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Activated Protein C Attenuates Systemic Lupus Erythematosus and Lupus Nephritis in MRL-Fas(lpr) Mice

Julia Lichtnekert,1 Khader Valli Rupanagudi,1 Onkar P. Kulkarni, Murthy Narayana Darisipudi, Ramanjaneyulu Allam,2 and Hans-Joachim Anders

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease leading to inflammatory tissue damage in multiple organs (e.g., lupus nephritis). Current treatments including steroids, antimalarials, and immunosuppressive drugs have significant side effects. Activated protein C is a natural protein with anticoagulant and immunomodulatory effects, and its recombinant version has been approved by the U.S. Food and Drug Administration to treat severe sepsis. Given the similarities between overshooting immune activation in sepsis and autoimmunity, we hypothesized that recombinant activated protein C would also suppress SLE and lupus nephritis. To test this concept, autoimmune female MRL-Fas(lpr) mice were injected with either vehicle or recombinant human activated protein C from week 14–18 of age. Activated protein C treatment significantly suppressed lupus nephritis as evidenced by decrease in activity index, glomerular IgG and complement C3 deposits, macrophage counts, as well as intrarenal IL-12 expression. Further, activated protein C attenuated cutaneous lupus and lung disease as compared with vehicle-treated MRL-Fas(lpr) mice. In addition, parameters of systemic autoimmunity, such as plasma cytokine levels of IL-12p40, IL-6, and CCL2/MCP-1, and numbers of B cells and plasma cells in spleen were suppressed by activated protein C. The latter was associated with lower total plasma IgM and IgG levels as well as lower titers of anti-dsDNA IgG and rheumatoid factor. Together, recombinant activated protein C suppresses the abnormal systemic immune activation in SLE of MRL-Fas(lpr) mice, which prevents subsequent kidney, lung, and skin disease. These results implicate that recombinant activated protein C might be useful for the treatment of human SLE. The Journal of Immunology, 2011, 187: 3413–3421.
endothelial protein C receptor (EPCR) (13). Activated protein C also had protective effects on chronic inflammatory bowel disease by reducing intestinal microvascular inflammation (14). Given these beneficial effects of activated protein C on multiple hyperinflammatory and autoimmune disease states, we speculated that activated protein C may also suppress SLE and lupus nephritis. To address this question, we treated autoimmune and nephritic MRL-Fas(lpr) mice with recombinant human activated protein C and characterized the therapeutic effects on systemic autoimmunity, lupus nephritis, and other organ manifestations of SLE.

Materials and Methods

Mice and experimental protocol

Eight-week-old female MRL-Fas(lpr) mice were obtained from Harlan Winkelmann (Borchen, Germany) and kept under pathogen-free and normal housing conditions in a 12-h light and dark cycle. Water and standard chow (Ssniff, Soest, Germany) were available ad libitum. At the age of 14 wk, groups of 15 mice each started to receive either 5 mg/kg body weight mouse recombinant human activated protein C (Xigris; Lilly Deutschland) or vehicle PBS (50 mM Tris, 150 mM NaCl [pH 7.6]) only by i.p. injection on alternative days. All mice were sacrificed by cervical dislocation at the end of the study period. All experiments were performed according to German animal protection laws and had been approved by the local government authorities.

Morphological evaluation

Kidneys and lungs from all mice were fixed in 10% buffered formalin, processed, and embedded in paraffin. Two-micrometer paraffin sections for periodic acid-Schiff (PAS) stains were prepared from lung and kidney tissue samples following routine protocols as described (15). The severity of the renal lesions was graded using the indices for activity and chronicity as described for human lupus nephritis (16). IgG and Mac-2 immunostaining staining was performed as described using the following primary Abs: anti-mouse Mac-2 (Cedarlane Laboratories, Toronto, Ontario, Canada) and anti-mouse IgG (Caltag Laboratories, Burlingame, CA). Immunostaining was performed on frozen sections using anti-mouse C3c (Nordic Immunological Laboratories, Tilburg, The Netherlands). The severity of the peribronchial inflammation was graded semiquantitatively from 0–4 as described (17).

Plasma cytokines

For the detection of IgG and its isotypes, plates were precoated with anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3, and IgM (Bethyl Laboratories, Montgomery, TX), and sera were applied at dilutions of 1:10,000 to 1:50,000. The assay was developed with HRP-labeled goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3, and IgM Abs (Bethyl Laboratories). A standard curve was derived with purified mouse, IgG, IgG1, IgG2a, IgG2b, IgG2c, IgG3, and IgM Abs (Bethyl Laboratories). Anti-nuclear Abs (ANA) were detected by indirect immunofluorescence using HEp-2 cells as described (18). In brief, ELISA plates were coated with poly-L-lysine and then with 1 μg/ml DNA (purified from mouse tails after proteinase K digestion and isopropanol precipitation) in SSC buffer. Next, the plates were incubated with 1:2000 diluted serum samples, and the assay was developed with HRP-labeled goat anti-mouse IgG (Bethyl Laboratories). Plasma levels of rheumatoid factor were estimated by ELISA as described (19). Endogenous levels of activated protein C was estimated in plasma samples using an ELISA kit for Activated Protein C from Usn Life Science (Wuhan, China).

Renal function analysis

Mouse albumin was estimated in urine samples using a Mouse Albumin ELISA Quantitation set (Bethyl Laboratories). Mouse creatinine was estimated in urine and serum samples using a Creatinin FS kit (DiaSys
Diagnostic Systems, Holzheim, Germany). Mouse blood urea nitrogen (BUN) was estimated in plasma samples using a Urea FS kit (DiaSys Diagnostic Systems).

Flow cytometry

Cell suspensions were stained with the following Abs from BD Pharmingen (Heidelberg, Germany): anti-mouse CD4-allophycocyanin, CD8-PerCP, CD3-FITC, CD25-PerCP, CD45R-allophycocyanin, CD11c-FITC, B220-Alexa Fluor 647, IgD-FITC, IgM-PE, CD23-PE, CD21-FITC, CD40-FITC, κL Chain-PE, and FITC-BrdU (catalog number 559615).

The following Abs were procured from Serotec (Oxford, U.K.): F4/80-allophycocyanin, CD19-FITC, and MHC class II (MHC II)-FITC. Siglec-H is from eBioscience (San Diego, CA). Intracellular IFN-γ and IL-17A staining was performed as described elsewhere (20). Briefly, splenocytes were isolated by standard protocols and incubated with PMA (5 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Calbiochem) for 5 h. After 30 min, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. After washing, cells were stained for CD3 and CD4 (see above). After permeabilization of the cells with Cytofix/Cytoperm (BD Biosciences), cells were stained with IFN-γ–PE (eBioscience) and IL-17A–Alexa Fluor 647 (BD Biosciences).

Real-time PCR

Total RNA was isolated from spleen and kidney tissue using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 1 μg total RNA by using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). SYBR Green I Dye detection system was used for quantitative PCR (qPCR) on LightCycler 480 (Roche Diagnostics, Mannheim, Germany). The sequences of the gene-specific primers (500 nM; Metabion, Martinsried, Germany) used are listed in Table II. All reactions were performed in triplicate, and negative controls contained no template DNA. We used 18S rRNA as an endogenous control for normalization. To verify that the primer pair produced only a single product, a dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 60–95°C. Data were analyzed using the comparative threshold cycle (ΔΔCt) method.

Statistical analysis

Data are expressed as means ± SD. The statistical differences between two groups were performed using a Student t test, and comparison of three groups was performed using one-way ANOVA. A p value <0.05 was considered statistically significant. All statistical calculations were performed using commercially available statistical software GraphPad Prism (version 5.0; GraphPad, San Diego, CA).

Results

Recombinant activated protein C suppresses renal pathology in MRL-Fas(lpr) mice

We addressed our hypothesis by observing MRL-Fas(lpr) mice until the first signs of lupus nephritis occurred (i.e., albuminuria of >3 μg/dl, which was evident in almost all animals at 14 wk of age). Note that serum levels of activated protein C in MRL-Fas(lpr) mice were significantly lower compared with MRL wt mice at 14 wk of age (Supplemental Fig. 1). Albuminuric MRL-Fas(lpr) mice were randomized to two treatment regimen with i.p. injections given on alternate days for 5 wk. One group was treated with recombinant activated protein C at a dose of 5 mg/kg in PBS, whereas the other group received PBS (vehicle) injections only. At 18 wk of age, vehicle-treated MRL-Fas(lpr) mice developed diffuse proliferative glomerulonephritis, which was associated with diffuse mesangial matrix expansion, profound mesangial cell proliferation, and focal segmental glomerulosclerosis. Cellular crescents and global glomerulosclerosis were often seen (Fig. 1A).

### FIGURE 2. Kidney qPCR and glomerular macrophages.

A, Renal mRNA expression of IL-12p40, IL-6, and CCL2/MCP-1 in MRL-Fas(lpr) mice was determined by quantitative real-time PCR (Table II). Data are expressed as a mean ratio to the respective 18S rRNA mRNA expression level ± SEM. B, Renal sections of 18-wk-old MRL-Fas(lpr) mice were stained with an anti–Mac-2 Ab. Representative images show Mac-2–positive glomerular macrophages and quantification of Mac-2–positive glomerular macrophages. Images are representative for 15 mice in each treatment group and 5 mice in MRL wt group. Data are expressed as means ± SEM. Original magnification ×100. *p < 0.05, **p < 0.01 versus vehicle group (wt MRL, n = 5; vehicle, n = 15; activated protein C [APC], n = 15).
activated with extensive glomerular IgG and complement factor C3c deposits presenting in mesangial and capillary staining patterns altogether, indicating diffusely proliferative lupus nephritis as the renal manifestation of SLE in MRL-Fas(lpr) mice (Fig. 1A, 1B). Treatment with recombinant activated protein C suppressed lupus nephritis as documented by a significant reduction of the lupus nephritis activity index that encompasses glomerular cell proliferation, matrix deposition, leukocyte infiltration, focal necrosis, crescent formation, and tubulointerstitial inflammation (Fig. 1B). Glomerular crescents and globally sclerotic glomeruli were no longer observed (Fig. 1A). This improvement of renal structure was associated with a trend toward less proteinuria and BUN levels. Serum creatinine levels in our model were low and not significantly reduced in activated protein C-treated mice (Supplemental Fig. 2). Together, recombinant activated protein C attenuates lupus-like renal pathology in MRL-Fas(lpr) mice.

Recombinant activated protein C reduces renal immune complex disease and intrarenal inflammation in MRL-Fas(lpr) mice

In SLE, renal damage and dysfunction result from immune complex glomerulonephritis, in which glomerular deposits of immune complexes activate the components of the classical complement pathway, which finally leads to the assembly of the C5b-9 multimer membrane attack complex (21). The favorable effects of recombinant activated protein C treatment on markers of active lupus nephritis were associated with a significant reduction of glomerular IgG deposits as well as the deposition of complement factor C3c, a marker of intraglomerular complement activation (Fig. 1A). Local complement activation has the potential to activate (and eventually kill) renal cells; therefore, we next evaluated the intrarenal expression of proinflammatory mediators. Recombinant activated protein C treatment reduced mRNA expression levels of the proinflammatory cytokines IL-6 and IL-12 p40 and of the chemokine CCL2/MCP-1 (Fig. 2A), which correlated with a significant reduction of Mac2-positive glomerular macrophages (Fig. 2B). Together, we conclude that activated protein C treatment reduces SLE-associated immune complex glomerulonephritis, glomerular complement activation, and intrarenal inflammation.

Recombinant activated protein C improves lung disease and cutaneous lupus in MRL-Fas(lpr) mice

Is the therapeutic effect of recombinant activated protein C limited to renal manifestations of SLE? To address this question, we carefully monitored the skin of MRL-Fas(lpr) mice throughout the duration of the study and analyzed the lungs upon sacrifice. Skin disease is the most common organ manifestation in SLE (1). Treatment with recombinant activated protein C significantly delayed the onset of facial dermatitis as well as of neck ulcerations as compared with vehicle-treated MRL-Fas(lpr) mice (Fig. 3A). During that period, all vehicle-treated MRL-Fas(lpr) mice developed skin disease characterized by typical lesions in the facial area (Fig. 3B). In contrast, among activated protein C-treated MRL-Fas(lpr) mice, <20% developed skin lesions. Autoimmune lung disease is another manifestation of SLE (22). Vehicle-treated MRL-Fas(lpr) mice displayed focal areas of peribronchial and perivascular lymphocyte infiltrates. Such infiltrates were significantly decreased in 18-wk-old activated protein C-treated MRL-Fas(lpr) mice (Fig. 3D). This was evaluated by a semiquantitative lung injury score ranging from 0–3 (Fig. 3D). Thus, recombinant activated protein C protects MRL-Fas(lpr) mice not only from lupus nephritis but also from autoimmune lung disease and cutaneous lupus.

Recombinant activated protein C suppresses the abnormal humoral immunity in MRL-Fas(lpr) mice

As treatment with recombinant activated protein C reduced glomerular immune complex deposition, the question arises whether activated protein C modulates systemic autoimmunity and lupus autoantibody production in MRL-Fas(lpr) mice. At the age of 18 wk, MRL-Fas(lpr) mice displayed significant hypergammaglobulinemia, which was significantly reduced by activated protein C treatment (Fig. 4A). This effect related especially to IgG of the IgG2a and IgG3 isotype, which are known to activate complement

FIGURE 3. Recombinant activated protein C improves lung disease and cutaneous lupus in MRL-Fas(lpr) mice. A, The plot shows the percentage of mice developing skin lesions during the treatment period. Mice of both groups were regularly checked for cutaneous lupus manifestations, which typically occur in the facial or neck/back area and were less common in activated protein C-injected mice. The image shows representative mice from both groups at 18 wk of age (B). C, Lung sections of 18-wk-old MRL-Fas(lpr) mice of all groups were stained with PAS. Note the peribronchial and perivasculapillary lymphocyte infiltrates were less in activated protein C-injected mice when compared with vehicle-injected mice. Images are representative for 15 mice in each treatment group and 5 MRL wt mice. Original magnification ×100. Morphometry was used to quantify the peribronchial and perivascular inflammation by using a score ranging from 0–3 and scored semi-quantitatively as described in Materials and Methods section. Data represent mean scores ± SEM. **p < 0.01 versus vehicle group.
Activated protein C reduces autoantibodies in MRL-Fas(lpr) mice. Plasma levels of total IgG (A) and total IgM (B) were determined by ELISA. C, ANA staining patterns on Hep2 human epithelial cells for serum, derived from MRL wt, vehicle-treated, and activated protein C-treated MRL-Fas(lpr) mice at 1:200 dilution. Original magnification $\times 1000$. Plasma levels of total IgG against dsDNA (D) or rheumatoid factor (E) were determined by ELISA. Data are expressed as means $\pm$ SEM ($n$ = 15 each treatment group; $n$ = 5 MRL/wt). *$p < 0.05$, **$p < 0.01$ versus vehicle group.

in murine lupus nephritis (Table I). Plasma IgM levels were affected the same way (Fig. 4B). Furthermore, activated protein C reduced the amount of circulating lupus autoantibodies as evidenced by a marked reduction in diffuse ANA staining intensity on Hep2 cells as well as quantitative plasma anti-dsDNA IgG and rheumatoid factor (anti-IgG) measured by ELISA (Fig. 4C–E). Serum IgG as well as autoantibodies derive from Ig-producing plasma cells. Therefore, we performed flow cytometry for CD138 and $\kappa$ L chain-positive plasma cells in spleen of both treatment groups. Activated protein C treatment significantly reduced the absolute numbers of total plasma cells (Fig. 5A). To analyze the effect of activated protein C on short-lived and long-lived plasma cells, we injected BrdU in mice 5 d before sacrifice, which allowed us to differentiate short- from long-lived plasma cells. Activated protein C treatment also significantly reduced the short- and long-lived spleen plasma cells (Fig. 5B). Similarly, follicular B cell (CD23highCD21low), marginal zone B cells (CD23lowCD21high), and mature B cells (IgM IgD) were reduced by activated protein C treatment (Fig. 5C), whereas other major spleen T cell populations (i.e., CD4 T cells, CD8 T cells, and CD4/CD8 double-negative T cells) remained unaffected (Table I). Interestingly, activated protein C treatment reduced the Th1 and Th17 polarization of T cells (Table I). Together, treatment with recombinant activated protein C specifically reduces B cells and plasma cells as well as Th1 and Th17 polarization of T cells, which decreases overproduction of Igs and lupus autoantibodies causing lupus nephritis in MRL-Fas(lpr) mice.

Activated protein C treatment suppresses the activation of APCs and systemic inflammation in MRL-Fas(lpr) mice

The activation of activated protein C is a major stimulus for B cell proliferation and autoantibody production in SLE (23, 24). Therefore, we analyzed the activation of spleen dendritic cells by flow cytometry in MRL-Fas(lpr) mice of both treatment groups. Activated protein C treatment significantly reduced the total numbers of CD11c cells including CD4/CD11c and CD8/CD11c cells (Fig. 6A). Activated protein C treatment also reduced dendritic cell activation as demonstrated by significant reduction in CD11c-positive cells that were also positive for the activation markers CD40 or MHC II (Fig. 6B). In addition, activated protein C decreased expression marker CD40 in CD11c/CD4 and CD11c/CD8 cells (Supplemental Fig. 3). Consequently, spleens of activated protein C-treated MRL-Fas(lpr) mice expressed much lower mRNA levels of several proinflammatory cytokines such as IL-6, IL-12p40, and CCL2/MCP-1 (Fig. 7) using primers listed in Table II. This finding was mirrored by lower plasma protein levels of these cytokines (Fig. 8). Spleen mRNA and plasma proteins levels of TNF remained unaffected by activated protein C treatment (Figs. 7, 8). Thus, treatment with recombinant activated protein C reduced the activation of splenic dendritic cells as well as systemic inflammation in MRL-Fas(lpr) mice.

**Discussion**

Recombinant activated protein C was recently shown to suppress a number of hyperinflammatory and autoimmune states; hence, we had hypothesized that recombinant activated protein C may also suppress SLE and lupus nephritis. Our experiments using autoimmune and nephritic MRL-Fas(lpr) mice now document that recombinant human activated protein C can suppress lupus

Table I. Phenotype of SLE in MRL-Fas(lpr) mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle</th>
<th>Activated Protein C</th>
<th>$p$ Value by $t$ Test</th>
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<tr>
<td>FACS (cells/spleen [millions])</td>
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<tr>
<td>CD3+CD4+</td>
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<td>45.17 ± 7.09</td>
<td>0.3873</td>
</tr>
<tr>
<td>CD3+CD8+</td>
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<tr>
<td>CD3+CD4−CD8−</td>
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<tr>
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<td>Th17 cells</td>
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<td>0.0340 ± 0.007</td>
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<td>Serum IgG isotypes</td>
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<tr>
<td>IgG1</td>
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<td>IgG3</td>
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nephritis as well as other organ manifestations of SLE such as cutaneous lupus and lung disease. Lupus nephritis is triggered by glomerular immune complex deposits that activate complement, increased intrarenal expression of proinflammatory cytokines and chemokines, and subsequent recruitment of macrophages and other immune cells that contribute to renal inflammation and damage. Therefore, active lupus nephritis in humans and MRL-Fas(lpr) mice is characterized by diffuse mesangioproliferative glomerulonephritis and often associated with fibrinoid tuft necrosis, focal adhesions, and crescent formation. In this study, we used Drotrecogin alfa activated (Xigris; Lilly Deutschland), a human recombinant activated protein C that was shown to be effective in mice. Xigris (Lilly Deutschland) had a neuroprotective effect in murine neuronal and brain endothelial cell injury models (25) and mice. Xigris (Lilly Deutschland) had a neuroprotective effect in murine neuronal and brain endothelial cell injury models (25) and prevented septic-like reactions after injection of histones into mice (26). In sepsis, it was initially thought that activated protein C has antithrombotic and profibrinolytic functions, but recently, it has been postulated that cytoprotective effects are responsible for reduced mortality. Treatment with activated protein C variants with diminished anticoagulant activity could still have cytoprotective effects in a mouse model of sepsis (27). This suggests that the effects of activated protein C in MRL-Fas(lpr) mice are due to cytoprotective signaling properties. In addition, we could detect lower serum activated protein C levels in MLR-Fas(lpr) mice compared with MRL wt mice, indicating a suppression of the protein C pathway during SLE. In septic patients, reduced activated protein C levels have been associated with an increased risk of death (28, 29).

Aberrations of monocyte/macrophage phenotype and function are increasingly recognized in SLE and animal models of the disease. Cao et al. (30) showed that the anti-inflammatory activity of activated protein C on macrophages is dependent on integrin CD11b/CD18, but not on EPCR. They showed that CD11b/CD18-bound activated protein C, facilitated cleavage and activation of PAR-1, leading to enhanced production of sphingosine 1-phosphate and suppression of the proinflammatory response of activated macrophages (30). Activated protein C decreases the release of the MIP-1α from the monocytic cell line THP-1 and from human monocytes of septic patients (31). Activated protein C also inhibits LPS-induced nuclear translocation of NF-κB, TNF-α, IL-1β, IL-6, and IL-8 production in the THP-1 cells (32, 33).

Treatment with recombinant activated protein C over a period of 5 wk suppressed all of the aforementioned histopathological abnormalities of lupus nephritis. Renal function parameters like proteinuria, BUN, and serum creatinine showed nonsignificant trends toward improvement of secretory and barrier functions, indicating that recombinant activated protein C is not as potent as pulse dosing with cyclophosphamide, which we previously reported to almost completely abrogate kidney disease in this model (34). What are the mechanisms by which activated protein C treatment suppresses kidney disease in MRL-Fas(lpr) mice? Activated protein C can elicit direct cytoprotective effects on glomerular endothelial cells and podocytes by inhibiting the intrinsic mitochondrial apoptosis pathways via PAR-1 and EPCR in mice with diabetic nephropathy (13). This effect could contribute to the protective effects on glomerular damage in MRL-Fas(lpr) mice. Recombinant activated protein C has been reported to prevent microvascular inflammation in autoimmune bowel disease (14). Microvascular inflammation is a central element of infectious as well as autoimmune tissue damage and mediated by the activation of endothelial cells. A recent report first described that extracellular histones, which are released from dying cells, elicit direct killing effects on endothelial cells followed by microvascular inflammation, a process that is inhibited by recombinant activated protein C, because the proteolytic activity of activated protein C degrades histones to immunologically inactive peptides (26).

**FIGURE 5.** Activated protein C affects plasma cells and B cells in MRL-Fas(lpr) mice. FACS analysis of the absolute number of total spleen plasma cells (A) as well as short- and long-lived spleen plasma cells (B). C. FACS analysis of the absolute number of the follicular B cells, marginal zone B cells, and mature B cells from spleen. Data are expressed as means ± SEM. \(n=10\) each treatment group. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) versus vehicle group.

**FIGURE 6.** Activated protein C suppresses dendritic cell activation in MRL-Fas(lpr) mice. Spleen cell suspensions were prepared for flow cytometry by using specific Abs that identify subsets of dendritic cells. A, FACS analysis of the absolute numbers of CD11c, CD4/CD11c, and CD8/CD11c cells. B, FACS analysis of total numbers of CD11c-positive cells that were also positive for the activation markers CD40 or MHC II. Data are expressed as means ± SEM \(n=10\) each treatment group. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) versus vehicle group.
of extracellular histones likely contribute also to lupus nephritis, at least in areas of focal necrosis, because histones are an essential element of neutrophil extracellular traps that were recently shown to mediate glomerular inflammation in anti-neutrophil cytoplasmic autoantibody-associated renal vasculitis (35).

Activated protein C is a serine protease derived from its inactive zymogen protein C, and the fact that kidney, skin, and lung disease were all improved similarly suggests that recombinant activated protein C might suppress systemic disease mechanisms of SLE. In fact, our analysis revealed that activated protein C treatment affected many elements of autoimmunity. For example, lupus nephritis and most other tissue pathologies in SLE develop secondary to immune complex disease involving a polyclonal expansion of autoreactive B cells, plasma cells, and anti-nuclear autoantibody production (36). Activated protein C significantly reduced all of these elements of humoral autoimmunity as well as subsequent glomerular IgG deposits, indicating that protection from severe lupus nephritis is due to a systemic suppression of immune complex disease. The proliferation of autoreactive B cells in lupus depends on autoantigen-presentation and costimulatory signals from dendritic cells (23, 24). Therefore, it is of note that recombinant activated protein C suppressed the activation of spleen dendritic cells, which is an important determinant of SLE disease activity (23, 24, 37). The activation of dendritic cells also contributes to systemic inflammation; hence, we attribute the activated protein C-mediated suppression of plasma cytokine levels to its capacity to suppress dendritic cells. Pretreatment with activated protein C

![FIGURE 7. Spleen qPCR. Spleen cytokine and chemokine mRNA expression in MRL-Fas(lpr) mice. The levels of spleen mRNA expression were determined for the IL-12p40, IL-6, CXCL10, CCL2, and TNF by quantitative real-time PCR. Data are expressed as mean ± SEM (MRL/wt, n = 5; vehicle, n = 15; activated protein C [APC], n = 15). *p < 0.05, **p < 0.01 versus vehicle group.](http://www.jimmunol.org/)

![FIGURE 8. Plasma cytokine ELISA. Plasma protein levels of IL-12p40, IL-6, MCP-1, and TNF were estimated by ELISA. Data are expressed as means ± SEM (n = 15 each treatment group; n = 5 MRL/wt). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle group. ND, not detectable.)](http://www.jimmunol.org/)
in isolated macrophages activated in vitro with LPS showed less release of IL-6, whereas activated protein C-pretreated T cells activated with CD3/CD28 showed inhibition of NF-κB signaling (11). The signaling effects of activated protein C on B cells are still unknown. A recent study discovered that the activation of EPCR on the specific CD8+ subset of spleen dendritic cells is sufficient to suppress immune responses during sepsis and that activated protein C has additional immunosuppressive effects on other dendritic cells in an EPCR-independent manner (38). Our own data are in line with these observations, as recombinant activated protein C reduced the activation of CD4+–positive and CD8+–positive spleen dendritic cell subsets in MRL-Fas(lpr) mice. As such, we assume that activated protein C did not have direct effects on T and B cells, but rather that these are secondary to activated protein C-mediated suppression of dendritic cell activation. Interestingly, recombinant activated protein C did not have an effect on spleen T cell counts, and its suppressive effect was limited to Th1 and Th17 T cells, two effector T cell subpopulations that play a dominant role in the cellular component of autoimmunity in human SLE as well as in SLE of MRL-Fas(lpr) mice (20, 39). Also, in a mouse model of multiple sclerosis, the amelioration of disease after activated protein C treatment was accompanied with inhibition of Th1 and Th17 cytokines (11). We have recently shown that cyclophosphamide or mycophenolate mofetil cause broad T cell ablation in MRL-Fas(lpr) mice, like in humans, which should be a causative factor for the infectious complications that are associated with such therapies (27, 40). In this regard, recombinant activated protein C may have a better toxicity profile as T cell ablation was not observed. The most frequent toxicity that occurred in the recombinant activated protein C sepsis trials was bleeding (7, 41), notifying activated protein C’s toxicity profile as T cell ablation was not observed. Therefore, recombinant activated protein C appears to be suitable to block the abnormal immune activation of SLE and thereby prevent lupus nephritis and other lupus manifestations. As recombinant activated protein C (Xigris) is a U.S. Food and Drug Administration-approved drug, it should be feasible to test whether recombinant activated protein C is also effective in suppressing human SLE.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplementary figures

**Figure 1. Plasma APC levels in lupus and wildtype mice.** Plasma levels of Activated protein C was quantified in wildtype MRL and in MRL-Fas(lpr) mice. **p<0.01. (Wild type MRL n=5, MRL-Fas(lpr) n=15).**

**Figure 2. Proteinuria, blood urea nitrogen and serum creatinine levels in MRL-Fas(lpr) mice.** Proteinuria was determined at 18 weeks of age (A). Urinary albumin/creatinine ratio is a functional marker of damage to the glomerular filtration barrier. Albumin/creatinine ratio is reduced substantially in APC-injected mice when compared to vehicle-injected mice. (B) BUN levels at 18 weeks of age were substantially, but not significantly reduced in APC-treated mice. (C) Serum creatinine levels were low in MRL-Fas(lpr) mice. There was no significant reduction in APC-treated mice. Data are expressed as means ± SEM (Vehicle n=15, APC n=15).

**Figure 3. Dendritic cell activation in MRL-Fas(lpr) mice.** FACS analysis of total numbers of CD11c/CD4 or CD11c/CD8 positive cells that were also positive for the activation markers CD40. Data are expressed as mean percentages of all splenocytes ± SEM (n=10 each treatment group). *p<0.05, **p<0.001 versus vehicle group.