Circulating CSF-1 Promotes Monocyte and Macrophage Phenotypes that Enhance Lupus Nephritis

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ABSTRACT

Macrophages mediate kidney disease and are prominent in a mouse model (MRL-Faslpr) of lupus nephritis. Colony stimulating factor-1 (CSF-1) is the primary growth factor for macrophages, and CSF-1 deficiency protects MRL-Faslpr mice from kidney disease and systemic illness. Whether this renoprotection derives from a reduction of macrophages and whether systemic CSF-1, as opposed to intrarenal CSF-1, promotes macrophage-dependent lupus nephritis remain unclear. Here, we found that increasing systemic CSF-1 hastened the onset of lupus nephritis in MRL-Faslpr mice. Using mutant MRL-Faslpr strains that express high, moderate, or no systemic CSF-1, we detected a much higher tempo of kidney disease in mice with the highest level of CSF-1. Furthermore, we uncovered a multistep CSF-1-dependent systemic mechanism central to lupus nephritis. CSF-1 heightened monocyte proliferation in the bone marrow (SSClowCD11b/H11001), and these monocytes subsequently seeded the circulation. Systemic CSF-1 skewed the frequency of monocytes toward "inflammatory" (SSClowCD11bLy6Chigh) and activated populations that homed to sites of inflammation, resulting in a more rapid accumulation of intrarenal macrophages (CD11bCSF-1Ror CD68) that induced apoptosis of tubular epithelial cells, damaging the kidney. In humans, we found increased levels of CSF-1 in the serum, urine, and kidneys of patients with lupus compared with healthy controls. Furthermore, serum and urine CSF-1 levels correlated with lupus activity, and intrarenal CSF-1 expression correlated with the histopathology activity index of lupus nephritis. Taken together, circulating CSF-1 is a potential therapeutic target for lupus nephritis.


Identifying molecules that mediate experimental lupus nephritis may uncover therapeutic targets and biomarkers. MRL-Faslpr mice develop a systemic autoimmune disease akin to human lupus nephritis and thus are a powerful tool to probe for molecules that regulate kidney disease in these patients.1,2 Kidney disease in MRL-Faslpr mice is rapid, progressive, and predictable. Moreover, the time frame is sufficiently slow to tease apart the pathogenesis, and sufficiently fast to be efficient. Thus, these mice are a powerful tool to probe for therapeutic targets and biomarkers in human lupus nephritis.

Macrophages (Mø) regulate kidney disease.4 Mø originate from pluripotent stem cells in the bone marrow that differentiate into mature monocytes (Mo), which enter the blood stream and traffic to...
the kidney. Growing evidence implicates Mø as mediators of lupus nephritis because intrarenal Mø (CD68⁺, F4/80⁺) increase with advancing disease in MRL-Fas⁺/− mice. Mø require the colony stimulating factor-1 (CSF-1), their principle growth factor, to differentiate, survive, and multiply. Our prior studies indicate that CSF-1 is central to lupus nephritis. Implanted cells generating CSF-1 into the kidney of MRL-Fas⁺/− mice incites local Mø-rich inflammation. Moreover, CSF-1 deficient mice (Csf1op/op;MRL-Fas⁺/−) are protected from kidney disease and systemic illness. However, the Csf1op/op;MRL-Fas⁺/− mice are frail and have skeletal abnormalities and numerous other defects. Thus, it is possible that the effect of deleting CSF-1 on lupus in MRL-Fas⁺/− mice is, at least in part, not directly related to the reduction of Mø. Moreover, CSF-1 generating cells implanted into the kidney induce inflammation that is restricted to the area adjacent to the implant site.

Thus, the systemic effect of CSF-1 during the initiation and progression of Mø-dependent lupus nephritis remains unclear.

Understanding the effect of circulating and tissue CSF-1 expression is key to designing a therapeutic treatment. CSF-1 is expressed in the circulation and is upregulated in the kidney in MRL-Fas⁺/− mice with lupus nephritis. Intrarenal CSF-1 expression occurs during inflammation and expression is largely limited to tubular epithelial cells (TECs). Moreover, the rise in circulating CSF-1 precedes intrarenal CSF-1 expression and is bimodal in MRL-Fas⁺/− mice. CSF-1 is upregulated in neonates, declines to normal levels, and then progressively rises with advancing kidney disease in MRL-Fas⁺/− mice. Moreover, an increase in CSF-1 in the circulation precedes overt kidney pathology in MRL-Fas⁺/− mice. However, it is not clear whether CSF-1 in the circulation, apart from intrarenal CSF-1, is central to the progression of lupus nephritis in MRL-Fas⁺/− mice. Therefore, we propose to test the hypothesis that systemic CSF-1 hastens the progression of Mø-rich lupus nephritis. Furthermore, we hypothesize that circulating CSF-1 increases the frequency of circulating Mø (SSClowCD11b⁺), which are more readily recruited to the kidney and, in turn, induce injury.

Finally, preclinical studies are a first step in identifying therapeutic targets and biomarkers for lupus nephritis and require validation in humans. Therefore, we propose to test the hypothesis that CSF-1 is upregulated in the circulation, urine, and kidneys of patients with active lupus nephritis.

RESULTS

The CSF-1 Transgene Drives Disease-Related Tissue Expression of CSF-1 in TgC/+;MRL-Fas⁺/− Mice

To verify that the CSF-1 promoter/first intron-driven full-length CSF-1 transgene used to overexpress CSF-1 restored disease-related tissue expression of CSF-1, we created TgC/+;Csf1op/op;MRL-Fas⁺/− mice, in which the only source of CSF-1 was encoded by the TgC transgene. We compared CSF-1 in the serum and tissues and the restoration of the phenotypic features characteristic of MRL-Fas⁺/− mice (kidney, skin disease, lymphadenopathy, and splenomegaly) in this transgenic mouse during the disease progression (1.5, 3.0, and 5.0 mo of age) with MRL-Fas⁺/− mice. Csf1op/op;MRL-Fas⁺/− mice deficient in CSF-1 were used as negative controls. In the TgC/+;Csf1op/op;MRL-Fas⁺/− mouse, CSF-1 expression in the serum, kidney, and other tissues (skin, salivary gland, lung, skin, spleen, and lymph nodes), as well as kidney pathology, lymphadenopathy, and splenomegaly were restored to the values of age-matched MRL-Fas⁺/− mice [Supplemental Figure 1 (3 mo data) and 1.5 and 5.0 mo data (not shown)]. Similarly, skin, salivary gland, and lung pathology in TgC/+;Csf1op/op;MRL-Fas⁺/− mice were restored to MRL-Fas⁺/− levels (data not shown). Thus, the TgC transgene, previously shown to direct normal tissue-specific and temporal expression of CSF-1, drives the disease-related tissue expression of CSF-1 in TgC/+;Csf1op/op;MRL-Fas⁺/− mice in a manner mimicking the expression of CSF-1 in nontransgenic MRL-Fas⁺/− mice.

The CSF-1 Transgene Increases CSF-1 in the Serum, Kidney, and Other Tissues in MRL-Fas⁺/− Mice

To overexpress CSF-1 in MRL-Fas⁺/− mice, we constructed TgC/+;MRL-Fas⁺/− mice in which CSF-1 is expressed from the wild-type CSF-1 gene and the TgC transgene. To determine whether this strategy amplified CSF-1 expression, we compared CSF-1 levels in the serum, kidney, and other tissues in wild-type CSF-1 gene and the TgC transgene. To determine whether an increase in CSF-1 promotes kidney disease, we compared the time-related renal pathology and function in MRL-Fas⁺/− mice progressively rose in the serum (Figure 1A) and tissues (Figure 1B) with advancing age and disease (1.5, 3.0, and 5.0 mo of age). The increase in CSF-1 in the TgC/+;MRL-Fas⁺/− was, as anticipated, higher than in MRL-Fas⁺/− mice. By comparison, serum CSF-1 levels in age-matched MRL-++ and normal B6 mice remained unchanged (dotted line, Figure 1A). Thus, we have constructed mutant MRL-Fas⁺/− strains with varying levels of CSF-1 expression in the serum and kidneys: TgC/+;MRL-Fas⁺/− (high), MRL-Fas⁺/− (intermediate), and Csf1op/op;MRL-Fas⁺/− (none).

CSF-1 Exacerbates Kidney Disease and Shortens Survival in MRL-Fas⁺/− Mice

To determine whether an increase in CSF-1 promotes kidney disease, we compared the time-related renal pathology and function in TgC/+;MRL-Fas⁺/−, MRL-Fas⁺/− (WT), and Csf1op/op;MRL-Fas⁺/− mice. The severity of renal pathology (glomerular, interstitial, perivascular) in TgC/+;MRL-Fas⁺/− mice was increased at 1.5 and 3.0 mo of age as compared with WT mice (Figure 2A1). Renal disease in the WT and TgC/+;MRL-Fas⁺/− mice was equivalent by 5 mo of age. The progression in renal pathology is consistent with an increased loss of renal function (BUN, albuminuria/creatinine ratio) in the TgC/+;MRL-Fas⁺/− mice (Figure 2A2). The Csf1op/op;MRL-Fas⁺/− mice remained protected from renal disease and MRL-++ and B6 kidneys did not have any renal pathology between 1.5 and 5.0 mo of age. Because CSF-1 is increased with advancing age and is higher in TgC/+;MRL-Fas⁺/− mice.
than WT mice, we conclude that the tempo of kidney disease is accelerated by amplifying CSF-1 expression in MRL-Faspr mice.

Because increasing CSF-1 accelerates the tempo of kidney disease in MRL-Faspr mice, we hypothesized that the rate of mortality is accelerated in TgC/+;MRL-Faspr mice compared with WT mice. As hypothesized, TgC/+;MRL-Faspr mice had an accelerated rate of mortality compared with WT mice (Figure 2B). Taken together, increasing CSF-1 in MRL-Faspr mice in the circulation (Figure 1A) and kidney (primarily in TECs and to a lesser extent within glomeruli) (Figure 2C) hastens the tempo of kidney disease and shortens survival.

CSF-1 Increases Intrarenal Mø in MRL-Faspr Mice

To determine whether the intrarenal Mø in MRL-Faspr mice are CSF-1-dependent, we examined the time-related intrarenal accumulation of CD68+ cells in MRL-Faspr mice with varying levels of CSF-1. The number of CD68+ cells progressively increased in the TgC/+;MRL-Faspr and WT mice from 1.5 to 5.0 mo of age. However, TgC/+;MRL-Faspr mice accumulated more CD68+ in the kidney than WT mice (Figure 3A). In contrast, CD68+ cells were rare in Csf1op/op;MRL-Faspr kidneys (Figure 3A). Furthermore, we detected an increase in the leukocyte populations of B220+ (largely expressed by double-negative T cells21), CD4+, and CD8+ known to accompany Mø that was proportional to the level of CSF-1 in nephritic MRL-Faspr mice (Supplement Figure 2). Of note, the magnitude of double-stranded DNA antibodies (dsDNA Abs) did not rise along with increasing CSF-1 levels and the CSF-1-dependent increase in renal disease (Supplement Figure 3). Thus, increasing CSF-1 hastens the accumulation of intrarenal Mø (CD68+) and the spectrum of leukocytes characteristic of MRL-Faspr kidney disease.

CSF-1 Elevates the Proliferation of SSClowCD11bhigh Cells in the Bone Marrow Resulting in a Rise in Circulating Mø in MRL-Faspr Mice

To determine whether the CSF-1-dependent accumulation of intrarenal Mø (CD68+) in MRL-Faspr mice resulted from a rise of circulating Mo (SSClowCD11bhigh), we evaluated the frequency and total number of circulating SSClowCD11bhigh leukocytes in TgC/+;MRL-Faspr and WT mice. We detected an enhanced frequency of SSClowCD11bhigh leukocytes in the circulation of TgC/+;MRL-Faspr mice compared with WT mice (Figure 3B1, Supplement Figure 4A). To determine whether the heightened SSClowCD11bhigh leukocytes in the blood resulted from enhanced SSClowCD11bhigh leukocyte proliferation in the blood or bone marrow (BM), we evaluated the frequency and total number of proliferating (Brdu+) SSClowCD11bhigh leukocytes in both locations at 24 and 48 h. We detected a rise in SSClowCD11bhigh cell proliferation in the BM (Figure 3C), but not in the blood (Figure 3B2, Supplement Figure 4B), proportional to the level of CSF-1 (TgC/+;MRL-Faspr > WT > Csf1op/op;MRL-Faspr mice). We wish to point out that adult Csf1op/op;MRL-Faspr mice are not monocytopenic (data not shown). Taken together, this suggests that increasing CSF-1 elevates circulating Mo (SSClowCD11bhigh) as a result of heightened BM Mo proliferation.

CSF-1 Shifts the Circulating Mo toward the “Inflammatory,” Activated Phenotype in MRL-Faspr Mice

Our findings thus far indicate that CSF-1 increases circulating Mo and thereby provides a larger pool of cells to be recruited into the kidney. However, there are two functional subsets among blood Mo (SSClowCD11bhigh): a short-lived Ly6ChighCX3CR1lowCCR2+ subset that is actively recruited to inflamed tissues and a Ly6ChighCX3CR1highCCR2+ “resident” subset characterized by CCR1-dependent recruitment to noninflamed tissues.6,23 To determine if an increased CSF-1 expression shifted the circulating Mo (SSClowCD11bhigh) to the actively recruited “inflammatory” Mo phenotype, we probed for the expression of these Mo subsets in the circulation of TgC/+;MRL-Faspr, WT, and Csf1op/op;MRL-Faspr mice. We detected a higher frequency and total number of Ly6ChighCX3CR1high cells in the circulation and kidney of TgC/+;MRL-Faspr mice compared with WT mice (Figure 3D, Supplement Figure 4C). Moreover, we detected a decrease in Ly6ChighCX3CR1low subsets in the circulation and kidneys of Csf1op/op;MRL-Faspr compared with TgC/+;MRL-Faspr.
CSF-1 Fosters the Recruitment of Activated Mo (CD45.1\(^{\text{+}}\)CSF-1R\(^{\text{+}}\)) into the Kidney that Induces Apoptosis in MRL-Fas\(^{\text{pr}}\) Mice

To determine whether increasing CSF-1 in the kidney more actively recruits Mo (CD11b\(^{\text{+}}\)CSF-1R\(^{\text{+}}\)), we injected EGFP\(^{\text{+}}\) BM cells into MRL-Fas\(^{\text{pr}}\) mice expressing varying levels of CSF-1 (TgC\(^{+/+}\);MRL-Fas\(^{\text{pr}}\), WT, and Csf1\(^{op/op}\);MRL-Fas\(^{pr}\)). Most of these BM Mo (SSC\(^{\text{low}}\)CD11b\(^{\text{high}}\)) derived from MRL-Fas\(^{pr}\) mice are Ly6C\(^{\text{high}}\) (78% ± 4.3), a finding that is similar to nonlupus susceptible mice.\(^6\) Dependent on their milieu in the circulation, the Ly6C expression on Mo will be downregulated, resulting in an increased recruitment to inflamed tissue.\(^6\) We detected an increase in hematopoetic cells (CD45.1\(^{\text{+}}\)) expressing the CSF-1R promoter EGFP in the kidneys of MRL-Fas\(^{pr}\) with the highest level of CSF-1 (TgC\(^{+/+}\);MRL-Fas\(^{pr}\) > WT > Csf1\(^{op/op}\);MRL-Fas\(^{pr}\)), but not in the blood at 24 h posttransplant (Figure 4A, Supplement Figure 6A). This increase in EGFP\(^{+}\) leukocytes in the kidney was 50% higher in TgC\(^{+/+}\);MRL-Fas\(^{pr}\) compared with WT mice, 80% higher in WT compared with Csf1\(^{op/op}\);MRL-Fas\(^{pr}\) mice, and 90% higher in TgC\(^{+/+}\);MRL-Fas\(^{pr}\) compared with Csf1\(^{op/op}\);MRL-Fas\(^{pr}\) mice. Because cultured BM Mo derived from MRL-Fas\(^{pr}\) mice do not double until 48 h (Supplement Figure 7), it is likely that most EGFP\(^{+}\) BM in the kidney reflects recruitment rather than proliferation.\(^6\)

CSF-1 directly activates Mo (CD68\(^{+}\)),\(^2\,5\) and these Mo, in turn, release mediators that induce TEC apoptosis \textit{in vitro}.\(^2\,6\) Therefore, we hypothesized that the number of circulating Mo (SSC\(^{\text{low}}\)CD11b\(^{\text{high}}\)) and intrarenal activated Mo (CD68\(^{+}\)) and apoptotic TECs in TgC\(^{+/+}\);MRL-Fas\(^{pr}\) mouse is greater than WT mice. We detected a higher frequency and total number of activated (CD69\(^{+}\) and CD86\(^{+}\)) cells in TgC\(^{+/+}\);MRL-Fas\(^{pr}\) mice with increased levels of CSF-1 in the circulation (Figure 4B1, Supplement Figure 6B1) and the kidney (Figure 4B2, Supplement Figure 6B2). Moreover, in the circulation we detected a CSF-1-dependent shift to an increased population of activated Ly6C\(^{\text{high}}\)CD69\(^{+}\) and Ly6C\(^{\text{high}}\)CD86\(^{+}\) Mo (SSC\(^{\text{low}}\)CD11b\(^{\text{high}}\)) (Supplement Figure 8), MRL-Fas\(^{pr}\) mice at 3 and 5 mo of age that are primarily recruited to inflamed tissues.\(^9\) Similarly, the number of intrarenal apoptotic TECs rose in proportion to the level of CSF-1 in MRL-Fas\(^{pr}\) mice (Figure 4C). Taken together, enhancing CSF-1 expres-
sion in MRL-Faslpr mice increases the number of Mo seeded from the BM into the circulation; shifts these Mo toward an inflammatory and activated phenotype; and, in turn, hastens the rise of activated Mo in the kidney that induces TEC apoptosis.

Circulating CSF-1, apart from Intrarenal CSF-1, Increases Mo in the Kidney
To determine whether a rise in circulating CSF-1 alone, apart from upregulated intrarenal CSF-1, contributes to the CSF-1-dependent increase in intrarenal Mo (CD45.1+ CSF-1R+) in the kidney, we compared Csf1op/op;MRL-Faslpr mice injected with CSF-1 versus PBS for 2 d (Supplement Figure 9). Hence, the sole source of CSF-1 in the Csf1op/op;MRL-Faslpr mice was in the circulation. After adoptive transfer of EGFP+ BM cells (derived from MacGreen;MRL-Faslpr mice) into Csf1op/op;MRL-Faslpr mice, the frequency and total number of EGFP+ cells in the kidney was substantially greater in CSF-1 versus PBS-treated mice (Figure 5A). This suggests that increasing circulating CSF-1 enhances Mo accumulation in the kidney.

Circulating CSF-1 Enhances BM Mo Proliferation, the Frequency of Inflammatory Mo in the Circulation, and, in Turn, Mo Accumulation in MRL-Faslpr Kidneys
Consistent with the action of CSF-1 in increasing blood Mo (SSClowCD11bhigh), we detected an elevation in the frequency and total number of SSc-lowCD11b+ cells in the blood in Csf1op/op;MRL-Faslpr mice injected with CSF-1 compared with PBS (Figure 5B). Moreover, there was a robust increase in the frequency and total number of proliferating (Brdu+) SSc-lowCD11b+ cells in the BM in CSF-1 versus PBS-treated Csf1op/op;MRL-Faslpr mice (Figure 5C). Furthermore, we detected a considerable rise in the frequency and total number of Ly6ChighCD11b+ inflammatory Mo in the circulation and Ly6ChighF4/80+ activated Mo (F4/80+) in the kidney of CSF-1- versus PBS-treated mice (Figure 5D). Taken together, this suggests that elevation of circulating CSF-1 alone is responsible for increasing the number of Mo released from the BM into the circulation and for shifting the circulating Mo populations toward a Ly6Chigh activated Mo phenotype that is more readily recruited into the kidney.

CSF-1 is Expressed by TECs in Patients with Lupus Nephritis
To determine whether our findings in lupus nephritis in mice may be applicable to human lupus, we probed for CSF-1 expression in kidney sections in patients with lupus nephritis, in patients with lupus nephritis as compared with other kidney diseases (minimal-change and membranous GN), and in control patients (Table 1). Similar to the MRL-Faslpr phenotype, CSF-1 is more robustly expressed in lupus nephritis as compared with other kidney diseases {minimal-change and membranous GN} with far fewer intrarenal leukocytes (CD68: 36 ± 8 versus 10 ± 3; CD3: 18 ± 5 versus 4 ± 1, respectively, n = 10/group). Note CSF-1 expression is specific because we did not detect staining using a goat IgG control, and CSF-1 staining was blocked by
preabsorption of the anti-CSF-1 antibody (Figure 6A). To determine whether elevated CSF-1 expression by TECs correlates with the activity and chronicity of the lupus nephritis, we evaluated the histopathological activity index and chronicity index. We determined that the CSF-1 expression in TECs correlates with the activity index but not with the chronicity index (Figure 6B). Thus, TECs are the principle source of CSF-1 in human lupus nephritis and the level of CSF-1 expression is an index of disease activity.

Figure 4. CSF-1 recruits Mo (CD45.1*CSF-1R*) into the kidney, increases activated Mo (SSC<sub>low</sub>CD11b<sub>high</sub>) in the circulation, activated Mo (CD68<sup>-</sup>) in the kidney and, in turn, apoptosis in MRL-Fas<sup>transgenic</sup> kidneys. (A) We transferred BM cells from MacGreen (EGFP<sup>-</sup>);MRL-Faslpr mice into TgC/+;MRL-Faslpr, MRL-Faslpr, and Csf1op/op;MRL-Faslpr mice and evaluated the recruitment of EGFP<sup>-</sup> cells into the kidney at 24 h. Data are representative of two separate experiments. (B) Mo (CD11b<sup>+</sup>) activation (CD86<sup>+</sup>) in the blood and Mø (CD68<sup>+</sup>) activation (CD86<sup>+</sup>,CD69<sup>+</sup>) in the kidney analyzed by flow cytometry in TgC/+;MRL-Faslpr<sup>transgenic</sup> versus PBS-injected Csf1op/op;MRL-Faslpr<sup>transgenic</sup> mice. (C) Rise in BM Mo proliferation in CSF-1<sup>transgenic</sup> versus PBS-injected Csf1op/op;MRL-Faslpr<sup>transgenic</sup> mice. (D) Increase in CD11b<sup>+</sup> expressing Ly6C<sup>high</sup> Mo in the blood and kidney in CSF-1<sup>transgenic</sup> versus PBS-injected Csf1op/op;MRL-Faslpr<sup>transgenic</sup> mice. (A through D) Data used mice at 3 mo of age and are mean ± SEM (flow cytometry). Representative plots are shown.

Figure 5. Circulating CSF-1 alone increases BM Mo proliferation; increases circulating, activated Mo (SSC<sub>low</sub>CD11b<sub>high</sub>) and intra-renal inflammatory activated Mo (F4/80<sup>+</sup>). (A) We transferred BM cells from MacGreen (EGFP<sup>+</sup>);MRL-Faslpr mice into TgC/+;MRL-Faslpr, MRL-Faslpr, and Csf1op/op;MRL-Faslpr mice and evaluated the recruitment of EGFP<sup>-</sup> cells into the kidney at 24 h. Data are representative of two separate experiments. (B) Mo (CD11b<sup>+</sup>) activation (CD86<sup>+</sup>) in the blood and Mø (CD68<sup>+</sup>) activation (CD86<sup>+</sup>,CD69<sup>+</sup>) in the kidney analyzed by flow cytometry in TgC/+;MRL-Faslpr<sup>transgenic</sup> versus PBS-injected Csf1op/op;MRL-Faslpr<sup>transgenic</sup> mice. (C) Rise in BM Mo proliferation in CSF-1<sup>transgenic</sup> versus PBS-injected Csf1op/op;MRL-Faslpr<sup>transgenic</sup> mice. (D) Increase in CD11b<sup>+</sup> expressing Ly6C<sup>high</sup> Mo in the blood and kidney in CSF-1<sup>transgenic</sup> versus PBS-injected Csf1op/op;MRL-Faslpr<sup>transgenic</sup> mice. (A through D) Data used mice at 3 mo of age and are mean ± SEM (flow cytometry). Representative plots are shown.
CSF-1 Is Increased in the Serum and Urine in Patients with Serologically Active Lupus Nephritis

To determine whether the pathologic effect of elevated systemic CSF-1 on lupus nephritis may be relevant in humans, we measured the concentrations of CSF-1 in serum and urine of patients with lupus nephritis, proteinuric patients with other kidney diseases (minimal-change and membranous GN), lupus patients without nephritis, and healthy controls. We detected a marked rise in CSF-1 in the serum and urine in patients with lupus nephritis, as compared with other kidney diseases and healthy controls.

Table 1. Characteristics of patients analyzed for CSF-1 expression in serum and urine

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gender (Male/Female)</th>
<th>Age (yr)*</th>
<th>C3/C4b (g/L)</th>
<th>dsDNAc &gt;200 (IU/ml)</th>
<th>Serum Creatinine (mg/dl)*</th>
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<tr>
<td>SLE with lupus nephritis</td>
<td>20</td>
<td>0/20</td>
<td>19.88 ± 4.68</td>
<td>&gt;0.9/&gt;0.1d</td>
<td>6 of 20</td>
<td>1.01 ± 0.27</td>
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<td>WHO Class IV</td>
<td>14</td>
<td>2/12</td>
<td>37.67 ± 5.07</td>
<td>&lt;0.9/&lt;0.1a</td>
<td>12 of 14</td>
<td>1.13 ± 0.17</td>
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<tr>
<td>SLE with lupus nephritis</td>
<td>7</td>
<td>0/7</td>
<td>37.51 ± 4.25</td>
<td>&gt;0.9/&gt;0.1d</td>
<td>2 of 7</td>
<td>1.38 ± 0.28</td>
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<tr>
<td>WHO Class III or V</td>
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<td>0/2</td>
<td>39.29 ± 6.12</td>
<td>&lt;0.9/&lt;0.1a</td>
<td>None</td>
<td>1.24 ± 0.17</td>
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<td>SLE without nephritis</td>
<td>10</td>
<td>0/10</td>
<td>45.34 ± 7.3</td>
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<td>Other kidney diseases</td>
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<td>40.14 ± 6.9</td>
<td>&lt;0.9/&lt;0.1a</td>
<td>8 of 10</td>
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<td>Healthy controls</td>
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<td>6/11</td>
<td>32.71 ± 2.86</td>
<td>&gt;0.9/&gt;0.1d</td>
<td>1 of 17</td>
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aData are mean ± SD.
bC3, complement 3 in the serum; C4 complement 4 in the serum.
cdsDNA, dsDNA Ab in the serum.
dC3/C4 normal.
eC3/C4 reduced.
SLE, systemic lupus erythematosus; WHO, World Health Organization; 190 × 113 mm (300 × 300 DPI).

Figure 6. CSF-1 expression is abundant in TECs from patients with lupus nephritis and correlates with the histopathologic activity. (A) We detected far more robust CSF-1 expression in the kidney of patients with lupus nephritis compared with minimal expression in other kidney diseases (minimal-change and membranous GN) with far fewer inflammatory infiltrates and with controls. Boxed area is enlargement. (B) CSF-1 expression in TECs correlates with the histopathology activity but not chronicity index in patients with lupus nephritis. Values are the mean ± SEM.
diseases and healthy controls (Figure 7A, Table 1). Moreover, CSF-1 was elevated in the serum, but not in the urine, in patients with lupus nephritis compared with patients with lupus without nephritis (Figure 7A, Table 1). To determine whether CSF-1 expression correlated with disease activity in lupus, we evaluated serologic parameters and renal function. Lupus activity parameters included low levels of serum C3 and C4 and high levels of serum anti-dsDNA Ab. We determined that circulating CSF-1 concentrations correlated with the low levels of C3 and C4 and elevated dsDNA Ab levels in the sera (Figure 7B). By comparison, we did not detect CSF-1 in the sera of healthy individuals. Furthermore, CSF-1 levels in the urine correlated with low, but not high, proteinuria/creatinine ratios in patients with serologically active lupus nephritis. This finding most likely reflects an increased loss of TECs that generate CSF-1 with advancing loss of renal function (Figure 7C1). Of note, CSF-1 was higher in patients with active lupus nephritis compared with those in remission (Figure 7C2). Taken together, a rise of CSF-1 expression in TECs and elevated CSF-1 in the serum and urine are indicators of active lupus nephritis.

**DISCUSSION**

CSF-1 may be a “master switch” regulating the multiorgan disease characteristic of MRL-Fas<sup>dp</sup> mice. The concept that CSF-1 expression in the circulation and tissues promotes lupus is in keeping with our prior studies. Using the CSF-1 transgenic MRL-Fas<sup>dp</sup> line that promotes lupus nephritis we have used in the study presented here, we previously detected a more rapid onset of cutaneous disease (discoid lupus) compared with WT mice. Moreover, sunlight exposure induces CSF-1 in keratinocytes in the epidermis that spills over into the circulation and, in turn, leads to the recruitment and activation of Mø (F4/80<sup>+</sup>CD68<sup>+</sup>) in the skin. Thus, CSF-1 sets in motion Mø-mediated events leading to the destruction of keratinocytes and discoid lupus. Because disease in MRL-Fas<sup>dp</sup> is a multiorgan disease involving kidney, skin, and other tissues, many tissues may contribute and respond to increasing circulating CSF-1 levels. Because our study indicates that circulating CSF-1 is instrumental in mediating renal injury, we speculate that CSF-1 generated within the kidney that is released into the circulation has an effect on cutaneous disease and other tissues, and vice versa. Thus, systemic CSF-1 expression may regulate multi-tissue injury in lupus.

We now report that CSF-1 regulates the generation of inflammatory Ly6<sub>C</sub>high Mo (SSC<sub>low</sub>CD11b<sub>high</sub>). Evidence suggests that inflamed tissues increase circulating CSF-1 and thereby stimulate the production of Mo (SSC<sub>low</sub>CD11b<sub>high</sub>) in the BM and their subsequent release into the circulation. When this is substantial, there is a skewing of the Mo population toward a higher frequency of Ly6<sub>C</sub>high cells. Our data suggest that this skewing toward circulating Ly6<sub>C</sub>high cells is CSF-1-dependent. This is based on the CSF-1-dependent increase in the immature Ly6<sub>C</sub>high Mo proliferation and the rise in circulating Ly6<sub>C</sub>high Mo that are more readily recruited to the kidney. The MRL-Fas<sup>dp</sup> kidney may be self-destructive. Because renal TECs are a rich source of CSF-1 during inflammation<sup>20</sup>, CSF-1 originating from the kidney may, in part, account for the rise in circulating CSF-1 that drives Mø...
(CD68+) to become activated and destroy the kidney. On the other hand, CSF-1 is increased in the circulation in neonates, well in advance of renal injury in MRL-Faslpr mice. Thus, it is possible that a circulating CSF-1-dependent mechanism leads to amplifying intrarenal CSF-1. Regardless, our findings indicate that CSF-1 is the conduit between the circulation and tissue that regulates the destiny of Mo and, in turn, inflammation in MRL-Faslpr mice.

CSF-1 levels in the serum and urine may be a biomarker for active lupus nephritis. This is consistent with a prior finding indicating that urinary CSF-1 (also known as M-CSF), regulated upon activation, normal T cell expressed and secreted protein, and monocyte chemoattractant protein-1 are increased during renal flares in patients with lupus. Our data agree with a report indicating that elevated CSF-1 in the sera of patients with lupus, including those with nephritis, correlates with disease activity. However, our findings extend prior reports because our data indicate that urinary and serum CSF-1 and CSF-1 levels in the kidney correlate with active lupus. Although our findings indicate that low serum C3/C4, an index of active lupus, have a high correlation with increasing serum CSF-1 concentrations, the correlation of high serum dsDNA Ab, another indicator of active lupus, is less impressive. This may be related to the extreme variability between dsDNA Ab commercial kits (10 kits tested, unpublished data). It is worth noting that urine CSF-1 correlates with moderate proteinuria (>1000 mg/24h), but not higher urinary protein levels. This is in keeping with the generation of CSF-1 at the onset of inflammation and the destruction of TECs, the major source of CSF-1, during persistent inflammation. Consistent with this concept, CSF-1 levels in TECs do not correlate with increasing histopathologic chronicity. Although urine and serum CSF-1 may be a biomarker for active lupus, it is unlikely that it is unique for lupus nephritis because CSF-1 is upregulated in the kidney after other forms of renal injury. For example, CSF-1 is expressed in TECs and to a lesser extent in glomeruli after acute kidney injury (after ischemia/reperfusion in mice and in patients with loss of renal function and tubular pathology postengraftment). Future studies will determine whether CSF-1 is a biomarker for active lupus nephritis. However, to more fully explore CSF-1 as a biomarker for lupus nephritis, follow-up studies will correlate the pathology findings with the clinical disease activity score (SLEDAI) for each patient and determine whether serum and urine CSF-1 levels longitudinally track with the progression of lupus nephritis.

Understanding the effect of circulating versus tissue CSF-1 has profound therapeutic implications. We have compelling evidence that CSF-1 in the circulation alone enhances Mo-dependent lupus nephritis in MRL-Faslpr mice. Injecting CSF-1 stimulates the proliferation of Mo (SSClowCD11bhigh) in the BM, which subsequently leads to a rise in circulating Mo populations, which in turn are more readily recruited to the kidney and induce injury. This is consistent with a study in which injecting CSF-1 accelerates the intrarenal accumulation of Mo and proteinuria in LPS-treated nonlupus susceptible mice. Within this context, our human translational findings indicate that the rise in circulating CSF-1 in these patients with lupus nephritis is not only a biomarker of disease activity but is instrumental in mediating lupus nephritis. This suggests that strategies to diminish circulating CSF-1 will halt escalating lupus nephritis in humans. Thus, purging CSF-1 from the circulation may tilt the balance of Mo back to a more mature population that is not readily recruited to tissues. Taken together, circulating CSF-1 is a potential therapeutic target and may be an attractive biomarker for lupus.

CONCISE METHODS

Mice
Mice heterozygous for the osteopetrotic mutation (Csfrpp) on the C57BL/6JxC3Heb/Fel-a/a background, BALB/c, C57BL/6 (B6), MRL/MpJ-+/+ (MRL-++), and MRL/MpJ-Faslpr/Faslpr (MRL-Faslpr) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The Csfrpp mutation was backcrossed onto the MRL-Faslpr background for ten generations. Transgenic TgFms-EGFP mice that express EGFP under the control of the Csfrpp promoter and first intron, were provided by Dr. D.A. Hume (University of Edinburgh, Edinburgh, Scotland). These mice were backcrossed onto the MRL-Faslpr background (N7) and are referred to as MacGreen;MRL-Faslpr mice. The TgN(FLCsf1)9Ers/+ mice expressing the Flcns1 mutation was backcrossed onto the MRL-Faslpr background. The TgN(FLCsf1)9Ers/+ mice expressing the Flcns1 transgene (full-length SSc1 gene driven by the Csfrpp promoter/first intron) were backcrossed onto the MRL-Faslpr and Csfrpp/MRL-Faslpr background (N7) and are referred to as TgCsfrpp/MRL-Faslpr mice. Transgenic mice in which lacZ expression is driven by the same Csfrpp promoter first intron sequence used to construct the transgene were backcrossed onto the MRL-Faslpr background (N7) to identify CSF-1 expressing cells in the kidney. Mice were bred and housed at Harvard Medical School. Only female mice were used. The use of mice in this study was reviewed and approved by the Standing Committee on Animals at the Harvard Medical School in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
CSF-1 ELISA
We measured mouse CSF-1 levels in the serum, urine, and tissue homogenates using an ELISA method. Undiluted serum and urine samples were analyzed directly. The tissues used for homogenates were from mice perfused with PBS through the heart to flush out CSF-1 in the circulation. Briefly, we homogenized tissue samples using a MixMill 300 (Qiagen, Valencia, CA). Samples were spun down and the supernatant fraction of the homogenates was used for the ELISA.

We determined the protein concentration of each sample (supernatant of homogenate) using the BCA Protein Assay Kit (PIERCE, Rockford, IL) and evaluated 20 μg of protein per tissue sample. To detect CSF-1 in human serum and urine, we used a human CSF-1 ELISA kit according to the manufacturer’s instructions. The ELISA antibodies and reagents were purchased from BD Bioscience (San Jose, CA).

Renal Histopathology
We fixed kidneys in 10% neutral buffered formalin for 24 h and stained paraffin sections (4 μm) with periodic acid–Schiff reagent. We evaluated kidney pathology as described previously. Briefly, we assessed glomerular pathology by scoring each glomerulus on a semiquantitative scale: 0 = normal [35 to 40 cells/glomerular cross section (GCS)]; 1 = mild [glomeruli with few lesions showing slight proliferative changes, mild hypercellularity (41 to 50 cells/GCS)]; 2 = moderate [glomeruli with moderate hypercellularity (51 to 60 cells/GCS, including segmental and/or diffuse proliferative changes, hyaline); 3 = severe [glomeruli with segmental or global sclerosis and/or severe hypercellularity (>60 cells/GCS), necrosis, and crescent formation]. We scored 20 gcs/kidney. Interstitial/tubular pathology was assessed semiquantitatively on a scale of 0 to 3 in 10 randomly selected high-power fields. We determined the largest and average number of infiltrates and damaged tubules and adjusted the grading system accordingly: 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Perivascular cell accumulation was determined semiquantitatively by scoring the number of cell layers surrounding most vessel walls (score: 0 = none, 1 = <5 cell layers, 2 = 5 to 10 cell layers, 3 = >10 cell layers).

Renal Function
We measured BUN, creatinine, and albuminuria as described previously.

Splenomegaly/Lymphadenopathy
Splenomegaly and lymph node weight were determined at the time of sacrifice by comparing the ratios of the tissue to body weight.

Immunostaining

Mouse Tissue.
Kidney tissue was processed and stained for the presence of CD68, CD4, CD8, B220, and anticleaved caspase-3 antibody (Asp175) (Cell- Signaling, Danvers, MA), as described previously for the kidney. To identify β-galactosidase encoded by lacZ (CSF-1 expression), we treated frozen kidney sections as described previously.

Human Tissue.
We deparaffinized and rehydrated serial sections (4 μm). Antigen retrieval was performed by immersion in citrate buffer followed by blocking endogenous peroxidase activity and nonspecific binding of avidin and biotin as described previously. We incubated these sections with a primary antibody, goat anti-human CSF-1 antibody (N-16; Santa Cruz, CA), and we detected the primary antibody by incubation with biotinylated rabbit anti-goat Ab followed by development with 3’3-diaminobenzidine (Vector; Burlingame, CA). We verified staining specificity by replacing the primary antibody with goat IgG (eBioscience, San Diego, CA) and by preabsorbing with peptide for CSF-1 antibody (Santa Cruz). We determined the percentage of positive TECs in ten randomly selected high-power fields.

Flow Cytometry
We prepared and stained single-cell suspensions from kidneys, blood leukocytes, and BM Mo as described previously. We collected 0.5 × 10^6 to 1.0 × 10^7 total kidney cells and 0.5 × 10^5 to 1.0 × 10^6 blood leukocytes/BM Mo using a FACS-Calibur (Becton Dickinson, San Jose, CA) and analyzed the data using Flowjo software (Tree Star, Palo Alto, CA). The BM Mo and circulating Mo were characterized by gating on the SSC^low/CD11b^high population.

Antibodies
We used the following antibodies from eBioscience (San Diego, CA) for FACS analysis: FITC-conjugated anti-CD4 (L3T4), anti-CD8 (5 to 6.7), and anti-CD11b (M1/70) band anti-CD45.2 (104); phycoerythrin (PE)-conjugated anti-CD4, anti-CD45.2, anti-CD69 (H1.2F3), and anti-CD86 (GL1); PE-Cy5-conjugated anti-CD8; and allophyocyanin-conjugated anti-CD4, anti-CD45.2, and anti-F4/80 (BM8). We used FITC- and allophyocyanin-conjugated anti-CD68 antibody (FA11) and PE-conjugated Ly6C (Serotec, Oxford, United Kingdom), purified CX3CR1 (2A9-1; MBL, Woburn, MA), and purified rabbit anti-mouse CCR2 (NovusBiologicals). For the secondary PE- or allophyocyanin-conjugated Ab, we used goat-anti-rabbit from Jackson ImmunoResearch Laboratories (West Grove, PA) and biotin-conjugated rabbit anti-goat antibody (Vector Laboratories, Burlingame, CA). To detect biotin-conjugated secondary antibodies, we used streapavidin PE or allophyocyanin (Jackson ImmunoResearch Laboratories, West Grove, PA).

BrdU Treatment

In Vivo.
We injected mice with BrdU (2 mg/mouse intraperitoneally, every 12 h, Sigma, St. Louis, MO) for 24 and 48 h before sacrifice. BrdU^+ cells were analyzed by flow cytometry with an anti-BrdU antibody (eBioscience, San Diego, CA).

In Vitro.
We incubated freshly isolated blood leukocytes for 6, 12, and 24 h in media containing BrdU (1 mg/ml) and analyzed the BrdU^+ cells by flow cytometry.

Adoptive Transfer
We isolated BM from MacGreen;MRL-Faslpr mice and adoptively transferred these EGFP^+ cells (2 × 10^5) by intravenous injection into the tail. We sacrificed these mice after 24 and 48 h and prepared the kidney and blood samples to detect EGFP^+ cells using flow cytometry.
as described previously.41 We adoptively transferred EGFP+ BM cells into Csf-1<sup>−/−</sup>;MRL-Faslpr<sup>−/−</sup> mice. These mice were then injected with rCSF-1 (50 μg/kg body wt) every 12 h, and sacrificed after 24 and 48 h.

**BM-Derived Mø**

We isolated BM Mø as described previously.43,44 When the cultured cells were confluent, we incubated cells for 24 h without CSF-1 to synchronize the cells, before proceeding with specific stimulations.

**Proliferation**

BM Mø (1 × 10⁶ well) were stimulated with mouse CSF-1 (10 ng/ml, Preprotech, Rocky Hill, NJ) for 24 and 48 h. We analyzed proliferation using the MTT colorimetric assay (Roche, Palo Alto, CA) according to the manufacturer’s instructions.

**Serum, Urine, and Renal Biopsy Samples**

Human kidney sections with a confirmed pathologic diagnosis lupus nephritis type III, IV, and V (according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis<sup>45</sup> and activity and chronicity index<sup>27,28</sup> or minimal-change GN and membranous GN were provided by the Department of Pathology, Rush University Medical Center, Chicago, Illinois. Urine and serum samples were collected at the Department of Medicine, Johannes-Gutenberg University Mainz, Germany. Serum and urine samples were stored at −20°C. The use of the samples was reviewed and approved by the Standing Committee for Clinical Studies of the Johannes-Gutenberg University in adherence to the Declaration of Helsinki. Samples were taken from patients attending the outpatient clinic who fulfilled at least four of the American College of Rheumatology criteria for the classification of systemic lupus erythematosus after informed consent. The characteristics of the patients are summarized in Table 1. The clinical parameters (C3/C4; dsDNA, creatinine, and proteinuria) were evaluated at the Johannes-Gutenberg University Mainz, Germany. Note that we define C3/C4 levels according to the following criteria: C3 (high >0.09g/L; low C3<0.09; and C4 (high C4>0.01; low C4<0.01). The control human kidney tissue was taken from uninvolved areas of nephrectomies for malignancy and preimplantation donor kidney biopsies.

**Statistical Analysis**

The data representing the mean ± SEM were prepared using GraphPad PRISM version 4.0. We used the nonparametric Mann-Whitney U test to evaluate P values. For correlation analysis, we used the Spearman correlation calculation. For statistical evaluation of survival, we used the log rank test.

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**DISCLOSURES**

None.

**REFERENCES**


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