Fractalkine expression and CD16⁺ monocyte accumulation in glomerular lesions: association with their severity and diversity in lupus models

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¹First Department of Internal Medicine, Nara Medical University, Kashihara, Nara; and ²Division of Pathogenomics, Department of Pathology, and ³Department of Bioregulatory Medicine, Ehime University Graduate School of Medicine, Toon, Ehime, Japan

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Nakatani K, Yoshimoto S, Iwano M, Asai O, Samejima K, Sakan H, Terada M, Hasegawa H, Nose M, Saito Y. Fractalkine expression and CD16⁺ monocyte accumulation in glomerular lesions: association with their severity and diversity in lupus models. Am J Physiol Renal Physiol 299: F207–F216, 2010. First published April 21, 2010; doi:10.1152/ajprenal.00482.2009.—Fractalkine (Fkn) is expressed on injured endothelial cells and is a membrane-bound chemokine that attracts cells expressing its receptor, CX3CR1, including CD16⁺ monocytes (CD16⁺ Mos). To clarify the role played by Fkn in the development of glomerular lesions in lupus nephritis, we examined Fkn expression and CD16⁺ Mo accumulation induced in experimental C.B-17/Inc-scid/scid (SCID) lupus model mice by injection of IgG₁-producing hybridoma clones obtained from MRL/lpr mice. Glomerular Fkn expression and accumulation of CD16⁺ Mos were semiquantitatively evaluated using laser capture microdissection and real-time PCR. Injection of the 2B11.3 and 7B6.8 clones induced formation of glomerular proliferative and wire-loop lesions, respectively. Immunohistological analysis of the localization of Fkn and CD16⁺ Mos revealed that Fkn expression and CD16⁺ Mo accumulation were markedly elevated in glomerular lesions induced by 2B11.3, whereas no elevation was detected in those induced by 7B6.8. In addition, to examine the contribution of glomerular Fkn to the development of proliferative lesions, L cells producing an Fkn antagonist (Fkn-AT) were transplanted into SCID mice exhibiting proliferative lupus nephritis (DPLN). Taken together, our findings suggest that Fkn and CD16⁺ Mo accumulation are partially associated with the severity and diversity of histology of lupus nephritis.

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the deposition of immune complexes (ICs) and autoantibodies leads to the activation of complement systems and subsequent inflammation (17). IC-induced glomerulonephritis in SLE is termed lupus nephritis and is a common complication in SLE (5). Similarly, MRL/lpr mice spontaneously develop a lethal glomerulonephritis with an increase in circulating ICs, autoantibody production, and cytokine abnormalities (3, 20). The glomerular lesions seen in MRL/lpr mice consist of diffuse cell proliferative and/or wire-loop-like lesions that resemble the various histological patterns observed in human lupus nephritis (3, 20). These lesions are characterized by the deposition of ICs and are associated with increases in serum levels of rheumatoid factors and anti-DNA and anti-glycoprotein 70 autoantibodies, which are thought to play major roles in the histopathogenesis of lupus nephritis (6, 16, 22).

The membrane-spanning protein fractalkine (Fkn) is a member of the CX3C family of chemokines and contains a conserved CX3C domain atop a mucin-like stalk in its extracellular region (4). Fkn is expressed at very low levels by resting endothelial cells but undergoes marked upregulation following stimulation of the cells by cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (4). Fkn is also known to function as a cellular adhesion molecule and to attract cells expressing its receptor, CX3CR1 (4, 12). The Fkn-CX3CR1 interaction has been implicated in the pathogenesis of several renal diseases, including lupus nephritis, acting through the accumulation and/or activation of CX3CR1-positive cells. For instance, proinflammatory CD16⁺-positive monocytes (CD16⁺ Mos) express CX3CR1 and appear to preferentially recruit to vessel walls through the chemoattractant property of Fkn (1, 2). Moreover, we previously showed that the expression of Fkn and accumulation of CD16⁺ Mos within glomeruli are enhanced in human proliferative lupus nephritis, suggesting the interaction between CD16⁺ Mos and Fkn-expressing cells, and plays an important role in the pathogenesis of human lupus nephritis (26).

We also previously found that IgG₃ production plays a critical role in the development of glomerulonephritis in MRL/lpr mice (25), which prompted us to develop two nephritogenic IgG₁-producing hybridoma clones, 7B6.8 and 2B11.3, from unmanipulated MRL/lpr mice. These clones respectively induce two different types of glomerular lesions when injected in C.B-17/Inc-scid/scid (SCID) lupus model mice: a wire-loop-like lesion and an endocapillary proliferative lesion (14). In the present study, we investigated the levels of Fkn expression and CD16⁺ Mos accumulation in glomerular lesions induced by clones 2B11.3 and 7B6.8 to assess the role played by Fkn in the generation of glomerular lesions in lupus nephritis. We also established a system for producing an Fkn antagonist (Fkn-AT) in vivo by transplanting L cells expressing the antagonist in mice. We then used this system to evaluate the contribution made by Fkn to the development of lupus nephritis in vivo.

Materials and Methods

Mice. MRL/lpr and MRL/Mp⁺/⁺ (MRL/+⁺) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME); C.B-17/Inc-scid/scid (SCID) mice were from Japan Clea (Tokyo, Japan). All mice were housed under conditions free of specific pathogens in the Animal Research Institute of Nara Medical University. All procedures involving mice were carried out in accordance with the National...
Institutes of Health guidelines for the care use of live animals and were approved by the Nara Medical University Animal Care Committee.

Hybridoma clones. IgG3 antibody-producing hybridoma clones, 2B11.3, 7B6.8, and 1G3, derived from an unmanipulated MRL/lpr mouse were used in this study (14). When injected in SCID mice, the 2B11.3 and 7B6.8 clones induced the endocapillary proliferative and wire-loop types of glomerular lesions, respectively. The 1G3 clone does not induce any glomerular injury (14).

Injections of hybridomas. Hybridoma clones (1 × 10⁷ cells) were intraperitoneally injected in SCID mice. In our earlier study, SCID mice injected with either the 2B11.3 or 7B6.8 clone developed significant glomerular lesions after 15–25 days (21). In the present study, therefore, serum samples and kidneys were collected from mice under ether anesthesia 15–25 days after the injection. We also killed mice under ether anesthesia 10–15 days after the injection to collect serum samples and kidneys at a predisease stage characterized by the deposition of IgG3 along the glomerular capillary walls without development of histopathological glomerular lesions.

Histopathological and immunohistochemical examinations. For histopathology, tissue samples were fixed with 10% formalin in 0.01 mol/l phosphate buffer (pH 7.2) and embedded in paraffin. They were then stained with hematoxylin and eosin (H&E) or periodic acid–Schiff for examination under a light microscope. The glomerular lesions in SCID mice injected with 2B11.3 clones were graded from zero to three, based on the severity of the glomerular cell proliferation and inflammatory cells infiltration; grade 0, normal; grade 1, mild; grade 2, moderate; and grade 3, severe. Index of glomerular lesions means the average grading of 20–30 glomeruli. For immunostaining, pieces of kidney were frozen in 22-oxacalcitriol compound (Miles, South Bend, Elkhart, IN), after which frozen sections were cut and fixed with cold acetone, and endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide for 15 min. Thereafter, the tissue sections were incubated with biotin-labeled goat anti-murine Fkn polyclonal antibody (R&D Systems, Minneapolis, MN) for 2 h at room temperature, and Fkn expression was detected using an avidin-biotin-peroxidase system (LSAB+ kit; Dako, Carpentaria, CA). Pre-immune biotin-labeled goat serum served as a negative control. The phenotype of the infiltrating cells was also analyzed in frozen sections using the avidin-biotin-peroxidase method with a rat anti-mouse CD16 monoclonal antibody (Pharmingen, San Diego, CA). For staining IgG3, fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG3 antibodies (ICN ImmunoBiologicals, Lisle, IL) were used.

Tissue preparation and laser-capture microdissection. Frozen kidney sections were cut transversely into 7- to 10-μm sections using a cryostat, mounted on uncoated glass slides, and stored at −80°C until use. Tissue dehydration and staining were then accomplished using Histogen laser-capture microdissection (LCM) frozen-section staining reagents as instructed by the manufacturer (Arcturus Engineering, Mountain View, CA) (19, 24). Briefly, sections were fixed and sequentially dehydrated in 75%, 95%, and 100% ethanol followed by 100% xylene. The sections were then air-dried and stored in a desiccator at room temperature until used for LCM; care was taken to ensure that LCM was completed within 1 h after the slides were placed in the desiccator. Two or three sections from each sample were stained with H&E to verify the quality of the sections. LCM was then carried out using a PixCell II LCM System (Acturus Engineering). Targeted glomeruli were selectively captured from each section, and a total of 50–100 glomeruli were obtained from each specimen (typically from 6–8 slides). Targeted glomeruli contained lesions characterized by 1) pure mesangial proliferation in 4-wk-old MRL/lpr mice (Fig. 1A); 2) endocapillary proliferation with inflammatory cell infiltration and segmental wire-loop-like lesions in 20-wk-old MRL/lpr mice (Fig. 1B); 3) pure endocapillary proliferation with inflammatory cell infiltration in SCID mice injected with the 2B11.3 clone (see Fig. 3A); 4) little inflammatory cell infiltration, despite IgG3 deposition in mesangial areas and along the capillary walls in SCID mice injected with 2B11.3 clone at the predisease stage (see Fig. 5A); and 5) hyaline deposits along capillary walls and thrombi composed in part of hyaline within capillaries of SCID mice injected with 7B6.8 clone (see Fig. 3B).

RNA isolation and real-time PCR assay. Total RNA preparations were produced from 50–100 glomeruli obtained using LCM with a Pico pure RNA isolation kit (Arcturus Engineering) (19). Total RNA was extracted from the captured cells by incubating the LCM caps in extraction buffer for 30 min at 42°C, after which the RNA was purified using preconditioned MiraCol (Arcturus Engineering) purification columns. Total RNA was also extracted from primary cultured cells using TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer’s procedure. First-strand cDNA was then made from the total RNA using a SuperScript Preamplification System (Invitrogen, Carlsbad, CA) with random hexamers. For real-time PCR, 1 μl of each first-strand reaction product was amplification using appropriate primers and corresponding fluorescent probes designed by the Applied Biosystems ‘‘Assay-on-Demand’’ service for mouse and human Fkn (assay ID: Mm00436454_m1, Hs00171086_ml) and mouse and human β-actin (assay ID: Mm00607939_m1, Hs00239933_g1). Fkn-to-β-actin mRNA ratios were calculated for each sample.

Culture of human umbilical vein endothelial cells and primary murine mesangial cells with TNF-α and IL-1β. Human umbilical vein endothelial cells (HUVECs) and their culture medium were from Clonetics (Walkersville, MD). The cells were grown at 37°C on gelatin (Sigma Chemical)-coated flasks (Costar, New York, NY). Primary cultures of murine mesangial cells (MCs) were obtained from outgrowths of isolated mouse renal glomeruli, as described previously (15). The cells were grown at 37°C on plastic petri dishes (Nunc, Roskilde, Denmark) in RPMI 1640 medium containing 20% FCS. Once the cells were semiconfluent, they were passaged after trypsinization. HUVECs and MCs at passages 3–8 were grown to confluence in six-well culture plates and then incubated for 6 h in fresh serum-free RPMI containing TNF-α (Wako Pure Chemical Industries, Osaka, Japan) and IL-1β (Wako Pure Chemical Industries), as induced in the individual experiments.

Construction of expression vectors encoding murine NH2-terminal truncated Fkn (Fkn-AT). We used the following specific primers for murine Fkn (23) to amplify fragments containing functional Fkn coding regions (Fkn analog): 5′-ACTCCAGCATGGTCTCCTCCTG-3′ (forward) and 5′-CTCTACTGTTGACCATTTTATGAGG-3′ (reverse). The reverse primer contained the stop codon. Total cellular RNAs were extracted from murine spleen cells, with or without phytohemagglutinin stimulation, as described previously (10). The aforementioned fragments were then amplified by RT-PCR using an RNA PCR kit (Takara Shuzo, Kyoto, Japan) as described previously (10). Forward primers for an NH2-terminal truncated Fkn analog were prepared using 21-base oligonucleotide sequences corresponding to a truncated Fkn analog in which four amino acid residues were removed from the NH2-terminus (Fkn-AT). To convert the truncated analogs into their secretory forms, a 67-bp oligonucleotide containing the signal sequence from human interferon-β (18) (5′-CATGACCAAACAGTGTCCTCCTACATT-GCTCTCCGTGTGGCTCCTCCACTACGCTTCCCATG-3′) was added to the beginning of each forward primer. The amplified fragments were then cloned into the Eco RI site of the pCXN2 vector.

Cell transfection and selection of Fkn-AT transfectants. Mouse L cells (mouse fibroblastic cells (L292) at passages 10–15, purchased from the ATCC) were transfected with 10 μg of pCXN2 vector carrying the Fkn truncated analog gene (Fkn-AT) using Lipofectamine (Life Technologies) as described previously (11). Clones showing the strongest expression of this gene were then selected for further study. Mouse L cells were also transfected with 10 μg of an empty pCXN2 vector, as a control.

Chemotaxis assay. Chemotaxis by murine spleen cells was assayed in Transwell culture chambers with polycarbonate membranes (6.5-mm diameter, 5-μm pore size) (Costar, Cambridge, MA), as
described previously (10). Aliquots (100 μl) of cells (5 × 10⁶/ml) suspended in RPMI 1640–0.5% BSA were added to the upper wells while murine Fkn was added to the lower wells to a final concentration of 5 μg/ml. The cells were then allowed to migrate for 2 h at 37°C in a 5% CO₂ incubator, after which the filters were fixed with 1% glutaraldehyde in PBS for 30 min and stained with 0.5% toluidine blue overnight. Cell migration was quantified by counting the cells in the lower chamber and those adhering to the underside of the polycarbonate filter. In blocking assays, the cells were pretreated with 25 or 50 ng/ml Fkn-AT derived from conditioned medium from L cells transfected with pCXN2 vector carrying the Fkn-AT for 30 min at 37°C before their addition to the upper chambers. As a control, the cells were pretreated with an equivalent volume of conditioned medium from L cells transfected with an empty pCXN2 vector for 30 min at 37°C before their addition to the upper chambers. Each assay was performed in triplicate. Fkn-AT/control pretreated cell migration count ratios were calculated in each concentration of Fkn-AT.

Injection of transfected cells and hybridomas. Transfected L cells (1 × 10⁷ cells) were subcutaneously injected in SCID mice, and 5 days later a hybridoma clone (1 × 10⁷ cells) was injected intraperitoneally. On day 25 after the first injection, serum samples were collected from the mice under ether anesthesia, and the kidneys, heart, lungs, liver, pancreas, and salivary glands were removed for histopathological examination.

Measurement of Fkn-AT and serum IgG3, blood urea nitrogen, and albumin using ELISAs. To measure levels of Fkn-AT in culture supernatants conditioned by transfected L cells and in serum of SCID mice injected of transfected L cells, we prepared 96-well Maxisorp plates (Nalge Nunc) containing goat anti-murine Fkn polyclonal antibody. As described previously, the Fkn-AT concentrations in the

Fig. 1. Representative photomicrographs showing immunohistochemical staining for fractalkine (Fkn) and the presence of CD16⁺ monocytes (CD16⁺ Mos) within glomerular lesions in MRL/lpr mice and MRL/Mp⁺/⁻ (MRL/+⁻) mice. Periodic acid-Schiff (PAS) staining of glomerular lesions in 4-wk-old MRL/lpr mice (A), 20-wk-old MRL/lpr mice (B), and 20-wk-old MRL/+ mice (C) (magnification, ×400). Fkn (D–F) and CD16 (G–I) staining in glomerular lesions in 4-wk-old MRL/lpr mice (D and G), 20-wk-old MRL/lpr mice (E and H), and 20-wk-old MRL/+ mice (F and I).

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serum of Fkn-AT-expressing mice were corrected by subtracting the value obtained in control mice (9). The concentration of IgG3 in serum was determined using a mouse IgG3 ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX). The concentration of blood urea nitrogen (BUN) and albumin in serum was determined by the Bromcresol green method (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis. Numerical results were expressed as means ± SD. Student’s paired t-test was used for normally distributed variables. When comparing groups, one-way ANOVA was used, followed by post hoc t-test with Fisher’s protected least significant difference adjustment. For variables having a skewed distribution, we used Kruskal-Wallis ANOVA by ranks, with Bonferroni’s adjustment. Values of P < 0.05 were considered significant.

RESULTS

Fkn mRNA expression and CD16⁺ Mos accumulation in the glomeruli of MRL/lpr mice. We found that MRL/lpr mice exhibit progressively worsening renal damage that first became noticeable when the mice were about 12 wk old and that the glomerular lesions exhibited regular variation with progression of the disease. Immunohistochemical and electron microscopic analyses revealed that the lesions were initiated by Ig deposition in the subendothelial, subepithelial, and mesangial regions, which was followed by segmental mesangial proliferation. At age 4 wk, glomeruli remained nearly intact or showed slight mesangial proliferation. By 20 wk, however, endocapillary proliferative lesions characteristic of severe inflammatory cell infiltration had formed, causing the glomeruli to assume a lobular structure that was associated with segmental lesions with wire-loop and/or hyaline thrombus formation (Fig. 1).

We initially used immunohistochemistry to analyze glomerular Fkn expression and CD16⁺ Mos accumulation and found that there was a significant increase in both glomerular Fkn expression and CD16⁺ Mos accumulation in MRL/lpr mice by the time they were 20 wk old (Fig. 1). We then carried out real-time PCR using glomerular RNA collected by LCM to analyze glomerular Fkn expression and CD16⁺ Mos accumulation in three groups (2B11.3, 7B6.8, and 1G3: 1.22 ± 0.31, 1.19 ± 0.36, and 1.52 ± 0.41 mg/ml).

In a second set of experiments, we analyzed the relationship between levels of Fkn expression and the histopathology of these monoclonal antibody-induced glomerular lesions in serum BUN levels were markedly elevated in mice injected with 2B11.3 or 7B6.8 clone (72.3 ± 30.5 mg/dl, n = 7, P < 0.05 and 68.4 ± 32.4 mg/dl, n = 5, P < 0.05, respectively) compared with those injected with 1G3 clone (22.6 ± 7.5 mg/dl, n = 4). There was no significant difference in serum BUN levels (P = 0.83) between mice injected with 2B11.3 clone and those injected with 7B6.8 clone. Moreover, there was a marked reduction in serum albumin levels in mice injected with 2B11.3 or 7B6.8 clone (1.7 ± 0.9 mg/dl, n = 7, P < 0.05 and 1.8 ± 0.7 mg/dl, n = 5, P < 0.05, respectively) compared with those injected with 1G3 clone (3.4 ± 0.5 mg/dl, n = 4 ). Again, there was no significant difference in serum albumin levels between mice injected with 2B11.3 clone and those injected with 7B6.8 clone (P = 0.84).

In a second set of experiments, we analyzed the relationship between levels of Fkn expression and the histopathology of these monoclonal antibody-induced glomerular lesions in
SCID mice. In the glomerular lesions induced by 2B11.3, Fkn expression was detected in mesangial areas and along capillary walls, whereas it was not detected in glomerular lesions induced by 7B6.8 or 1G3 (Fig. 3). 2B11.3 clone also induced significantly \((P < 0.005)\) greater expression of Fkn mRNA in glomerular lesions than did 7B6.8 or 1G3 clone (Fig. 4A). Likewise, CD16\(^+\) Mo counts were significantly \((P < 0.001)\) higher in glomerular lesions induced by 2B11.3 clone than in those induced by 7B6.8 or 1G3 clone (Figs. 3 and 4B).

We defined the predisease glomeruli in SCID mice injected with 2B11.3 clone as containing few infiltrating inflammatory cells, despite deposition of IgG3 in mesangial areas and along capillary walls (Fig. 5, A and B). At the predisease stage, serum BUN and albumin levels were normal (BUN and albumin: 25.4 ± 8.7 and 3.0 ± 0.8 mg/dl, \(n = 7\)) in SCID mice injected with 2B11.3 clone. Immunohistochemical analysis revealed no expression of Fkn protein in predisease glomeruli (Fig. 5C), and Fkn mRNA levels were about the same as in glomeruli from control mice (Fig. 5D). Thus Fkn expression was not substantially increased in predisease glomeruli in SCID mice injected with 2B11.3 clone. Levels of TNF-\(\alpha\) and IL-1\(\beta\) mRNA were slightly higher in glomerular lesions induced by 7B6.8 clone than in glomeruli from control mice, but the difference was not significant (TNF-\(\alpha\): \(P = 0.188\), IL-1\(\beta\): \(P = 0.078\)) (Fig. 5D).
Fkn mRNA expression induced in HUVECs and murine MCs by combined stimulation with TNF-α and IL-1β. We next examined the effects of TNF-α and IL-1β on expression of Fkn mRNA in HUVECs and murine MCs. Addition of TNF-α plus IL-1β to HUVECs cultured strongly and dose-dependently increased expression of Fkn mRNA within 6 h (Fig. 6, A and B) and had a similar effect in murine MCs (Fig. 6, C and D). This suggests that Fkn expression is likely induced in glomerular endothelial and MCs in response to combined stimulation by TNF-α and IL-1β.

Production of Fkn-AT in SCID mice. In this study, we prepared L cell transfectants that expressed and secreted Fkn-AT (Fig. 7A) that significantly inhibited Fkn-stimulated chemotaxis of murine spleen cells (Fig. 7C). When L cells were injected in SCID mice, those receiving Fkn-AT-producing cells showed high serum levels of Fkn-AT by day 25 after injection while those receiving control L cells did not (Fig. 7B).

Effect of Fkn-AT on glomerular lesions in SCID mice injected with 2B11.3 clone. The proliferative glomerular lesions in SCID mice injected with 2B11.3 clone appeared to be significantly less severe in mice pretreated with Fkn-AT-producing L cells than in those pretreated with control cells [Fkn-AT and control (index of glomerular lesions): 1.25 ± 0.12 and 2.54 ± 0.29 (P < 0.01) (Fig. 8, A, B, and G)]. In addition, there was a significant (P < 0.01) reduction in glomerular infiltration by CD16⁺ Mos in mice treated with Fkn-AT-producing L cells compared with control (Fkn-AT vs. control: 0.13 ± 0.03 vs. 0.66 ± 0.29, P < 0.05) (Fig. 8, C, D, and H). Moreover, the elevation of serum BUN and hypoalbuminemia was strongly inhibited in mice pretreated with Fkn-AT-producing L cells compared with control (Fkn-AT vs. control: BUN, 35.3 ± 2.9 vs. 58.8 ± 11.6 mg/dl; Alb, 3.01 ± 0.33 vs. 1.75 ± 0.24 mg/dl; n = 5, P < 0.05). On the other hand, when SCID mice were pretreated with Fkn-AT-producing L cells, glomerular wire-loop lesions in mice injected with 7B6.8 clone showed no significant change from control [Supplemental Fig. 1, A and B (Supplemental data for this article can be found on the American Journal of Physiology: Renal Physiology website.)]. Likewise, serum BUN and albumin levels in mice pretreated with Fkn-AT-producing L cells did not significantly differ from control (Fkn-AT vs. control: BUN, 55.9 ± 11.5 vs. 58.2 ± 11.4 mg/dl, P = 0.76; albumin, 1.83 ± 0.27 vs. 1.57 ± 0.23 mg/dl, P = 0.13).

The glomerular deposition of IgG3 in mice injected with Fkn-AT-producing L cells and those injected with control L cells appeared similar when the mice were injected with 2B11.3 clone (Fig. 8, E and F) or 7B6.8 clone (Supplemental Fig. 1, E and F). No IgG3 was detected elsewhere in the kidney, nor was any detected in the lung or liver. Moreover, there was no significant difference in serum IgG3 levels between mice injected with Fkn-AT-producing cells and those injected with control cells (2B11.3 clone: Fkn-AT vs. control, 0.72 ± 0.31 vs. 0.78 ± 0.38 mg/ml; 7B6.8 clone: Fkn-AT vs. control, 0.81 ± 0.42 vs. 0.84 ± 0.34 mg/ml).

DISCUSSION

In this study, we demonstrated Fkn expression and CD16⁺ Mos accumulation within proliferative glomerular lesions, but not in wire-loop-like lesions, in SCID lupus model mice injected with IgG3-producing hybridoma clones derived from an MRL/lpr mouse. In addition, we found that Fkn-AT mitigated the progression of proliferative glomerular lesions as well as glomerular infiltration by CD16⁺ Mos in these mice.

Fkn is a mononuclear cell-directed, cell surface-anchored chemokine that likely plays an essential role in the recruitment and accumulation of CX3CR1-expressing leukocytes, especially monocytes/macrophages, under conditions of high blood flow like that in glomerular capillaries (7, 12). CD16⁺ Mos are thought to express higher levels of CX3CR1 than CD16⁻ Mos and also produce high levels of proinflammatory cytokines (2, 8, 27). We previously reported that both Fkn expression and CD16⁺ Mos accumulation are markedly elevated in patients with proliferative lupus nephritis (ISN/RPS classes III and IV) (26), but it remained uncertain whether Fkn played any significant role in the pathogenesis of glomerular lesions in lupus nephritis. For that reason, our first set of experiments was undertaken to assess the degree to which the level of Fkn expression in glomeruli was related to the severity of glomerular lesions in MRL/lpr mice, which are regarded as a good model of proliferative lupus nephritis. An earlier study showed
that renal expression of Fkn was upregulated in MRL/lpr mice during the development of renal damage (13), but the relation between the severity of the glomerular lesions and the level of Fkn expression was unclear. We therefore semiquantitatively analyzed expression of Fkn mRNA in glomeruli using LCM, which enabled us to rapidly isolate selected glomeruli from MRL/lpr mice, based on the degree of injury. We found significant levels of Fkn expression and CD16<sup>+</sup>Monos accumulation in the glomeruli of 20-wk-old MRL/lpr mice, suggesting that Fkn expression and CD16<sup>+</sup>Monos accumulation contribute to the progression of glomerular lesions in these animals. However, glomerular lesions of 20-wk-old MRL/lpr mice were histopathologically very heterogeneous, and it is unclear which glomerular lesions of MRL/lpr mice are actually associated with Fkn expression and CD16<sup>+</sup>Monos accumulation.

Our second set of experiments was undertaken to further clarify the association between Fkn expression with the observed diversity of glomerular lesions using a simplified lupus nephritis model in which glomerular lesions were induced with monoclonal antibodies derived from an MRL/lpr mouse. In SCID mice injected with IgG<sub>3</sub>-producing hybridomas, both Fkn expression and CD16<sup>+</sup>Monos counts were markedly elevated in endocapillary proliferative glomerular lesions, but not in wire-loop lesions. This may mean that the histopathological variation in glomerular lesions seen in MRL/lpr mice is a reflection of the expanded B cell clones and the pathogenic potencies of different antibodies they express.

To determine whether the observed increase in Fkn expression was a cause or a result of inflammatory cell infiltration in the glomeruli of SCID mice injected with IgG<sub>3</sub>-producing hybridoma clone 2B11.3; we also evaluated glomerular Fkn expression at a predisease stage, during which glomerular deposition of IgG<sub>3</sub> was detected, but inflammatory cells and/or proliferative lesion were not. We found that Fkn expression was not yet upregulated in
endocapillary proliferative glomerular lesions at the predisease stage, but levels of two proinflammatory cytokines, TNF-α and IL-1β, were already elevated. By contrast, levels of these two proinflammatory cytokines were not significantly elevated in the glomeruli of SCID mice injected with 7B6.8 clone. In vitro, Fkn mRNA expression in HUVECs and MCs increased in response to combined stimulation with TNF-α and IL-1β; moreover, we recently observed that 2B11.3 antibodies do not induce Fkn expression by cultured endothelial cells (data not shown). Taken together, these findings suggest that Fkn expression is induced in response to the production of inflammatory cytokines such as TNF-α and IL-1β triggered by complement activation and/or accumulation of circulating leukocytes in the glomeruli of SCID mice injected with 2B11.3 clone and that it is not primarily induced by the deposition of 2B11.3 antibodies. Inflammatory cytokines released from infiltrating leukocytes may induce Fkn expression by cultured endothelial cells (data not shown).

**Fig. 6.** Effects of cytokines on expression of Fkn mRNA. Expression of Fkn mRNA levels in human umbilical vein endothelial cells (HUVECs) (A and B) and murine mesangial cells (MCs) (C and D) induced by the combination of TNF-α (A and C) and IL-1β (B and D) at the indicated concentrations. Bars depict means ± SE in arbitrary units. *P < 0.001 vs. 0 pg/ml. #P < 0.01 vs. 0 pg/ml.

**Fig. 7.** ELISAs of Fkn antagonist (Fkn-AT) and inhibition of chemotaxis. A: levels of Fkn-AT in L cell culture supernatant. B: levels of Fkn-AT in serum from SCID mice collected 25 days after injection with L cells (n = 5). C: inhibition of murine spleen cell chemotaxis by Fkn-AT produced by L cells. Chemotaxis was assayed after murine spleen cells were pretreated with the indicated concentration of Fkn-AT for 30 min at 37°C. Experiments were performed in triplicate. Values are means ± SD; bars depict means ± SE. *P < 0.005 vs. control L cells.
expression to accelerate further accumulation of leukocytes, such as CD16⁺ Mos, thereby exacerbating the proliferative glomerular lesions of lupus nephritis.

These findings prompted us to speculate that we could mitigate the proliferative glomerular lesions in this lupus model mouse by using an Fkn-AT to reduce the infiltration of inflammatory cells such as CD16⁺ Mos. To test that idea, we developed a system to overproduce Fkn-AT in the circulation by transplanting Fkn-AT-producing L cells in SCID mice. The antagonist consisted of an Fkn truncation mutant in which the four NH₂-terminal amino acid residues were removed. This molecule was previously shown to be a potent Fkn-AT and to ameliorate the progression of lupus nephritis in MRL/lpr mice (10). We found that Fkn-AT protected against the development of glomerular endocapillary proliferative lesions induced by clone 2B11.3 in SCID mice while markedly reducing glomerular CD16⁺ Mos accumulation. However, Fkn-AT did not protect against the development of glomerular wire-loop lesions induced by clone 7B6.8. It thus appears that, by stimulating CD16⁺ Mos accumulation, Fkn contributes to the development of endocapillary proliferative glomerular lesions induced by monoclonal antibodies raised in SCID mice, which completely lack T and B cells but have a normal granulomonocyte system. That said, other pathways that do not involve Fkn-CD16⁺ Mos interaction may also contribute to lupus nephritis, for example, glomerular wire-loop lesions induced by clone 7B6.8. Further study will be needed to clarify these pathways with the goal of complete amelioration of lupus nephritis.

In conclusion, Fkn expression and CD16⁺ Mos accumulation in glomeruli is associated with the histopathology of glomerular lesions in MRL/lpr mice. Fkn appears to mainly affect the progression of proliferative glomerular lesions of lupus nephritis and does not trigger lesion formation. Fkn-AT may thus represent potentially useful tools for targeting leukocytes such as CD16⁺ Mos, which mediate the glomerular injury seen in lupus nephritis.

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DISCLOSURES
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