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FLI1 Levels Impact CXCR3 Expression and Renal Infiltration of T Cells and Renal Glycosphingolipid Metabolism in the MRL/lpr Lupus Mouse Strain

Kamala P. Sundararaj,* Thirumagal Thiagarajan,* Ivan Molano,* Fahmin Basher,† Thomas W. Powers,‡ Richard R. Drake,‡ and Tamara K. Nowling*

The ETS factor Friend leukemia virus integration 1 (FLI1) is a key modulator of lupus disease expression. Overexpressing FLI1 in healthy mice results in the development of an autoimmune kidney disease similar to that observed in lupus. Lowering the global levels of FLI1 in two lupus strains (Flit1+/−) significantly improved kidney disease and prolonged survival. T cells from MRL/lpr Flit1+/− lupus mice have reduced activation and IL-4 production, neuraminidase 1 expression, and the levels of the glycosphingolipid lactosylceramide. In this study, we demonstrate that MRL/lpr Flit1+/− mice have significantly decreased renal neuraminidase 1 and lactosylceramide levels. This corresponds with a significant decrease in the number of total CD3+ cells, as well as CD4+ and CD44+CD62L− T cell subsets in the kidney of MRL/lpr Flit1+/− mice compared with the Flit1+/− nephritic mice. We further demonstrate that the percentage of CXCR3+ T cells and Cxcr3 message levels in T cells are significantly decreased and correspond with a decrease in renal CXCR3+ cells and in Cxcl9 and Cxcl10 expression in the MRL/lpr Flit1+/− mice compared with the Flit1+/− nephritic mice. Our results suggest that the levels of FLI1 in MRL/lpr mice may be protective against development of nephritis in part through downregulation of CXCR3, reducing renal T cell infiltration and glycosphingolipid levels. The Journal of Immunology, 2015, 195: 5551–5560.

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Abbreviations used in this article: FLI1, Friend leukemia virus integration 1; FTICR, Fourier transform ion cyclotron resonance; GSL, glycosphingolipid; LacCer, lactosylceramide; NE, nuclear extract; Neu1, neuraminidase 1; PBST, PBS plus 0.05% Tween 20; P/R, promoter/reporter.

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their infiltration of the kidney and on renal GSL metabolism. Taken together, our results suggest that intrinsic FLI1 levels in T cells play an important role in their migration to the kidney in part by regulating the expression of CX3CR3 and renal chemokine expression. The decreased renal inflammatory infiltration and chemokine expression by reducing FLI1 likely reduces GSL metabolism. A more solid understanding of the role and mechanisms by which FLI1 mediates T cell function and renal disease in lupus will be invaluable in identifying pathways and molecules that may serve as potential targets for therapeutic intervention, as well as provide better insight of disease progression in general.

Materials and Methods

Mice

All animal experiments and methods of euthanasia were approved by the Ralph H. Johnson Veterans Affairs Medical Center Institutional Animal Care and Use Committee. Mice were housed and maintained under pathogen-free conditions at the Ralph H. Johnson Veterans Affairs Medical Center Animal Care Facility (Charleston, SC). MRL/lpr FLI1+/− and FLI1+/+ mice (6) and C57BL/6 FLI1+/− and FLI1+/+ mice (16) were obtained from the Jackson Laboratory (Bar Harbor, ME) were used for matings every generation to avoid any genetic drift in the colonies. Age-matched animals of both genders were used in experiments.

LacCer quantification and neuraminidase activity assays

LacCer was measured quantitatively by the Lipidomics Core at the Medical University of South Carolina using 2 mg kidney homogenates as described previously (15). The Amplex Red Neu assay kit (Invitrogen, Grand Island, NY) was used to measure Neu enzyme activity in 50 μg kidney homogenate following the manufacturer’s instructions (15) and is presented in arbitrary units.

MALDI–Fourier transform ioncyclotron resonance imaging of kidney sections

Direct profiling of LacCer expression in kidney tissue sections was performed using MALDI–Fourier transform ioncyclotron resonance (FTICR) imaging mass spectrometry, as we described previously (15).

Semiquantitative RT-PCR assays

RNA was prepared from isolated T cells or kidney using the RNaseasy kit (Qiagen, Hilden, Germany) following the manufacturer’s directions and cDNA generated using 0.5–1 μg RNA using the iScript cDNA synthesis kit (Bio-Rad and Hercules, CA). Real-time PCR was performed with the cDNA using the LightCycler 480 SYBR Green I Master kit and LightCycler 480 II (Roche, Indianapolis, IN). Primers used for real-time PCR include: Neul, forward, 5′-ACGATGTAAGACAGAGATGTC-3′, reverse, 5′-GTCGTCCTTACTCCAACAACT-3′; Cxcl9, forward, 5′-GTCATTGGAAGCTGAT-3′, reverse, 5′-CCCATCAATCGGAGAGGT-3′; Cxcl10, forward, 5′-GCCCTGAAGTCCGTCCTTTCC-3′, reverse, 5′-GGTTCCCTTCTCCCACTCTA-3′; Cxcl11, forward, 5′-GACGGTCCGCTGCAACTG-3′; Cxcl11, reverse, 5′-GGGTTCCTCGAACTCCAGG-3′, and β-actin, forward, 5′-AGGATGTGCTGTGGTCT-3′, reverse, 5′-GTCCTGCTTGGTACCTAC-3′. Relative message levels of Neul, Cxcl9, Cxcl10, and Cxcl11 were calculated using the ΔΔCT method. Relative expression after normalizing to β-actin or GAPDH was similar and the β-actin-normalized values were presented. The ΔΔCT from one MRL/lpr FLI1+/− mouse was set to one and all other mice compared with that mouse (n, number of animals analyzed).

Immunofluorescence and Immunohistochemistry

Frozen and OTC-embedded kidneys were sectioned at 5 μm. Sections were washed with PBS plus 0.05% Tween 20 (PBST) and blocked with 5% BSA in PBST. Sections were fixed with 10% phosphate-buffered formalin, washed with PBS plus 0.05% Tween 20 (PBST) and blocked with 5% BSA in PBST. Sections were washed with PBST/1% BSA and incubated with a rabbit anti-LacCer (Bioryt, San Francisco, CA) in PBST/5% BSA or rabbit anti-CXCR3 (Novus Biologicals, Littleton, CO). Anti-LacCer was detected with anti-rabbit FITC (SouthernBiotech, Birmingham, AL) and nuclei were detected by addition of DAPI. Anti-CXCR3 was detected with anti-rabbit HRP and nuclei were detected by addition of Gill’s hematoxylin. Images of sections were taken using a Nikon Eclipse 80i microscope, DS camera, and software (Nikon Instruments, Melville, NY) at the magnifications indicated.

T cell isolation

T cells were isolated using Pan T Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany) as described previously (14). This method removes the CD3+CD4+CD8+2B20+ (double-negative) T cell population that accumulates in the MRL/lpr strain. T cells were analyzed after isolation by flow cytometry and were 90–95% pure. Approximately 1–4% of the isolated T cells in the MRL/lpr strain were CD4+CD8+.

Flow cytometry

Kidneys were harvested from euthanized MRL/lpr (MRL Fli1+/− or MRL/lpr Fli1+/+ (MRL Fli1+/+) mice, rinsed in PBS, minced, and digested with collagenase, type I (Calbiochem, La Jolla, CA). Digested kidneys were passed over a 40-μm cell strainer washed in cold PBS and resuspended in PBS, 1% FBS, 0.1% sodium azide (flow buffer). Primary T cells were isolated from spleen, as described above, and resuspended in flow buffer. Cells were blocked with Fc receptor block, labeled with fluorophore-conjugated Abs, and analyzed by flow cytometry. Fluorophore-conjugated Abs included anti-mouse CD3, CD4, CD8, CD62L, and CD44 Abs (BD Biosciences, San Jose, CA). For the kidney analyses, cells were gated on CD3 and then analyzed for CD4 and CD8 staining or CD4 and CD62L staining. Cell counts represent the total number of cells expressing a marker as calculated by multiplying the percentage of cells expressing a specific marker by the number of total cells in the sample. Flow was initially performed on cells from perfused and nonperfused kidneys. There were no significant differences in the percentages of the T cell subsets obtained between perfused and nonperfused kidneys for CD3+ T cells; therefore, perfusion was not performed on subsequent analyses. Data from the perfused and nonperfused kidneys were averaged and are presented.

Western immunoblot

Whole-cell extracts were prepared by incubating cells with RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM DTT, 10 mM EDTA, 1 mM PMSE) plus 1 μl each of protease and phosphatase inhibitor mixes (Sigma-Aldrich, St. Louis, MO) for 1 hour at 4˚C. The pGL3 Renilla construct (Switchgear Genomics/Active Motif, Carlsbad, CA) contains a Renilla luciferase reporter element (Firefly luciferase). Whole-cell extracts were prepared by incubating cells with RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM DTT, 10 mM EDTA, 1 mM PMSE) plus 1 μl each of protease and phosphatase inhibitor mixes (Sigma-Aldrich, St. Louis, MO) for 1 hour at 4˚C. The pGL3 Renilla construct (Switchgear Genomics/Active Motif, Carlsbad, CA) contains a Renilla luciferase reporter element (Firefly luciferase).

Transfections

The murine MS1 endothelial cell and human Jurkat T cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained according to their recommendations. The mouse Neu promoter/reporter (P/R) construct containing the −435 to −11 region of the promoter (pGL3 mNeu −435) was described previously (14). The pSG hCXCR3 P/R construct (Switchgear Genomics/Active Motif, Carlsbad, CA) contains the human CXCR3 768-bp proximal promoter region driving Renilla expression. MS1 cells were seeded at 2 × 105 cells per well in a six-well plate in DMEM supplemented with 5% FBS the day before transfection. The pGL3 mNeu −435 or control (pGL3 basic) constructs (1 μg) were transfected into the MS1 cells with increasing amounts of the pcDNA3 flag-tagged FLI1 (Flag FLI1) expression vector (17) using FuGENE (Promega, Madison, WI). Jurkat cell transfections were performed as previously described (18) using 0.5 μg pSG hCXCR3 and increasing amounts of FLI1 expression vectors pSG5 FLI1 or pSG5 FLI1DDB (FLI1 DNA binding mutant) (12) or pcDNA3 Flag FLI1, as indicated in the figures. Cells were stimulated with 10 ng/ml PMA and 100 ng/ml ionomycin 4 h after transfection and cells were harvested 24 h after transfection. Total molar amount of DNA was kept constant from well to well as calculated by multiplying the percentage of cells expressing a specific marker by the number of total cells in the sample. Flow was initially performed on cells from perfused and nonperfused kidneys. There were no significant differences in the percentages of the T cell subsets obtained between perfused and nonperfused kidneys for CD3+ T cells; therefore, perfusion was not performed on subsequent analyses. Data from the perfused and nonperfused kidneys were averaged and are presented.

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EMSAs
EMSAs were performed essentially as described (17). Nuclear extracts (NE) were prepared from Jurkat T cells stimulated with 10 ng/ml PMA and 100 ng/ml ionomycin (Sigma-Aldrich) for 24 h. Probes, annealed Cy5.5-labeled oligonucleotides containing putative conserved ETS binding sites of the hCXCR3 promoter (shown in Fig. 6E), were incubated with 10 μg NE in binding buffer (10 mM HEPES, 10 mM Tris-HCl, 100 mM NaCl, 15 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 1 μg poly(dI:dC)). Specific binding was identified by preincubating the NE with 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 1 μg poly(dI:dC). EMSAs were synthesized by Integrated DNA Technologies (Coralville, IA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). All EMSAs were performed at least twice with two independent preparations of NE. Representative gels are shown.

Adoptive transfer of MRL/lpr T cells
T cells were isolated as described above from spleens of three 7- to 8-wk-old MRL/lpr Fli1+/+ and Fli1−/− mice, combined and resuspended in sterile 1× PBS. A total of 8 × 10⁶ cells were transferred by tail vein injection into 7- to 8-wk-old MRL/lpr Fli1+/+ and Fli1−/− mice. Three transfers were performed: group 1, four Fli1+/+ mice received Fli1+/+ T cells; group 2, four Fli1−/− mice received Fli1+/+ T cells; and group 3, eight Fli1−/− mice received Fli1−/− T cells. Urine and serum were collected prior to transfer and 2, 4, 6, 8, and 12 wk after transfer. Urine was collected during a period of 24 h using metabolic cages. Recipient mice were euthanized 12 wk after transfer (19–20 wk of age) and kidneys were collected.

Statistical analysis
Statistical analyses were performed using Prism software (GraphPad Software, La Jolla, CA) at a 95% confidence level. Real-time PCR differences were analyzed using two-way ANOVA adjusting for multiple comparisons (Sidak). Adjusted t values are presented and all error bars represent SD.

Results
Elevated renal LacCer levels in glomeruli of MRL/lpr Fli1+/+ mice are decreased in MRL/lpr Fli1−/− mice
MRL/lpr Fli1+/+ mice have FLI1 levels that are 50% of those in MRL/lpr Fli1−/− mice and have significantly improved disease (6). We recently demonstrated that renal and urine LacCer levels, Neu1 levels, and/or Neu1 activity are elevated in MRL/lpr (Fli1+/+) mice (16–18 wk of age) and human lupus patients with nephritis compared with nonnephritic lupus mice and patients, respectively, or compared with healthy controls (15). To determine whether renal LacCer levels in MRL/lpr Fli1+/+ mice, which have improved renal pathology and function, are reduced compared with Fli1+/+ mice, we measured LacCer levels in kidney homogenates from 16- to 18-wk-old MRL/lpr Fli1+/+ and Fli1−/− mice. The Fli1−/− mice tended to have decreased levels of each of the major LacCer species expressed in the kidney (C16, C22, C24, and C24:1) and a significant reduction in total LacCer levels compared with the Fli1+/+ mice (Fig. 1A). LacCer levels across the tissue were assessed on renal sections by MALDI-FTICR imaging. As we demonstrated previously (15), LacCer species C16, C24, and C24:1 were observed to be higher across the tissue in the MRL/lpr Fli1+/+ kidneys compared with healthy C57BL/6 kidneys, whereas the LacCer levels in the Fli1−/− kidneys appear to have intermediate levels, higher than the C57BL/6 and lower than the Fli1+/+ kidneys (Fig. 1B and data not shown).

In addition to changes in LacCer, the kidneys of MRL/lpr Fli1+/+ mice had significantly decreased Neu1 expression compared with Fli1+/+ mice at 16–18 wk of age (Fig. 2A). However, a significant decrease in Neu1 expression did not result in a significant decrease in Neu1 activity at either 11 wk (pre-early disease) or 18 wk (diseased) of age (Fig. 2B), which may be due to activity of the other Neu1s (Neu2, 3, and 4) that may be unaffected by FLI1. Because FLI1 can regulate Neu1 transcription in T cells (14), it may similarly regulate Neu1 in the kidney. Endothelial cells are the only renal cell type shown to express FLI1 in the kidney (8, 19). Therefore, we transfected the mouse endothelial cell line, MS1, with a Neu1 PR construct and increasing amounts of a FLI1 expression vector. Unlike in T cells, FLI1 had no effect on Neu1 promoter activity in MS1 endothelial cells (data not shown). Taken together, these results indicate that globally reducing FLI1 levels in MRL/lpr mice decreases renal Neu1 and LacCer levels, but likely not due to FLI1 regulation of Neu1 expression in the kidney. Intense LacCer staining is observed in the mesangial region of human lupus renal biopsies (12), and we demonstrate in the present study specific LacCer staining in the glomeruli of MRL/lpr mice (Fig. 1C). T cell infiltration was shown to occur predominantly in the glomerular region in MRL/lpr kidneys (20). Because MRL/lpr Fli1−/− mice exhibit reduced immune cell infiltration of the kidney, including T cells (10), reduced T cell infiltration may be one mechanism by which reducing FLI1 levels impacts GSL metabolism in the kidney.

CD4+ and activated T cells are significantly reduced in MRL/lpr Fli1−/− kidneys
To further examine the effects of FLI1 on T cell infiltration in the kidney, we quantified T cell subsets in the kidneys of MRL/lpr Fli1+/+ and Fli1−/− mice by flow cytometry. Abs to CD3, CD4, CD8, CD44, and CD62L were used to stain single-cell suspensions of kidneys from 14- to 16-wk-old MRL/lpr mice. The overall numbers of T cells (all CD3+ cells) were significantly decreased, as were the numbers and percentage of CD4+ T cells (Fig. 3). The percentage of CD3+ cells that were CD8+ or CD4+CD8− double-negative T cells were slightly increased in the Fli1+/+ mice compared with the Fli1−/− mice, but there were no differences in their overall numbers. The decrease in the percentage of CD4+ T cells and increase in percentage of CD8+ T cells in the Fli1+/+ kidneys is likely due to the decrease in the numbers of CD4+ T cells and not due to an increase in the number of CD8+ T cells. Moreover, both the number and percentage of activated CD3+ cells (CD44+CD62L−; Fig. 3) in the kidneys were significantly decreased in the Fli1−/− compared with the Fli1+/+ mice. These results indicate that reducing FLI1 levels in MRL/lpr mice specifically decreases the renal infiltration of CD4+ T cells, as well as decreases the number of activated T cells in the kidney.

CXCR3 expression in T cells and CXCR3 ligands in the kidney are significantly reduced in MRL/lpr Fli1−/−
We next analyzed whether FLI1 regulates expression of chemokine receptors as a possible mechanism by which FLI1 impacts T cell migration to the kidney. Flow analysis was performed on spleen cells isolated from 18-wk-old MRL/lpr mice to examine expression of various chemokine receptors. Of the chemokine receptors analyzed (CCR4, CCR6, CCR7, CXCR3, CXCR4, CXCR5), CXCR3 was the most abundant on both CD4+ and CD8+ populations (data not shown). We then performed flow cytometry on T cells isolated from mice negatively isolated from spleens of 11- and 18-wk-old Fli1+/+ and Fli1−/− mice. Between 11 and 18 wk of age the percentage of CXCR3+ T cells significantly increased (p = 0.0165) in the Fli1+/+ mice whereas no increase was observed in the Fli1−/− mice (Fig. 4A). The percentage of CXCR3+ T cells in the Fli1+/+ mice was significantly higher compared with Fli1−/− mice at 18 wk of age (p = 0.0132). Differences in the percentage of CXCR3+ T cells between Fli1+/+ and Fli1−/− mice at 18 wk of age were observed in both the CD4+ and CD8− subsets, with the differences in CD8− cells being significant (Fig. 4B).

We then analyzed renal sections and observed reduced CXCR3 staining in the MRL/lpr Fli1−/− compared with Fli1+/+ mice.
Of the CXCR3 ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), mRNA levels of Cxcl9 and Cxcl10 are more highly expressed than Cxcl11 in the kidneys of MRL/lpr mice (21, 22). Measures of renal message levels showed a significant decrease of Cxcl9 and Cxcl10 in the Fli1+/−/2 compared with Fli1+/+ mice at 18 wk of age (Fig. 5B). Taken together, these results suggest that decreased T cell infiltration of the kidney in MRL/lpr Fli1+/−/2 mice may be due in part to decreased CXCR3 expression in T cells and decreased renal expression of its ligands Cxcl9 and Cxcl10.

**FIGURE 1.** The level of LacCer is significantly reduced in MRL/lpr Fli1+/−/2 kidneys. (A) Individual species and total levels of the major LacCer species in the kidney were measured in renal cortices from 16- to 18-wk-old MRL/lpr Fli1+/−/2 and Fli1+/+ mice. (B) MALDI-TOF images of C16-LacCer expression in renal sections from 16- to 18-wk-old C57BL/6 and MRL/lpr Fli1+/−/2 and Fli1+/+ mice. Sections from three mice of each strain were analyzed with representative images presented. (C) Immunofluorescence for LacCer in renal sections from 16- to 18-wk-old MRL/lpr Fli1+/−/2 mice. Sections from three mice were analyzed with representative images presented.

(Fig. 5A). Of the CXCR3 ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), mRNA levels of Cxcl9 and Cxcl10 are more highly expressed than Cxcl11 in the kidneys of MRL/lpr mice (21, 22). Measures of renal message levels showed a significant decrease of Cxcl9 and Cxcl10 in the Fli1+/−/2 compared with Fli1+/+ mice at 18 wk of age (Fig. 5B). Taken together, these results suggest that decreased T cell infiltration of the kidney in MRL/lpr Fli1+/−/2 mice may be due in part to decreased CXCR3 expression in T cells and decreased renal expression of its ligands Cxcl9 and Cxcl10.

**FIGURE 2.** Neu1 expression is significantly reduced in MRL/lpr Fli1+/−/2 kidneys. (A) Neu1 expression measured by real-time PCR in reverse-transcribed RNA isolated from renal cortices of 18-wk-old MRL/lpr Fli1+/−/2 and Fli1+/+ mice. (B) Neu activity measured in extracts from renal cortices of 11- and 18-wk-old MRL/lpr Fli1+/−/2 and Fli1+/+ mice.
FLI1 regulates CXCR3 expression in T cells

Next, we determined whether FLI1 regulates CXCR3 in T cells. Cxcr3 message levels in T cells from the 16- to 18-wk-old mice were decreased in MRL/lpr Fli1+/− T cells compared with the Fli1+/+ T cells, with the differences approaching significance (Fig. 6A). Importantly, T cells with reduced levels of FLI1 from healthy, non-autoimmune-prone (C57BL/6 Fli1+/− ) mice had significantly decreased Cxcr3 message levels compared with T cells from Fli1+/+ mice (p = 0.0153; Fig. 6B), indicating that FLI1 may regulate Cxcr3 transcription. We then transfected the Jurkat T cell line with a human CXCR3 P/R (hCXCR3) construct and a FLI1 expression vector (pcDNA Flag FLI1). Both isoforms of FLI1 are expressed endogenously in Jurkat cells (p50 and p48), and the transfected Flag FLI1 migrates slower than the endogenous FLI1 due to the presence of the Flag epitope (Fig. 6C, blot). Exogenous FLI1 resulted in an increase in expression of the endogenous protein, which was expected based on prior observations that FLI1 regulates Cxcr3 transcription. We then transfected the Jurkat T cell line with a human CXCR3 P/R (hCXCR3) construct and a FLI1 expression vector (pcDNA Flag FLI1). Both isoforms of FLI1 are expressed endogenously in Jurkat cells (p50 and p48), and the transfected Flag FLI1 migrates slower than the endogenous FLI1 due to the presence of the Flag epitope (Fig. 6C, blot). Exogenous FLI1 resulted in an increase in expression of the endogenous protein, which was expected based on prior observations that FLI1 regulates Cxcr3 transcription. We then transfected the Jurkat T cell line with a human CXCR3 P/R (hCXCR3) construct and a FLI1 expression vector (pcDNA Flag FLI1). Both isoforms of FLI1 are expressed endogenously in Jurkat cells (p50 and p48), and the transfected Flag FLI1 migrates slower than the endogenous FLI1 due to the presence of the Flag epitope (Fig. 6C, blot). Exogenous FLI1 resulted in an increase in expression of the endogenous protein, which was expected based on prior observations that FLI1 regulates Cxcr3 transcription.

The hCXCR3 promoter activity increased in response to Flag FLI1 in a dose-dependent manner (Fig. 6C). This effect of FLI1 may be the result of direct binding to the hCXCR3 promoter, as transfection with an expression vector for an FLI1 DNA–binding mutant (pSG5 FLI1 DBM) failed to activate the hCXCR3 promoter compared with wild-type FLI1 (pSG5 FLI1) (Fig. 6D). The pSG5 (SV40 driven) vector does not express as highly as the pcDNA (CMV driven) vector, accounting for the relative fold differences in hCXCR3 promoter activation by wild-type FLI1 in Fig. 6C and 6D (compare 1.6 μg pcDNA Flag FLI1 to 1.6 μg pSG5 FLI1).

To further determine whether FLI1 binds directly to the hCXCR3 promoter, EMSAs were performed. Putative ETS binding sites, identified by the core binding sequence (GGAA) in the human CXCR3 proximal promoter sequence, were chosen for EMSA analyses based on conservation with the mouse sequence. Three highly conserved putative ETS binding sites were identified (a1, a2, and c2) (Fig. 6E). Site a2 has the highest homology with the canonical FLI1 binding site ACCGGAA(G/A)(T/C) (23). Probes encompassing a1, a2, and c2 (underlined in the sequence in Fig. 6E) were incubated with NE prepared from stimulated Jurkat T cells. Specific binding was demonstrated by addition of excess cold-specific sequences compared with cold nonspecific (scrambled) sequences (data not shown). Although we identified bands that were a result of specific binding, neither FLI1 nor ETS1 was observed to bind these probes following addition of anti-FLI1, anti-ETS1, or normal rabbit IgG Abs (Fig. 6E). Overexpressed FLI1 in NE from Flag FLI1–transfected Jurkat cells (as in Fig. 6C) also failed to bind to these probes (data not shown). These results suggest that although FLI1 requires its DNA binding domain to fully activate the hCXCR3 promoter, FLI1 may regulate hCXCR3 from a less conserved and/or noncanonical FLI1/ETS binding site or by indirect regulation.

FLI1 levels in transferred T cells impact renal T cell infiltration in recipients

The contribution of FLI1 levels in MRL/lpr T cells to T cell migration to the kidney and progression of nephritis was analyzed in an adoptive transfer experiment. To avoid extrinsic effects of disease on T cell function prior to transfer, CD3+ T cells were negatively isolated from spleens of MRL/lpr Fli1+/− and Fli1−/− mice at 7–8 wk of age (nonnephritic) and transferred...
into 7- to 8-wk-old MRL/lpr Fli1+/+ and Fli1+/- mice. Control groups included Fli1+/+ mice receiving Fli1+/- T cells and Fli1+/- mice receiving Fli1+/- T cells, with the experimental group being Fli1+/- mice that received Fli1+/+ T cells. Urine was collected prior to transfer and every two weeks after transfer for a total of 12 wk. Kidneys were examined at the endpoint. All of the Fli1+/- mice that received Fli1+/- T cells had 5- to 10-fold more renal CD4+ T cells compared with the Fli1+/- mice that received Fli1+/- T cells (Fig. 7A, 7B). Interestingly, the number of CD4+ T cells in the kidneys of all the Fli1+/- mice that received Fli1+/+ T cells reflected the T cell numbers in the Fli1+/- to Fli1+/- transfer (Fig. 7A, 7B), suggesting that intrinsic levels of FLI1 in T cells impact T cell migration to the kidney independent of extrinsic levels of FLI1.

No differences were observed in IgG or C3 deposition between any of the transfer groups (data not shown). This was not unexpected, as significant differences in renal IgG and C3 deposition were not observed between MRL/lpr Fli1+/- and Fli1+/- mice previously (6). We measured proteinuria to determine whether the transfer of Fli1+/- T cells had an effect on renal disease in Fli1+/- mice. Although proteinuria steadily increased during the 12 wk of the study in the Fli1+/- mice that received Fli1+/- T cells and remained nearly undetectable in the Fli1+/- mice that received Fli1+/- T cells as expected, only two of the recipients in the experimental group had an increase similar to those observed in the Fli1+/- to Fli1+/- group (Fig. 7C). No significant differences in renal pathology between the Fli1+/- group that received Fli1+/- T cells and the control Fli1+/- group that received Fli1+/- T cells were observed (data not shown). Taken together, these results suggest that intrinsic FLI1 levels are important in promoting T cell migration to the kidney in MRL/lpr mice. However, a one-time transfer of Fli1+/- T cells likely was not sufficient to exacerbate nephritis in Fli1+/- mice or T cell–extrinsic effects of FLI1 also are important in disease progression.

**FIGURE 4.** The percentages of CXCR3+ T cells are significantly decreased in 18-wk-old MRL/lpr Fli1+/- and Fli1+/- mice. (A) Percentages of CXCR3+ cells were quantified by flow cytometry in T cells negatively isolated from spleens of 11- and 18-wk-old MRL/lpr Fli1+/- and Fli1+/- mice (n = 4–6 for each group). Example of flow data are shown to the right of the graph. (B) Percentages of CD4+ and CD8+ T cells that are CXCR3+ in the 18-wk-old samples analyzed in (A).

**FIGURE 5.** Renal CXCR3+ cells are reduced and expression of CXCR3 ligands Cxcl9 and Cxcl10 is significantly decreased in MRL/lpr Fli1+/- mice. (A) Renal sections from 16- to 18-wk-old MRL/lpr Fli1+/- and Fli1+/- mice were analyzed for CXCR3+ cells by immunohistochemistry. CXCR3 was detected with HRP (brown) and nuclei were stained with hematoxylin (purple). Negative control (Neg Control) is secondary only. Original magnification ×40. Sections are representative of three mice analyzed from each strain. (B) Cxcl9 and Cxcl10 message levels were measured by real-time PCR in reverse-transcribed RNA isolated from renal cortices of 18-wk-old MRL/lpr Fli1+/- and Fli1+/- mice.
Discussion

MRL/lpr mice that have reduced levels of FLI1 (Fli1+/-) have improved disease and survive significantly longer than do Fli1+/+ mice (6). FLI1 expression is highly expressed in the adult spleen, including mature T and B cells. There are no differences in lymphadenopathy or the percentages of CD4+, CD8+, or memory/activated (CD44+CD62L2) T cells in the spleens of nephritic MRL/lpr Fli1+/+ mice compared with MRL/lpr Fli1+/- mice, yet improved renal disease in the Fli1+/- mice is characterized by decreased infiltration of immune cells in the kidney (6). Moreover, MRL/lpr Fli1+/- T cells show reduced migration in vitro compared with wild-type MRL/lpr Fli1+/+ mice (10). In the present study, we show that MRL/lpr Fli1+/- mice have significantly decreased numbers of CD3+ cells, specifically CD4+ and activated/memory (CD44+CD62L2) CD3+ subsets, in the kidney compared with MRL/lpr Fli1+/- mice. Previously, we demonstrated that Fli1 levels in MRL/lpr T cells do not have an effect on apoptosis (14). Taken together, these results support a role for FLI1 in T cell activation/migration rather than on T cell development, survival, and/or differentiation.

FLI1 is expressed in kidney, but at 14-fold lower levels compared with spleen at the message level (24, 25), and it is only detected in endothelial cells of the kidney (8, 19). MRL/lpr Fli1+/+ immune cells, including T cells, do not readily migrate to the kidney when transferred into MRL/lpr Fli1+/- mice (10). Chemokines Ccl2 (Mcp-1), Ccl3 (Mip-1a), Ccl4 (Mip-1b), and Ccl5 (Rantes) are significantly decreased at the message level in the kidneys of MRL/lpr Fli1+/- compared with the Fli1+/+ mice, and FLI1 was demonstrated to regulate the promoter activity of several different cytokine genes in endothelial cells (8, 9, 12, 13). In this study, we demonstrated that the expression Cxcl9 and Cxcl10, CXCR3 ligands, also is significantly decreased in the kidneys of MRL/lpr Fli1+/- compared with Fli1+/+ mice. These results suggest that FLI1 regulation of chemokine levels in renal endothelial cells mediates immune cell infiltration to the kidney. However, MRL/lpr Fli1+/- immune cells show decreased migration in vitro toward...
CCL2 and CCL5 (10), suggesting that intrinsic FLI1 levels also impact immune cell migration. The mechanisms responsible for this effect of FLI1 are unknown, as the percentages of CD3+ cells expressing receptors for CCL2 and CCL5 were not significantly decreased in the Fli1+/− mice (10).

To identify possible mechanisms by which FLI1 intrinsically mediates T cell migration, we analyzed effects of FLI1 levels on chemokine receptors. We demonstrated that the percentage of CXCR3-expressing CD3+ T cells in the spleen of MRL/lpr mice significantly increases from 29% at 11 wk of age to ~40% at 18 wk of age. This increase was not observed in MRL/lpr Fli1+/− mice. The significantly reduced numbers of CD3+CD4+ and CD3+CD44+CD62L+ T cells in the kidney coincided with significantly reduced numbers of CXCR3+ T cells in the spleen and fewer CXCR3+ cells infiltrating the kidney in the Fli1+/− mice. The percentages of both CD4+CXCR3+ and CD8+CXCR3+ T cells were decreased in the spleens of Fli1+/− compared with the Fli1+/+ mice, but only the percentage of CD8+CXCR3+ T cells was significantly decreased. The greater percentage (and number) of CD4+ than CD8+ cells in the kidney may account for the fewer CD4+CXCR3+ cells (~18%) compared with CD8+CXCR3+ cells (~67%) in the spleen of Fli1+/− mice if CD4+CXCR3+ cells migrate more readily to the kidney than do CD8+CXCR3+ cells. This also may explain the lack of a significant decrease in CD4+CXCR3+ cells in the spleens of Fli1+/− compared with Fli1+/+ mice. Future analyses to measure the number of CD4+ versus CD8+ that are CXCR3+ in the kidney should address this question.

Alternatively, additional FLI1-regulated chemokine receptors or pathways may play a role in the migration of CD8+ versus CD4+ T cells to the kidney in MRL/lpr mice.

CXCR3 is important for T cell migration (26–28) and is implicated as an important molecule in the progression of lupus nephritis. The CXCR3 ligand CXCL10 is one of the first chemokines expressed in the kidney of MRL/lpr lupus mice (22). CXCL10 serum levels are elevated in lupus patients, renal infiltrating CD4+CXCR3+ T cells are abundant, and CXCR3 mRNA is detected in the urine of lupus patients with nephritis (30, 31). CXCR3 clearly plays an important role in mouse and human lupus, and our results indicate that FLI1 plays a role in regulating CXCR3 expression in T cells. CXCR3 ligands are potent stimulators of Ca²⁺ flux in activated naïve CD4+ and CD8+ T cells (26). We previously demonstrated that MRL/lpr Fli1+/− T cells had significantly decreased activation as measured by Ca²⁺ flux when stimulated through the TCR (14). As CXCR3 and CD3ε are spatially associated in the plasma membrane and exhibit signaling synergy (32, 33), it is possible that FLI1 plays a role in disrupting TCR signaling and Ca²⁺ flux by downregulating CXCR3 transcription in T cells to reduce T cell activation and migration in response to CXCR3 ligands.

Data presented in this study indicate that FLI1 may directly regulate Cxcr3 transcription. Cxcr3 message levels in Fli1+/− T cells were significantly decreased compared with Fli1+/+ T cells, and hCXCR3 promoter activity in Jurkat T cells was dose-dependently increased by FLI1 and required its DNA-binding domain. However, in vitro binding analyses of the most highly conserved ETS binding sites did not appear to bind FLI1. It is possible that FLI1 regulates Cxcr3 promoter activity through a nonconserved (such as a3, b1, or b2 shown in Fig. 6E sequence) and/or noncanonical ETS binding site that remains to be identified. The putative ETS sites in hCXCR3 tested for FLI1 binding were chosen based on numerous studies, including some from our laboratory, demonstrating that FLI1 binds and acts through conserved canonical ETS sites that are more proximal to the transcription start site (9, 12, 17, 34–41). However, we recently demonstrated that FLI1 also can regulate promoters through more distal sites (12, 13). Alternatively, FLI1 may indirectly regulate Cxcr3 transcription. We think that this is less likely because FLI1 activation of the hCXCR3 promoter required its DNA binding domain. Additional studies to identify the Cxcr3 promoter regions and specific sequences that are required for FLI1 activation are ongoing.

Previous adoptive transfer studies demonstrated that migration of MRL/lpr CD3+ cells to the kidney was reduced when either the donor CD3+ cells were Fli1+/− or the recipient animal was Fli1+/− (10). However, this study transferred total spleen cells from 18- to 24-wk-old MRL/lpr (active disease state for Fli1+/− mice) and examined infiltration 18 h after transfer. Thus, extrinsic effects of
disease on the T cells prior to transfer or effects of the other spleen cell types on the cotransferred T cells could have influenced their migration, and long-term effects were not examined. The adoptive transfer approach in this study was designed to avoid extrinsic effects of overt disease on the T cells prior to transfer by transferring T cells from prenephritic 7- to 8-wk-old donors into age-matched recipients and to examine effects over a longer period (12 wk). This experiment demonstrated that the number of T cells present in the kidney of Fli1+/- mice that received Fli1+/- mice was similar to Fli1+/- mice that received Fli1+/- T cells, further supporting an intrinsic role of FL11 on lupus T cell migration to the kidney. Only two of the Fli1+/- recipients of Fli1+/- T cells had significant increases in proteinuria compared with Fli1+/- recipients of Fli1+/- T cells. It is likely that a significant effect on proteinuria may not result from a one-time transfer. Alternatively, the continued presence of the recipient Fli1+/- T cells and/or the reduced chemokine expression in the kidney may provide a protective effect against the more pathogenic Fli1+/- T cells.

We recently demonstrated that MRL/lpr mice and human lupus patients with nephritis have significantly altered renal GSL levels compared with lupus counterparts without nephritis and healthy controls, including increased levels of renal and/or urine LacCer and Neu1 (15). Interestingly, we demonstrated in this study that MRL/lpr Fli1+/- mice have significantly decreased total LacCer levels and Neu1 expression levels compared with Fli1+/- mice. FL11 had no effect on Neu1 promoter activity in an endothelial cell line, suggesting that the decrease in Neu1 expression in the Fli1+/- kidney is not likely due to FL11 regulation of Neu1 in renal endothelial cells. Neu1 activity tended to be lower, but it was not significantly decreased in the Fli1+/- kidney. There are three other NEUs that are expressed in the kidney (Neu2, 3, and 4), and FL11 may not have an effect on the expression of the other NEUs. Therefore, the lack of a significant decrease in renal NEU activity, despite a significant decrease in Neu1 message, may be due to continued activity and/or compensation by the other NEUs. Renal LacCer elevation is observed at roughly the same time as major inflammatory infiltration in the MRL/lpr kidney (15). Based on our previous studies demonstrating that Fli1+/- T cells have decreased levels of LacCer and Neu1 and that FL11 can regulate Neu1 promoter activity in T cells (14), the decrease in renal infiltrating T cells in the Fli1+/- mice may explain, in part, the decrease in renal LacCer and Neu1 levels. Alternatively, or additionally, the decrease in renal GSL metabolism may be due to decreased local LacCer and Neu1 expression in response to decreased immune cell infiltration and cytokine expression. Studies aimed at identifying whether LacCer levels are decreased locally in renal cells and/or are due to decreased numbers of infiltrating Neu1/LacCer-expressing T cells in Fli1+/- are currently being pursued.

In summary, previous studies and the data presented in this study suggest that FL11 regulation of renal chemokine expression and T cell chemokine receptor expression both play an important role in reducing the number of renal-infiltrating T cells and other inflammatory cells to protect against and/or slow disease progression. FL11 likely mediates disease expression, in part, through direct regulation of CXCR3 to reduce T cell activation and migration and in part through downregulation of CXCR3 ligands Cxcl9 and Cxcl10 in the kidney. Additionally, reduced renal GSL metabolism in MRL/lpr Fli1+/- mice may be due to reduced numbers of T cells expressing LacCer/Neu1 and/or to reduced local LacCer/Neu1 expression in response to reduced T cell infiltration. Taken together, these results support a role for FL11 in mediating both intrinsic (Cxcr3 expression) and extrinsic (renal Cxcl9 and Cxcl10 expression) effects on T cell migration and disease expression (GSL metabolism). Further elucidation of the T cell–intrinsic and –extrinsic mechanisms will be important in fully understanding how FL11 mediates disease progression and identifying potential molecules and/or pathways as therapeutic targets.

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References


