Systemic lupus erythematosus (SLE) is characterized by the production of grouped sets of autoantibodies targeting mainly the U1 ribonucleoprotein (RNP) and/or Ro/La RNP particles. Intraparticle diversification of the autoimmune response is believed to occur via epitope spreading. So far, it is not known how the autoimmune response “jumps” from one particle to another. To the extent that the majority of nuclear autoantigens in SLE are RNA binding proteins and major epitopes were previously mapped within their RRM (RNA recognition motifs), conserved sequences within RRM could be involved in the intermolecular and interparticle diversification process of the autoimmune response. We investigated the potential of RRM of the La/SSB autoantigen to induce antibodies that cross-recognize components of the U1-RNP particle and therefore its capacity to produce interparticle epitope spreading. We immunized New Zealand white rabbits with a peptide corresponding to the epitope 145–164 of La/SSB (belonging to the RRM of La/SSB), attached in four copies on a scaffold carrier. Sera were drawn from 20 sera of patients with SLE and anti–U1-RNP antibodies and 26 sera of primary Sjögren syndrome patients with anti-La/SSB antibodies. All sera were evaluated for reactivity against the major epitope of La/SSB (pep349–364), the RNP antigen and the RRM-related epitope of La/SSB (pep145–164). Specific antibodies against pep145–164 were purified with immunoaffinity columns from selected sera. After the immunization of the animals with pep145–164, a specific IgG antibody response was detected, directed against the La/SSB autoantigen (wks 3–7), the immunizing peptide (wks 3–27), and the RNP autoantigen (wks 7–20). This response gradually decreased to low levels between postimmunization wks 27–42. Purified antibodies against pep145–164 recognized La/SSB and a 70-kD autoantigen in Western blot and exhibited significant reactivity in anti–U1-RNP ELISA. Depletion of anti-pep145–164 antibodies eliminated anti–U1-RNP reactivity from immunized rabbit sera but not from human sera. In addition, pep145–164 was recognized to a greater extent by autoimmune sera with anti-RNP reactivity compared with anti-La/SSB–positive sera, in contrast to pep349–364 of La/SSB, which was recognized almost exclusively by sera with anti-La/SSB reactivity. These data suggest that the RRM region of La/SSB can trigger interparticle B-cell diversification to U1-RNP-70 autoantigen via molecular mimicry. Identification of key sequences that trigger and perpetuate the autoimmune process is particularly important for understanding pathogenetic mechanisms in autoimmunity.
RNA Recognition Motif (RRM) of La/SSB: The Bridge for Interparticle Spreading of Autoimmune Response to U1-RNP

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Systemic lupus erythematosus (SLE) is characterized by the production of grouped sets of autoantibodies targeting mainly the U1 ribonucleoprotein (RNP) and/or Ro/La RNP particles. Intraparticle diversification of the autoimmune response is believed to occur via epitope spreading. So far, it is not known how the autoimmune response “jumps” from one particle to another. To the extent that the majority of nuclear autoantigens in SLE are RNA binding proteins and major epitopes were previously mapped within their RRM (RNA recognition motifs), conserved sequences within RRM could be involved in the intramolecular and interparticle diversification process of the autoimmune response. We investigated the potential of RRM of the La/SSB autoantigen to induce antibodies that cross-recognize components of the U1-RNP particle and therefore its capacity to produce interparticle epitope spreading. We immunized New Zealand white rabbits with a peptide corresponding to the epitope 145–164 of La/SSB (belonging to the RRM of La/SSB), attached in four copies on a scaffold carrier. Sera were drawn from 20 sera of patients with SLE and anti-U1-RNP antibodies and 26 sera of primary Sjögren syndrome patients with anti-La/SSB antibodies. All sera were evaluated for reactivity against the major epitope of La/SSB (pep349–364), the RNP antigen and the RRM-related epitope of La/SSB (pep145–164). Specific antibodies against pep145–164 were purified with immunoaffinity columns from selected sera. After the immunization of the animals with pep145–164, a specific IgG antibody response was detected, directed against the La/SSB autoantigen (wks 3–7), the immunizing peptide (wks 3–27), and the RNP autoantigen (wks 7–20). This response gradually decreased to low levels between postimmunization wks 27–42. Purified antibodies against pep145–164 recognized La/SSB and a 70-kD autoantigen in Western blot and exhibited significant reactivity in anti–U1-RNP ELISA. Depletion of anti-pep145–164 antibodies eliminated anti-U1-RNP reactivity from immunized rabbit sera but not from human sera. In addition, pep145–164 was recognized to a greater extent by autoimmune sera with anti-RNP reactivity compared with anti-La/SSB-positive sera, in contrast to pep349–364 of La/SSB, which was recognized almost exclusively by sera with anti-La/SSB reactivity. These data suggest that the RRM region of La/SSB can trigger interparticle B-cell diversification to U1-RNP-70 autoantigen via molecular mimicry. Identification of key sequences that trigger and perpetuate the autoimmune process is particularly important for understanding pathogenetic mechanisms in autoimmunity.

INTRODUCTION

Autoimmune diseases, caused by a breakdown in self tolerance, are characterized by the appearance of autoantibodies and autoreactive T lymphocytes. Systemic lupus erythematosus (SLE) is considered the prototypic systemic autoimmune disease disorder involving both humoral and cellular forms of adaptive immune response and affecting the skin, joints, kidneys, lungs, nervous system, serous membranes and virtually every organ in the body (1).

SLE is characterized by the production of autoantibodies to ribonucleoprotein (RNP) complexes. These autoantibodies often arise in 2 grouped sets targeting the U1-RNP complex and/or Ro/La particle (2). The U1-RNP particle is a major component of the spliceosome, catalyzing pre-messenger RNA (mRNA) splicing into mRNA. Together with the Sm proteins, U1-RNP contains specific proteins (RNP 70, RNP A and RNP C) that interact with the 164-nucleotide-long U1 RNA. The RNP-70 and RNP-A proteins bear classical RNA recognition motifs (RRMs) and bind directly to hYRNA, whereas Ro52, La/SSB and Ro60 autoantigens (4). La/SSB and Ro60 proteins possess RNA binding motifs (classical RRM and TROVE [telomerase-RO-vault-element], respectively) allowing their direct binding to hYRNA, whereas Ro52
participates in the complex via protein-protein interactions (2,5). The localization of Ro/La complexes is mainly cytoplasmic, but their assembly is performed in the nucleus (6,7).

The Ro/La RNP particle has been claimed to play an important role in the initiation of autoimmunity, because autoantibodies targeting this particle usually appear before clinical manifestations of SLE and earlier than anti-Sm and anti-nuclear RNP antibodies (mean 3.4 years versus 1.2 years) (8). The proportion of SLE patients with anti-Sm or anti-nuclear RNP antibodies increases dramatically in the year before diagnosis, indicating that appearance of these autoantibodies heralds the clinical onset of the disease (8). Specifically, the clinical onset of disease coincides not so much with the appearance of anti-Sm or RNP antibodies but with the cessation of the development of new autoantibody specificities. Indeed, the rate of appearance of new autoantibody specificities has been found to gradually increase until the diagnosis of SLE and to be halted afterward (8).

Diversification and augmentation of the autoimmune response is believed to occur via epitope spreading, a process whereby distinct and non-cross-reactive epitopes are created (9,10). Two types of epitope spreading have been described: intramolecular spreading, in which the autoimmune response spreads in epitopes within the same protein, and intermolecular spreading, which also involves other protein components physically associated within the same antigenic complex, such as a spliceosome and Ro/La particles. It is not yet known how the autoimmune response “jumps” from one particle to another. To the extent that the majority of nuclear autoantigens in SLE are RNA-binding proteins and major epitopes were previously mapped within their RRM motifs (in the case of La/SSB, RNP-A and RNP-70 autoantigens) (2), molecular similarity of conserved sequences within RRM could be involved in the intermolecular and interparticle diversification process of the autoimmune response. Here we report our exploration of the potential of the RRM of the La/SSB autoantigen to induce antibodies that recognize components of U1-RNP particle, and therefore its capacity to produce interparticle epitope spreading.

### MATERIALS AND METHODS

#### Human Sera

Sera were obtained from 72 patients with primary Sjögren syndrome (pSS) (11), 82 patients with SLE (12), 38 patients with rheumatoid arthritis (RA) (13) and 55 healthy subjects. All patients ful-
filled the American/European classification criteria (11–13). All sera had been previously screened for the presence of anti-Ro/SSA and anti-La/SSB autoantibodies by counterimmunoelectrophoresis (CIE) and immunoblot, as described previously (14).

**Peptide Synthesis and Purification**

The linear B-cell epitope of La/SSB that resides within its RRM 145TLHKAFKGSIFVVFDSIESA164, and the major epitope of La/SSB 349GSGKGKVQFQGKKTF364 were synthesized as multiple antigenic peptides (MAP) attached in four copies to the tetrameric MAP backbone and used for immunization and ELISA experiments. The irrelevant peptide IASRYDQL (corresponding to the sequence 250–257aa of Leismania glycoprotein gp63), selected because it has the same charge with pep145–164 at pH 7, was attached in MAP scaffold-like pep145–164 and used as control peptide (ctrl-pep). An additional peptide corresponding to the epitope 145TLHKAFKGSIFVVFDSIESA164 was synthesized in its free form and used for antibody purification and evaluation of their reactivity. All peptides were synthesized according to the solid-phase peptide synthesis procedure, purified with fast protein liquid chromatography and tested by mass spectrometry for confirmation of their sequence identity.

**Rabbit Immunization**

Ten New Zealand White female rabbits, 6±-8-wks-old, were immunized according to a previously described protocol (15), using 0.5 mg immunogen emulsified in complete Freund’s adjuvant (CFA) for the first injection and incomplete Freund’s adjuvant for the subsequent injections. Successive bleedings were performed before the immunizations of the animals at wks 0, 3, 7, 13, 20, 27, 38 and 42.

**Estimation of Anti–La/SSB and Anti–U1-RNP Reactivity**

Recombinant human La/SSB whole protein was constructed using the methodology that has been previously described by Troster et al. (16) and used in enzyme-linked immunosorbent assay (ELISA) according to a previously published protocol (17) with modifications in the coating conditions. More specifically, plates were coated with recombinant La/SSB in the presence of 6M urea to denature the autoantigen and to remove any RNA that could mask its RRM domains. The anti–U1-RNP reactivity was monitored by a commercially available ELISA based on affinity purified autoantigen (RNP ELISA; IBL, Hamburg, Germany). ELISA Assays with Synthetic Peptides

Specific antipeptide ELISA assays were developed and performed to detect the antibodies in human sera as well as in rabbit sera. Sera from all animals were tested against the peptides pep145–164, pep349–364 and ctrl-pep peptide. Sera from patients were tested for reactivity against the peptides pep145–164 and pep349–364; 96-well polystyrene plates (Costar, Corning, NY, USA) were coated with the solution of the coating peptide at a concentration of 5 μg/mL in carbonate-bicarbonate buffer (pH 9.6). Nonspecific-binding was blocked using blocking buffer consisting of bovine serum albumin 2% wt/vol in phosphate buffered saline (PBS) pH 7.4. Afterward, rabbit sera or human sera were added to blocking buffer in a dilution of 1:700 or 1:140, re-
spectively. After an incubation period of 2 h at room temperature, the ELISA plates were washed three times with PBS. Alkaline phosphatase–conjugated antirabbit IgG or antihuman IgG (Jackson Immunoresearch, West Grove, PA, USA), diluted 1:1400 in blocking buffer, was added to the assays with the rabbit or the human sera, respectively, and the plates were incubated for 1 h. Subsequently, the wells were washed and 100 μL p-nitrophenol substrate (Sigma-Aldrich, Munich, Germany) was added, and the absorbance was measured at 405 nm. The cutoff point for the assays with human sera was set as the mean optical density values plus three standard deviations of sera from 55 healthy individuals.

To exclude background binding and verify the coating efficiency in ELISA experiments, each plate was divided into two halves, one coated with pep145–164 and one coated with MAP carrier and blocked with albumin blocking buffer. None of the rabbit sera exhibited significant reactivity with MAP carrier or albumin. In the experiments done for human sera screening, 2 SLE sera demonstrated significant binding to MAP and/or albumin and they excluded from data analysis.

**Purification of Human Anti-pep145–164aa Antibodies**

Total IgG from the sera of three patients, containing autoantibodies to the La/SSB epitope 145–164, were purified by affinity chromatography using a protein-A Sepharose 4B column. IgG fractions were concentrated and dialyzed against PBS. A specific immunoaffinity column of cyanogen bromide (CNBr)–activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) was generated by standard methods, using 10 mg of the synthetic pep145–164. Anti-pep145–164 IgG antibodies were purified from the three human fractions of total IgG as well as from rabbit sera immunized with pep145–164 by using standard immunoaffinity procedure (elution was performed with 0.1 mol/L HCl-Gly, pH 2.7). Antibody concentration was measured using the Bradford assay.

**Western Blotting**

Rabbit thymus extract was first subjected to electrophoresis in 12% SDS-polyacrylamide gels and then transferred to nitrocellulose. The blotted strips were saturated in Tris-buffered saline, pH 7.5, containing 0.1% Tween (TBS-T) and 5% nonfat milk for 1 h at room temperature, and then incubated with rabbit antisera diluted 1:120 in TBS-T milk overnight at 4°C. After washing, strips were incubated with alkaline-phosphatase conjugated antibodies to rabbit IgG (1:1,100 in TBS-T milk). Afterward, the strips were washed and nitro-blue-tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate substrate (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to reveal positive reactions.

**RESULTS**

**Sequence and Structural Similarity between the RRM Belonging to Different Autoantigens**

According to our hypothesis, a consensus sequence within the RRM domain conserved in many autoantigens, might play a role as driver epitope for intermolecular and interparticle epitope spreading in systemic autoimmunity. We examined the sequence and structural similarity among the RRM motifs of several self-proteins. A homologous region was identified in the central part of the RRMs (Figure 1A). Although the degree of similarity in its primary structure was moderate, varying between 35% and 60% (Figure 1A), all RRM regions adopted a very similar tertiary structure (Figure 1B) (18–21) that could favor a potential cross-recognition by autoanti-

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**Figure 3.** Assessment of the reactivity of purified anti-pep145–164 antibodies from three patients and immunized rabbits. All purified antibodies recognized U1-RNP (A) and pep145–164 (B) in ELISA. Purified antibodies from two patients were also tested in Western Blot against HeLa extract (C) and found to recognize bands at 48kD and 70kD, corresponding to La/SSB and RNP-70 autoantigens.
bodies. The homologous region of La/SSB (aa145–164) had been previously characterized by our group as an SLE-associated B-cell epitope (14), serving also as a T-cell epitope (22) in rabbit immunization experiments.

**Antibodies Generated in Rabbits Immunized with the Epitope 145–164 of La/SSB React with the Cognate Protein and with the U1-RNP Autoantigen**

In our diversification model, the RRM of La/SSB may have been involved in the induction of the antispliceosomal autoimmune response via molecular mimicry. To investigate this possibility, we immunized New Zealand white rabbits with pep145–164 of La/SSB. Sera from rabbits were collected at different pre- and postimmunization time points and tested for reactivity against the immunizing peptide and a control peptide. It was found that 3 wks after the first immunization rabbits produced antibodies toward the immunizing peptide, which reached their maximum reactivity between wks 7 and 13. In contrast, no reactivity against the control peptide was observed (Figure 2A).

We also investigated whether antibodies against peptide pep145–164 of La/SSB were able to recognize the whole cognate protein and proteins present in the U1-RNP particle. It was found that the first-appearing antibodies, capable of recognizing the La/SSB protein (at wk 3 after the immunization) were progressively eliminated at wk 13. The development of anti-La/SSB antibodies was followed by the production of anti–U1-RNP antibodies at wk 7, which remained at high levels until wk 20 (Figure 2B). Therefore, immunization with an autoepitope of La/SSB autoantigen can produce antibodies capable of recognizing components of another autoantigenic complex, the U1-RNP.

To confirm the above findings the sequential sera from immunized rabbits were tested in Western blot against a rabbit thymus extract. At wk 7, a band at 70 kD appeared, which exactly followed the kinetics of anti–U1-RNP antibodies in terms of time and signal intensity (Figure 2C).

**Purification of Antibodies toward the Epitope 145–164 of La/SSB Reveals their Capacity to Cross-Recognize U1-RNP Autoantigen**

To gain further insight into the mechanism involved in the recognition of U1-RNP autoantigen by autoantibodies in our model, we coupled the latter peptide onto an immunoaffinity column and performed affinity purification of specific antipeptide antibodies from immunized rabbit and patient sera with anti-pep145-164 reactivity. It was found that anti-pep145–164 antibodies from immunized rabbits recognized not only the peptide against which they were purified (Figure 3A) but also the U1-RNP autoantigen (Figure 3B). This observation was also true for human sera from patients with SLE, demonstrating that specific antibodies that bind to a conserved epitope of La/SSB also have the capacity to cross-recognize U1-RNP autoantigen. Purified antibodies from patients with SLE were also tested in Western Blot against HeLa extract. Specific bands at molecular weights of 48 kD and 70 kD were detected (Figure 3C). Comparison with reference sera indicated that these bands most likely correspond to La/SSB and RNP-70 autoantigens. Since purified anti-pep145–164 antibodies recognize the U1-RNP antigen, we expect that purified anti-U1-RNP antibodies will also recognize pep145–164 of La/SSB. However, this experiment has not been done.

To examine if epitope recognition on U1-RNP autoantigen is expanded beyond the RRM homologous region, we investigated whether anti–U1-RNP reactivity of human and rabbit sera could be absorbed onto the RRM peptide (pep145–164 immunoaffinity column). It was found that after the depletion of anti-pep145–164 antibodies, sera of immunized rabbits lost their capacity to recognize U1-RNP autoantigen. In contrast, the serum of human study patient 1, which was subjected to the same immunoaffinity column, completely retained its anti–U1-RNP reactivity (Figure 4). These observations strongly indicate that immunized rabbit sera (tested at postimmunization wk 13) react with the homologous RRM region of U1-RNP but not with other epitopes on the same autoantigen. On the other hand human sera recognize multiple epitopes on U1-RNP autoantigen, and their anti–U1-RNP reactivity cannot be absorbed onto pep145–164. Additional evidence was obtained by competitive ELISA assays, which demonstrated that anti–U1-RNP reactivity of immunized rabbit sera could be inhibited (52%–59%) by incubation with pep149–164 peptide (data not shown).

**Prevalence of Anti-pep145–164 Antibodies in Autoimmune Sera**

We next examined the prevalence of antibodies against pep145–164 in differ-
ent autoimmune diseases, as well as in normal populations. Antibodies recognizing the epitope 145–164 of La/SSB were detected in 29% of SLE, 39% of SS, and 13% of RA sera, but not in sera from healthy individuals (Figure 5A). Analysis of the reactivities of the sera according to their autoantibody specificity in CIE demonstrated that although these antibodies were common in both anti-La/SSB–positive and anti–RNP-positive sera, their reactivity was higher in RNP-positive sera (t = 4.07, P = 0.0002), which occurs mainly in patients with SLE (Figure 5B).

To examine the autoantibody reactivity profile of the patient sera in more detail, we evaluated their reactivity against the pep145–164 of La/SSB and the pSS-related major epitope of La/SSB (pep349–364) with regard to their specificity in CIE. It was found that patient sera could be divided into 3 groups: (a) sera with anti-pep349–364 antibodies alone, (b) sera with both anti-pep145–164 and anti-pep349–364 antibodies and (c) sera with anti-pep145–164 antibodies alone (Figure 6A). Notably, all sera from group c were anti-RNP–positive sera, whereas sera belonging to groups (a) and (b) were anti-La/SSB–positive sera. Therefore, the existence of anti-pep145–164 without anti-pep349–364 antibodies is an exclusive feature of anti-RNP–positive sera. The autoantibody specificity of the sera was further confirmed by anti-La/SSB and anti–U1-RNP ELISA assays (Figure 6B).

**DISCUSSION**

The autoantibody diversification in SLE has been partially elucidated via the proposed mechanism of intra- and intermolecular spreading (9,10). This mechanism may explain the different clusters of autoantibodies in SLE (against spliceosome and hYRNPs), but not the sequential appearance of autoantibodies against autoantigens belonging to different macromolecular complexes. The results of the present study provide evidence that a conserved sequence within the RRM motif of the La/SSB autoantigen can induce antibodies that cross-recognize components of the U1-RNP particle.

We first noticed that the RRM regions of La/SSB and RNP-70 autoantigens differ significantly in their amino acid sequence, it is not surprising that a period of time was required in order for anti-pep145–164 antibodies to adapt their specificity to U1-RNP. The appearance of antibodies targeting U1-RNP might also be explained by a different mechanism, involving intramolecular spreading to another unidentified epitope of La/SSB followed by cross-recognition of U1-RNP. To elucidate the mechanisms, specific anti-pep145–164 antibodies were purified from immunized rabbits and found to cross-recognize directly the U1-RNP. Furthermore, to gain insight into human disease, we also purified specific anti-pep145–164, antibodies from SLE patient sera. These antibodies also recognized U1-RNP. Although both rabbit and human sera recognized the U1-RNP autoantigen, their reactivity could be discriminated on the basis of its ability to be absorbed onto pep145–164. Thus, immunized rabbit sera lost their reactivity after passage through the pep145–164 immunoaffinity column, but human sera did not. These results most likely reflect the limited epitope recognition in rabbits (owing to the cross-reaction with the RRM homologous region) and the multiple epitope recognition by human SLE sera. These findings are also consistent with previous studies reporting that limited epitope recognition on an autoantigen occurs when the immunizing peptide has a low degree of sequence similarity with it (23). In this case an additional step is required (for example, affinity maturation of antibodies in the presence of U1-RNP autoantigen) to gain diversification of immune response to additional epitopes of U1-RNP.

Antibodies against pep145–164 were detected in 39% of patients with pSS and in a small subgroup of patients with RA. However, anti-RNP–positive sera, which occur mainly in patients with SLE, demonstrated significantly higher reactivity for anti-pep145–164 antibodies as compared with anti-RNP–negative sera.
These sera did not possess reactivity against the epitope 349–364, which has been previously characterized as the major, associated with pSS, epitope of La/SSB (14). We should also note that these sera were found to be negative for anti-La/SSB antibodies in CIE, which most likely reflects either a limited epitope recognition within La/SSB autoantigen that is insufficient to produce an immunoprecipitation reaction in CIE or an epitope masking by hY-RNA bound in the RRM region of La/SSB in assays employing the antigen in its natural conformation (24).

Our experimental data strongly reinforce the hypothetical model proposed some years ago by Monneaux and Muller (9,25). In this model, the RRM consensus sequence can drive epitope spreading in spliceosomal proteins via molecular mimicry (25). In this regard, Monneaux and Muller demonstrated that immunization of rabbits with a peptide corresponding to the epitope 131–151aa within the RRM of RNP-70 could produce antibodies that recognize, in addition to the cognate protein, the components RNP-A, RNP-C and Sm-D of the U1-RNP particle (26). These antibodies had the capacity to cross-recognize peptides corresponding to the RRM(s) of different nuclear autoantigens, such as RNP-70, RNP-A and hnRNP-A2 (26). According to this model, the RRM initiates spreading of the immune response to the whole protein (intramolecular spreading), then spreading proceeds in an ordered manner to other proteins containing the RRM(s) and, finally, to proteins that do not contain any RRM but are also localized in the same particle (intermolecular spreading). To date, experimental data exist only for epitope spreading via RRM to the physically associated spliceosomal proteins. Our present data provide for the first time evidence that interparticle spreading of immune response via RRM can also occur. The proposed model can also be applied to other “driver” epitopes such as the proline-rich region PPPGMRPP that holds a cross-reactive epitope, present in several spliceosomal autoantigens and recently identified as an early target of RNP humoral autoimmunity in SLE (27). In this regard, in addition to intramolecular and intermolecular spreading, the autoimmune response diversifies via molecular mimicry with key sequences, common in autoantigens targeted in SLE.

Taken together, our data suggest that the RRM region of La/SSB can trigger interparticle B-cell diversification to U1-RNP, confirming the importance of the RRM region in the pathway of events leading to autoimmunity in SLE. Identification of key sequences that trigger and perpetuate the autoimmune process is particularly important for understanding the initial events in autoimmune response and designing specific therapeutic strategies capable of blocking the cascade of spreading (taking place during the development of autoimmune response).

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that
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