokine production to sorted naive T cells following ex vivo restimulation. Effector cells made IFN-γ, TNF-α, 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. 8*A*). 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, *Cxcl9* and *Cxcl10* (22). We show here, at the protein level, that the level of CXCL9 in MRL^{lpr} 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^{lpr} mice, mice lacking either gene have reductions in both glomerular and tubulointerstitial 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. Although the expression level of P-selectin correlates with the progression of nephritis in MRL^{lpr} 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 tubulointerstitial 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 downmodulates pathogenic autoantibody production and immune complex glomerulonephritis that arises in (NZB × NZW)F$_1$ lupus-prone mice (50). In concert with these observations, Kelley and colleagues (51) found that *Icos*$^{-/-}$ MRL^{lpr} 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 *Lmb1* 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^{lpr} 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^{lpr} 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-γ and TNF-α. 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-γ, 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 MHCII 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 efferent 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. 5*D*) 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 essential Ag-presenting role B cells play in the development of nephritogenic 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-B7RP-1 interactions may also be important in the pathogenic response in human lupus nephritis. Data from Ab-blocking experiments in (NZB $\times$ NZW)F$_1$ mice (50) and our genetic analysis of *Icos*$^{-/-}$ MRL^{lpr} mice provide direct data that ICOS-B7RP-1 interactions contribute to the inflammatory process in lupus nephritis.

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Disclosures

The authors have no financial conflict of interest.

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