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*J Immunol* 2012; 189:2054-2062; Prepublished online 13 July 2012; doi: 10.4049/jimmunol.1201097
http://www.jimmunol.org/content/189/4/2054

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/07/13/jimmunol.1201097.DC1

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Chronic Exposure to Staphylococcal Superantigen Elicits a Systemic Inflammatory Disease Mimicking Lupus

Vaidehi R. Chowdhary,* Ashenafi Y. Tilahun,† Chad R. Clark,† Joseph P. Grande,‡ andGovindarajan Rajagopalan†

Chronic nasal and skin colonization with superantigen (SAg)-producing Staphylococcus aureus is well documented in humans. Given that trans-mucosal and trans-cutaneous absorption of SAgs can occur, we determined whether chronic exposure to small amounts of SAg per se could activate autoreactive CD4+ and CD8+ T cells and precipitate any autoimmune disease without further external autoantigenic stimulation. Because HLA class II molecules present SAg more efficiently than do mouse MHC class II molecules, HLA-DQ8 transgenic mice were implanted s.c. with mini-osmotic pumps capable of continuously delivering the SAg, staphylococcal enterotoxin B (total of 10 µg/mouse), or PBS over 4 wk. Chronic exposure to staphylococcal enterotoxin B resulted in a multisystem autoimmune inflammatory disease with features similar to systemic lupus erythematosus. The disease was characterized by mononuclear cell infiltration of lungs, liver, and kidneys, accompanied by the production of anti-nuclear Abs as well as IFN-γ and IL-12. In conclusion, our study suggests that chronic exposure to extremely small amounts of bacterial SAg could be an etiological factor for systemic lupus erythematosus. The Journal of Immunology, 2012, 189: 2054–2062.

The Journal of Immunology
SAs is possible in *S. aureus* carriers. This could lead to activation of the autoreactive T and B lymphocytes that exist in those individuals. Because SAs can also activate APCs, either directly or indirectly, SAs might provide the necessary inflammatory milieu for continued expansion of pathogenic autoreactive clones, break immune tolerance, and, thereby, contribute to autoimmunity.

Human studies showed that *S. aureus* carriage is associated with certain autoimmune diseases, such as granulomatosis with polyangiitis, multiple sclerosis, and rheumatoid arthritis, through their SAs (22–26). However, no direct experimental evidence exists to prove that staphylococcal SAs (SSAs) by themselves (without the use of exogenous Ags) are capable of inducing any spontaneous autoimmune disease. Conventional laboratory mice are not suitable for such investigations because SSAg bind weakly to mouse MHC class II molecules. However, it is well established that SSAg bind more efficiently to human MHC (HLA) class II molecules (27). Therefore, we and other investigators showed that transgenic mice expressing HLA class II molecules, such as HLA-DQ6, -DQ8, or -DR3, mount a strong immune response to SSAgs and are excellent tools with which to study the immunopathogenesis of diseases caused by SAgs (15, 28–35). Because several additional knockout mice are available on the HLA-DQA background (15), using the HLA-DQ8 transgenic mouse model, we explored whether chronic exposure to extremely small nonlethal amounts of SSAg by itself can precipitate any autoimmune disease without immunization with any autoantigens.

**Materials and Methods**

**Mice**

HLA-DQ8 transgenic mice and HLA-DQ8 transgenic mice lacking CD4+ T cells (HLA-DQ8.CD4−), CD8+ T cells (HLA-DQ8.CD8−), STAT4 (DQ8.STAT4−), STAT6 (DQ8.STAT6−), and CD28 (DQ8.CD28−) were described previously (30). DQ8 transgenic mice deficient for IFN-γ (DQ8.IFN-γ−) were generated by standard mating and genotyping procedures. Briefly, HLA-DQA and IFN-γ-deficient mice on a B6 background (The Jackson Laboratory) were mated. Heterozygous offspring were intercrossed, and the F1 line. Mice were bred within the barrier facility of the Mayo Clinic Immunogenetics Mouse Colony and moved to a conventional facility after weaning. All of the experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

**Reagents, Abs, and flow cytometry**

Endotoxin-reduced, highly purified staphylococcal enterotoxin B (SEB; Toxin Laboratories, Sarasota, FL) was dissolved in PBS at 1 mg/ml and stored frozen at −80°C in aliquots. The purity of SEB was verified by SDS-PAGE followed by Coomassie blue staining, and the absence of certain other SSAgs was verified using staphylococcal enterotoxin identification visual immunoassay (3M Tecra Staph Enterotoxin ID Test; 3M, St. Paul, MN). The following Abs from BD Biosciences were used for flow cytometry: CD4+ T cells (HLA-DQ8.CD4−), CD8+ T cells (HLA-DQ8.CD8−), STAT4 (DQ8.STAT4−), STAT6 (DQ8.STAT6−), and CD28 (DQ8.CD28−) were described previously (30). DQ8 transgenic mice deficient for IFN-γ (DQ8.IFN-γ−) were generated by standard mating and genotyping procedures. Briefly, HLA-DQA and IFN-γ-deficient mice on a B6 background (The Jackson Laboratory) were mated. Heterozygous offspring were intercrossed, and their pups were typed for the absence of endogenous mouse MHC class II molecules, the absence of the IFN-γ gene, and the presence of transgenic HLA-DQ8 mice. Mice of the required genotype were intercrossed for several generations to establish the DQ8.IFN-γ− line. Mice were bred within the barrier facility of the Mayo Clinic Immunogenetics Mouse Colony and moved to a conventional facility after weaning. All of the experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

**Implantation of mini-osmotic pump**

Mini-osmotic pumps (Alzet, Cupertino, CA) capable of continuous constant delivery of liquids over 4 wk were filled with either PBS or SEB (10 µg dissolved in PBS) as per the supplier’s protocol. PBS- or SEB-filled pumps were implanted s.c. into surgically prepared experimental mice of either sex (8–12 wk old) under anesthesia as per standard procedure. Surgical incisions were closed with stainless steel wound clips, which were removed 7–10 d later.

**Serum cytokine quantification and histopathology**

Groups of HLA-DQ8 transgenic mice, implanted with either SEB or PBS pumps, were sacrificed at the end of the experimental period. At the time of sacrifice, blood was collected from the mice into serum separation tubes (BD Biosciences, San Jose, CA). The serum was separated and then stored frozen in aliquots at −80°C. Concentration of cytokines was determined in the serum samples with a multiplex bead assay using the manufacturer’s protocol and their software and hardware (Bio-Plex; Bio-Rad, Hercules, CA).

Tissues collected in buffered formalin were embedded in paraffin, cut, and stained with H&E per the standard procedure for histopathologic analysis (by J.P.G.). The extent of organ inflammation was semiquantitatively determined using a scoring system based on the percentage involvement of the tissue being examined, as described previously, with slight modifications (36–39) (Supplemental Fig. 4). For liver, H&E-stained sections were viewed under the low-power objective (10×), and the extent of inflammatory infiltration (periporal and perivenular) relative to the field size was scored from 0 to 4, with 0 indicating no infiltrates and 4 indicating >50% of the field showing infiltration. Lungs and kidneys were scored on a scale of 0 to 3 in a similar manner, with 3 representing the most intense infiltration (Supplemental Fig. 4). Several fields from each tissue section and tissues from three or four individual mice in each group were evaluated for scoring.

**Immunofluorescence**

Immediately following sacrifice as discussed above, tissues from mice were also collected in optimal cutting compound (Sakura Finetek Tissue-Tek) and stored frozen at −80°C. Five-micrometer sections were cut using a cryostat, fixed in cold acetone, and stained with fluorochrome-conjugated Abs as per standard techniques. Sections were mounted using SlowFade Gold Antifade Reagent with DAPI (Invitrogen) and were analyzed using an Olympus AX70 research microscope (Olympus America, Center Valley, PA). Images were acquired using an Olympus DP70 camera.

**Detection and quantification of autoantibodies**

Anti-nuclear Abs were determined using HEP-2 cells, per the manufacturer’s protocol (Bio-Rad), at 1 in 50 dilution. Binding of mouse autoantibodies was detected using a FITC-conjugated goat anti-mouse Ab (Jackson ImmunoResearch). Abs to dsDNA, Smith Ag (Sm), and ribonucleoprotein were determined using separate ELISA kits for each of these autoantigens (Alpha Diagnostic International, San Antonio, TX), following the protocol provided by the supplier.

**Statistics**

All analyses were performed using GraphPad Prism (version 3.0a; San Diego, CA). Parametric testing between two unmatched groups was performed by the unpaired *t* test; *p* values < 0.05 were considered significant.

**Results**

**Chronic immune activation with SEB results in production of autoantibodies, immune complex glomerulonephritis, and multitiorgan pathology**

All sera from HLA-DQ8 transgenic mice (8/8) implanted with SEB pumps contained anti-nuclear Abs when tested using HEP-2 cells (Fig. 1A), whereas sera from mice implanted with PBS pumps showed no positive staining (0/6 mice). Mice implanted with SEB pumps also had significantly elevated levels of anti-dsDNA and anti-Sm Abs but did not develop anti-ribonucleoprotein Abs (Fig. 1B). Kidneys from mice implanted with SEB pumps showed pathological changes in the glomeruli, consistent with the World Health Organization class II classification for human lupus nephritis. In addition, mononuclear cell infiltration into periglomerular, peri-vascular, and tubulointerstitial regions was seen (Fig. 1C). Lungs also showed infiltration with mononuclear cells in peribronchial and perivascular regions (Fig. 1C). Liver sections showed extensive perivenular, as well as portal, infiltration (Fig. 1C). As expected, organs from PBS pump mice were devoid of any inflammatory infiltrates (Fig. 1C). The mean organ pathology in SEB
pump-implanted HLA-DQ8 mice was 1.5 (Supplemental Fig. 4). Immunohistochemical staining showed that the organ infiltrates largely consisted of CD4+ T cells, followed by CD8+ T cells, few CD11b+ cells, and few to no B cells in the liver (Fig. 2A), kidneys (Fig. 2C), and lungs (data not shown). A significant majority of the CD4+ T cells expressed TCR Vβ8, indicating that these T cells...
were activated by SEB (Fig. 2B). Kidneys from mice implanted with SEB pumps also showed deposition of IgG and C3 complement in the glomeruli (Fig. 2D). Overall, chronic exposure to SEB elicited autoimmune production and a systemic inflammatory disease.

Chronic exposure to SEB results in expansion of TCR Vβ8+ T cells

HLA-DQ8 transgenic mice implanted with SEB pumps showed splenomegaly and had significantly elevated total splenocyte numbers compared with HLA-DQ8 mice implanted with PBS pumps (160 ± 20 and 83.8 ± 8.8 million splenocytes in SEB and PBS pump-implanted mice, respectively, n = 6). The percentages, as well as the absolute numbers, of total CD4+ T cells (Fig. 3A, 3B) were increased in SEB pump-implanted mice. The absolute numbers of SEB-reactive CD4+ TCR Vβ8+ T cells were increased by 10-fold, with no significant changes in the SEB nonreactive CD4+ TCR Vβ6+ T cell subset. With respect to the CD8+ T cell population, the total and TCR Vβ6+ CD8+ T cells were similar between PBS- and SEB-treated mice; surprisingly, the percentage and absolute numbers of TCR Vβ8+ CD8+ T cell subsets were significantly reduced in the spleens of SEB-treated mice. Although the CD8+ T cell numbers were lower in the spleens of SEB-treated mice, they were still seen in the organs (Fig. 2). This could be attributed to preferential migration of activated CD8+ T cells to the organs (40). Mice implanted with SEB pumps also had more B cells and macrophages in the spleens, but this difference was not statistically significant (Fig. 3C). We also enumerated Foxp3+ cells, a marker for regulatory T cells (Tregs), by flow cytometry. As shown in Supplemental Fig. 1, chronic stimulation with SEB also resulted in a significant expansion of total CD4+Foxp3+ T cells, as well as TCR Vβ8+ Foxp3+ Tregs, in the spleen.

Activation profiles of T cells

Concomitant with the observed pathology and increase in splenocyte count, the expression profiles of certain activation markers (such as CD44 and GL7) on T cells were appreciably increased in the SEB-treated group, suggesting the presence of significantly higher numbers of activated/memory T cells in these mice (Supplemental Fig. 1B). However, the expression profiles of PD-1 and several chemokine receptors, such as CXCR3, CXCR4, CXCR5, and CCR7, were not significantly different between PBS and SEB pump-implanted mice (data not shown).

Serum cytokine profile in mice chronically exposed to SEB shows a trend toward increased IL-12 p40

We showed on several occasions that acute exposure to a single dose of SEB (10 μg) through different routes results in a significant elevation in systemic levels of multiple cytokines/chemokines (SIRS), and such animals display symptoms of toxic shock syndrome (15, 28, 29, 31, 33). We anticipated that administration of the same dose of SEB chronically over a 4-wk period would not elicit a similar spike in systemic cytokine/chemokine levels. Consistent with this hypothesis, we did not see any marked elevation in the systemic levels of various cytokines and chemokines in mice implanted with SEB pumps. However, there was a trend toward an increase in serum levels of IL-12p40 (p = 0.06) (Supplemental Fig. 1C). As expected, mice implanted with SEB pumps also did not display any apparent symptoms of SIRS or toxic shock syndromes (e.g., hypothermia, weight loss, diarrhea, or mortality).

Critical role for CD4+ T cells in disease pathogenesis

SAgs are known to activate both CD4+ and CD8+ T cell subsets as long as they express the appropriate TCR Vβ. However, it is well known that CD4+ and CD8+ T cells perform different functions. Therefore, we next performed a series of experiments to delineate the roles of CD4+ and CD8+ T cell subsets in the pathogenesis of systemic inflammatory disease caused by chronic exposure to SEB. DQ8.CD8+ mice implanted with SEB pumps had pronounced inflammatory infiltrates in the liver (Fig. 4A), kidneys (Fig. 4B), and lungs (data not shown). Immunohistohemical analyses showed an intense infiltration of CD4+ T cells in the liver (Fig. 4A), kidney (Fig. 4B), and lungs (data not shown) in DQ8.CD8+ mice. In the kidneys, periglomerular and perivascular infiltrates were seen along with tubular necrosis (Fig. 4B). Surprisingly, DQ8.CD4+ mice showed little or no organ inflammation (Fig. 4) and only very few or no CD8+ T cells were seen in the organs of
DQ8.CD4˚ mice in concordance with minimal histopathological changes (Fig. 4, data not shown). The cumulative organ pathology in SEB pump-implanted DQ8.CD4˚ and DQ8.CD8˚ mice is shown in Supplemental Fig 4. In addition, in DQ8.CD8˚ mice, significant mononuclear cell infiltration could be seen in the thyroid and the heart (Fig. 5). In the heart, perivascular and diffuse mononuclear infiltration within the myocardium was seen (Fig. 5).

With respect to the T cell numbers in the spleen, SEB-treated DQ8.CD8˚ mice had significant splenomegaly (Supplemental Fig. 2A, inset) and showed significant expansion of TCR Vβ8+ CD4+ T cells. However, in SEB-treated DQ8.CD4˚ mice, although the total and TCR Vβ8+ CD8+ T cells were increased, the extent of splenomegaly and the fold increase in TCR Vβ8+ T cells were not as pronounced (Supplemental Fig. 2A). Sera from DQ8.CD4˚ and DQ8.CD8˚ mice implanted with PBS or SEB pumps were tested for anti-nuclear Abs using HEp-2 cells. Although sera from SEB-treated DQ8.CD8˚ mice showed strong reactivity to HEp-2 cells, sera from SEB-implanted DQ8.CD4˚ mice showed no reactivity. Overall, there was a good concordance with the ANA profiles and immunopathology findings (Supplemental Fig. 3A). Taken together, we conclude that, although SEB is capable of activating both CD4+ and CD8+ T cell subsets, CD4+ T cells are required for autoantibody production and to manifest the systemic inflammatory disease, whereas CD8+ T cells are dispensable.

**IL-12- and IFN-γ-dependent pathways play a major pathogenic role in multiorgan pathology following chronic stimulation with SEB**

We next determined the role of Th1 and Th2 cytokine pathways in disease pathogenesis using DQ8.STAT4˚ and DQ8.STAT6˚ mice. STAT4˚ mice are known to have major defects in Th1-type T cell responses and to have exaggerated Th2-type T cell responses. Conversely, STAT6˚ mice have major defects in mounting Th2-type T cell responses and, hence, mount exaggerated Th1-type T cell responses (41).

Interestingly, liver and kidney sections from SEB-treated DQ8.STAT6˚ mice showed extensive inflammation, similar to DQ8.CD8˚ mice, whereas DQ8.STAT4˚ mice showed little or no inflammation, similar to DQ8.CD4˚ mice (Fig. 6). The cumulative organ pathology in SEB pump-implanted DQ8.STAT4˚ and DQ8.STAT6˚ mice is shown in Supplemental Fig 4. Immunofluorescent staining revealed the presence of large numbers of CD4+ T cells and fewer CD8+ T cells or B cells in the liver and kidneys from
DQ8.STAT6° mice. In contrast, DQ8.STAT4° liver/kidney showed very little or no lymphocyte infiltration (Fig. 6, data not shown). In support of these findings, SEB-treated DQ8.STAT6° mice had profound splenomegaly (Supplemental Fig. 2B, inset), along with significant expansion of CD4+ TCR Vβ8+ T cells, whereas spleens from SEB-treated DQ8.STAT4° mice were not significantly enlarged and showed only a moderate increase in TCR Vβ8+ T cells (Supplemental Fig. 2B). Sera from DQ8.STAT6° mice implanted with SEB, but not PBS, pumps showed strong reactivity to HEp-2 cells, whereas sera from SEB-implanted DQ8.STAT4° mice showed no reactivity to HEp-2 cells, showing good concordance with the histopathological findings (Supplemental Fig. 3A).

The absence of autoantibodies and minimal multiorgan pathology in STAT4 knockout mice suggested that IL-12 plays a very important role in the immunopathogenesis. Because IFN-γ is the key Th1-type cytokine induced by the IL-12/STAT4 pathway, we next investigated the role of IFN-γ in the chronic systemic inflammatory disease using DQ8.IFN-γ° mice. As can be seen from Supplemental Fig. 3B, the absence of kidney and liver pathology in DQ8.IFN-γ° mice is consistent with the results in DQ8.STAT4° mice. Taken together, these data suggested that Th1 cytokines (IFN-γ) and STAT4-dependent proinflammatory signals are required for driving T cell expansion and autoantibody production and for eliciting immunopathology, whereas STAT6-dependent signals are anti-inflammatory.

CD28 costimulation is required for pathology

We next investigated the role of CD28 costimulation in eliciting organ pathology following chronic immune activation with SSAgs. Organ pathology was absent in DQ8.CD28° mice chronically exposed to SEB, similar to DQ8.CD4°, DQ8.STAT4°, and DQ8.IFN-γ° mice (Supplemental Fig. 3B), indicating that the disease is also dependent on CD28 costimulation. SEB-induced expansion of TCR Vβ8+ T cells was also severely curtailed in the absence of CD28 costimulation (data not shown), suggesting an important role for CD28 costimulation in this process.

Overall, our results showed that chronic exposure to the SSAg, SEB, resulted in a CD4+ T cell-dependent, CD28-dependent, IFN-γ-dependent, Th1-mediated multisystem autoimmune disease mimicking human systemic lupus erythematosus (SLE) (Fig. 7).

Discussion

SSAs, both bacterial and viral, are attractive candidates as inducers/propagators of autoimmunity because of their unique properties (42). Among them, SSAs are particularly important because S. aureus strains capable of producing SAg not only colonize apparently healthy individuals, they even produce SAg locally (12–14), thereby strongly substantiating the possibility that chronic or recurrent exposure to SSAg can occur naturally and contribute to autoimmunity. Several human studies support a role for S. aureus
carriage and their SAg in the etiopathogenesis of certain autoimmune diseases (22–26, 43, 44). However, a direct causal role for SSAsgs per se as triggers for autoimmune diseases has not been unequivocally established. Our study shows for the first time, to our knowledge, that chronic exposure to SSAgs by itself, without the administration of any exogenous Ags, is sufficient to trigger a systemic autoimmune inflammatory disease.

We postulate that, in our model, SEB released continuously from the mini-osmotic pump binds to HLA-DQ8 molecules on various APC and chronically activates Vβ8-bearing CD4+ and CD8+ T cells of diverse Ag specificities, including the self-reactive clones. When such SAg-primed, self-reactive T cells encounter their cognate self-antigens presented in a classical manner by professional APCs, they become effector cells, release proinflammatory cytokines (e.g., IFN-γ and IL-12), and cause immunopathology. They also provide help to autoreactive B cells to produce autoantibodies in the following manner (Fig. 7).

B cells are excellent presenters of SAg because they express high levels of MHC class II molecules. If the B cells presenting SEB happen to be self-reactive, then they would receive primary activation signals through their BCR when they bind to self-antigens (e.g., dsDNA, Sm) and, at the same time, receive costimulatory signals through CD40–CD40L and B7–CD28 pathways from the Vβ8-bearing CD4+ T cells that are concomitantly recognizing HLA-DQ8–SEB complexes presented by such B cells (45, 46). This would lead to full-fledged activation of these B cells and their differentiation into plasma cells, as well as production of autoantibodies. However, non-self-reactive B cells presenting the SAg will not undergo such activation and differentiation into Ab-producing plasma cells, because they fail to receive the primary activating signals through their BCR. Hence, there is a preferential activation and expansion of autoreactive T and B cells driven by SAg and self-antigens (Fig. 7). Because it is a characteristic of activated T cells to migrate into tissues in search of their cognate Ags (47), there is extensive infiltration of multiple organs with T lymphocytes in our model.

SAgs are known to directly signal through MHC class II molecules on APCs, such as macrophages and dendritic cells, resulting in IL-1 and IL-12 production, thereby driving the Th1 pathway (4, 48, 49). Increased production of IL-12 and other Th1 cytokines, such as IFN-γ, is well documented in systemic autoimmune inflammatory diseases, such as lupus (50). More pronounced im-

FIGURE 7. Model depicting how chronic exposure to SAg could elicit an autoimmune disease. Chronic colonization with SAg-producing S. aureus strains occurs in many individuals. Under suitable conditions, such colonizing strains produce one or more SAgs, which will be absorbed and bind to HLA class II molecules on B cells, as well as other APCs. This leads to activation of CD4+ and CD8+ T cells of diverse Ag specificities, including the self-reactive clones that are present in most individuals, as long as they express the appropriate TCR Vβ family preferred by those SAgs. Self-reactive T clones present among this population undergo additional rounds of activation when they encounter their cognate self-antigens presented in a classical manner by the professional APCs, amplifying autoimmunity. Because SAgs can directly signal through MHC class II molecules on professional APCs, they could induce IL-1 and IL-12 production, thereby skewing toward the proinflammatory Th1-type pathway. Self-reactive B cells presenting SAg would get signals through their BCR, as well as receive costimulatory signals through the CD40–CD40L and B7–CD28 pathways from the CD4+ T cells recognizing the SAg. Such B cells would proliferate, differentiate into plasma cells, and secrete autoantibodies. Non-self-reactive B cells presenting SAgs would fail to undergo such activation and differentiation into plasma cells, because they would not receive signaling through their BCR. Several bacterial (e.g., qualitative and quantitative nature of the SAg produced, chronicity of colonization), host (genetic and nongenetic factors, such as providing conducive milieu to the colonizing strains to elaborate SAg), and other environmental factors may determine the overall outcome.
munopathology in DQ8.8.8T6 mice and the absence of disease in DQ8.8.8T4 and DQ8.8FN-γ mice support this hypothesis. Because SAgs bind to HLA class II molecules with much higher affinity than to mouse MHC class II molecules, SAgs might be able to activate APCs and T cells more efficiently in humans (and in HLA class II transgenic mice), create a more proinflammatory milieu, and contribute to autoimmunity, rather than induce anergy. The unique signaling pathways used by SAgs may also promote the development of pathogenic T cells and render them less susceptible to Treg-mediated immune regulation (51).

SEB and other SAgs activate both CD4+ and CD8+ T cells. However, in our model, CD4+, but not CD8+, T cells are pathogenic in lupus nephritis, create more proinflammatory milieu, and contribute to autoimmunity, rather than induce anergy. The unique signaling pathways used by SAgs may also promote the development of pathogenic T cells and render them less susceptible to Treg-mediated immune regulation (51).

Acknowledgments
We are grateful to Prof. Chella David (Department of Immunology, Mayo Clinic) for critical reviews of the manuscript and to Dr. Shu Man Fu (Division of Clinical Rheumatology and Center for Immunology, Inflammation and Regenerative Medicine, University of Virginia, Charlottesville, VA) for critical comments.

Disclosures
The authors have no financial conflicts of interest.

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