Autoinhibitory regulation of S100A8/S100A9 alarmin activity locally restricts sterile inflammation

Thomas Vogl, … , Thomas Pap, Johannes Roth

*J Clin Invest.* 2018;128(5):1852-1866. [https://doi.org/10.1172/JCI89867](https://doi.org/10.1172/JCI89867).

Autoimmune diseases, such as psoriasis and arthritis, show a patchy distribution of inflammation despite systemic dysregulation of adaptive immunity. Thus, additional tissue-derived signals, such as danger-associated molecular patterns (DAMPs), are indispensable for manifestation of local inflammation. S100A8/S100A9 complexes are the most abundant DAMPs in many autoimmune diseases. However, regulatory mechanisms locally restricting DAMP activities are barely understood. We now unravel for the first time, to our knowledge, a mechanism of autoinhibition in mice and humans restricting S100-DAMP activity to local sites of inflammation. Combining protease degradation, pull-down assays, mass spectrometry, and targeted mutations, we identified specific peptide sequences within the second calcium-binding EF-hands triggering TLR4/MD2-dependent inflammation. These binding sites are free when S100A8/S100A9 heterodimers are released at sites of inflammation. Subsequently, S100A8/S100A9 activities are locally restricted by calcium-induced (S100A8/S100A9)$_2$ tetramer formation hiding the TLR4/MD2-binding site within the tetramer interphase, thus preventing undesirable systemic effects. Loss of this autoinhibitory mechanism in vivo results in TNF-α–driven fatal inflammation, as shown by lack of tetramer formation in crossing S100A9$^{-/-}$ mice with 2 independent TNF-α–transgene mouse strains. Since S100A8/S100A9 is the most abundant DAMP in many inflammatory diseases, specifically blocking the TLR4-binding site of active S100 dimers may represent a promising approach for local suppression of inflammatory diseases, avoiding systemic side effects.

Find the latest version:

[http://jci.me/89867/pdf](http://jci.me/89867/pdf)
Autoinhibitory regulation of S100A8/S100A9 alarmin activity locally restricts sterile inflammation

Thomas Vogl,1,2 Athanasios Stratis,1 Viktor Wixler,3 Tom Völler,1 Sumita Thurainayagam,1 Selina K. Jorch,1 Stefanie Zenker,1 Alena Dreiling,2 Deblina Chakraborty,1 Mareike Fröhling,3 Peter Paruzel,3 Corinna Wehmeyer,3 Sven Hermann,2,5 Olympia Papantonopoulou,6 Christiane Geyer,2,7 Karin Loser,2,8,9 Michael Schäfers,2,8,9 Stephan Ludwig,4,9 Monika Stoll,10 Tomas Leanderson,11 Joachim L. Schultz,6 Simone König,2 Thomas Pap,3 and Johannes Roth1,2,9

Institute of Immunology, and 1International Center for Clinical Research, University of Münster, Münster, Germany. 2Institute of Musculoskeletal Medicine and 3Institute of Molecular Virology, Zentrum für Molekularbiologie der Entzündung (ZMBE), University Hospital Münster, Münster, Germany. 4European Institute for Molecular Imaging, University of Münster, Münster, Germany. 5European Institute for Molecular Imaging, University of Münster, Münster, Germany. 6Genomics and Immunoregulation, Life & Medical Sciences–Institute (LIMES-Institute), University of Bonn, Bonn, Germany. 7Department of Clinical Radiology, 8Department of Dermatology, 9Cells-in-Motion Cluster of Excellence, and 10Institute of Human Genetics, Genetic Epidemiology, University of Muenster, Muenster, Germany. 11Department of Experimental Medical Science, Immunology Group, Lund University, Lund, Sweden.

Autoimmune diseases, such as psoriasis and arthritis, show a patchy distribution of inflammation despite systemic dysregulation of adaptive immunity. Thus, additional tissue-derived signals, such as danger-associated molecular patterns (DAMPs), are indispensable for manifestation of local inflammation. S100A8/S100A9 complexes are the most abundant DAMPs in many autoimmune diseases. However, regulatory mechanisms locally restricting DAMP activities are barely understood. We now unravel for the first time, to our knowledge, a mechanism of autoinhibition in mice and humans restricting S100-DAMP activity to local sites of inflammation. Combining protease degradation, pull-down assays, mass spectrometry, and targeted mutations, we identified specific peptide sequences within the second calcium-binding EF-hand of S100A8/S100A9 that form tetramers in the presence of calcium (16). There are many contradictory reports about the functional activities of S100A8 and S100A9. However, most published work neglects the possibility of functional variability of different S100 complexes. Describing the structural model for S100–TLR4...

Introduction

The term “sterile inflammation” describes inflammatory processes not triggered by microbial agents (1, 2), but rather induced by endogenous molecules released during tissue damage or cell stress (3). For these endogenous triggers, the terms alarmins or danger-associated molecular patterns (DAMPs) have been introduced (4). Many alarmins are primarily involved in intracellular homeostasis, but also act as extracellular danger signals triggering inflammation when released by damaged or activated cells (3, 5, 6). HMGB1, heat shock proteins, and certain S100 proteins belong to this family.

Many chronic inflammatory diseases are driven by systemic autoimmune mechanisms. However, the pattern of sterile inflammation in these diseases frequently shows a local and patchy distribution, e.g., circumscribed inflammatory plaques of psoriasis beside nonaffected skin that can be explained neither by systemic dysregulation of the adaptive immune system nor by distribution of autoantigens. It is therefore evident that additional tissue-derived signals such as alarmins are indispensable for local differences in the clinical manifestation of inflammation. However, molecular mechanisms responsible for local manifestation of inflammatory activity are barely understood.

S100A8 (also known as myeloid-related protein, MRP8) and S100A9 (MRP14) constitute up to 40% of cytosolic proteins in neutrophils and 5% in monocytes (7). They are released by activated phagocytes and promote inflammation as endogenous ligands of the TLR4 complex, but molecular models for these interactions are currently missing (8–12). S100A9-deficient mice revealed a pivotal role of these proteins for many inflammatory processes (13, 14). S100A8 and S100A9 are the most abundant alarmins during infections, arthritis, allergies, autoimmune diseases, and inflammatory bowel, pulmonary, or cardiovascular diseases (6, 15). S100A8/S100A9 usually exist as heterodimeric complexes (calprotectin) that form heterotetramers in the presence of calcium (16). There are many contradictory reports about the functional activities of S100A8 and S100A9. However, most published work neglects the possibility of functional variability of different S100 complexes. Describing the structural model for S100–TLR4...

Authorship note: TV and AS contributed equally to this work.
Conflict of interest: The authors have declared that no conflict of interest exists.
Submitted: July 29, 2016; Accepted: February 8, 2018.
interactions, we identified, for what we believe is the first time, an autoinhibitory feedback mechanism for S100 alamin functions locally restricting their inflammatory properties. S100A8/S100A9 released by activated phagocytes is only temporarily active in the local extracellular microenvironment due to a mechanism of autoinhibition that is induced by calcium-dependent tetramerization of S100A8/S100A9. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.

Results

S100A8/S100A9 tetramerization abolishes inflammatory activity. S100A8/S100A9 heterodimers associate to (S100A8/S100A9)2 heterotetramers, but not S100A8/S100A9 homodimers. Oligomer formation of 2 heterodimers blocks the ability of the heterodimer to bind to TLR4/MD2, thus restricting the activity of these alamins at local sites of inflammation. Loss of this control mechanism results in fatal TNF-α-driven inflammation in vivo. These properties define a specific TLR4-binding site on the active dimeric form of S100A8/S100A9 as a promising target for a very specific and locally restricted anti-inflammatory therapy minimizing systemic side effects.
Figure 1. S100A8/S100A9 tetramerization blocks inflammatory activity of the alarmin. (A and B) Effects of hS100A9 and hS100A8 homodimers or hS100A8/H100A9 (A), mS100A8 homodimer (B), or hS100A8/S100A9, hS100A8/S100A9N69A, and hS100A8/S100A9E78A (C) on TNF-α production by monocytes quantified by ELISA after 4 hours of incubation (>1 mM calcium in culture medium). (D) Representative SEC runs of human (grhS100A8/S100A9, granulocytic prepared human S100A8/S100A9 [red and green curves]; rechS100A8/S100A9, recombinant prepared human S100A8/S100A9; rechS100A8/S100A9E78A, recombinant prepared human S100A8/S100A9E78A; rechS100A8, recombinant prepared human S100A8; rechS100A9, recombinant prepared human S100A9) and murine (recmS100A8/S100A9, recombinant prepared murine S100A8/S100A9; recmS100A8, recombinant prepared murine S100A8) S100 proteins in the absence (black and red curves) and presence of 100 μM Ca2+ ions (blue and green curves) (3 independent experiments). (E) Induction of TNF-α and IL-8 mRNA in HEK293 cells expressing TLR4-MD2-CD14 and treated with either 5 or 10 μg/ml of rhS100A8/S100A9 (left), 1 or 5 μg/ml of rhS100A8/S100A9N69A (middle), or 1 or 5 μg/ml of rhS100A8/S100A9E78A (right) for 4 hours, as detected by qRT-PCR. Results are shown as relative to baseline expression in unstimulated cells. RPL was used as a housekeeping control gene. (F) Human monocytes were left untreated or stimulated with 5 μg/ml of either S100A8/S100A9, S100A9, or S100A8 or 1 ng/ml LPS for 4 hours. RNA was isolated and used for transcriptome analyses. The 1,000 most variable genes (FDR corrected, P < 0.05) within the data set were used for HC. Data were Z score–normalized and ranked according to changes in expression upon stimulus. Data represent mean ± SD of 5 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001, 2 tailed t test.
TTP KO (24, 25), we crossed S100A9−/− mice with the ihTNF-tg mouse strain, which displays features of psoriatic arthritis 3 to 6 weeks after TNF-α induction (26). Expression of human TNF-α was induced in adult mice by doxycycline (dox) for up to 8 weeks. Disease progression in ihTNF-tg S100A9−/− mice was significantly aggravated compared with that in ihTNF-tg mice, as shown by loss of body weight and grip strength as well as increased paw swelling. None of the control mice developed any
abnormalities after dox application (Figure 4, A–C). Employing optical imaging (OI) (fluorescence reflectance imaging [FRI]) using Cy5.5-labeled anti-S100A8 antibodies (27), we confirmed the recovery of S100A8 expression in vivo after TNF-α induction. Significant local tracer accumulation was observed in dox-treated ihTNF-tg S100A9−/− mice in paws and knee joints as well as in the skin (Figure 4D). The recovery of the S100A8 protein in ihTNF-tg S100A9−/− mice after dox stimulation was also confirmed by immunohistochemistry in the skin (Figure 4E), nail matrix (Figure 4F), and bone marrow as well as in bone marrow-containing metacarpal sections (Figure 4G and Supplemental Figure 5) and blood cells (Figure 4H). Histological analyses of distal interphalangeal (DIP) joints revealed a stronger hyperplasia of synovial tissue and cartilage destruction in ihTNF-tg S100A9−/− mice compared with that in ihTNF-tg mice (Supplemental Figure 6). Prolonged stimulation of isolated S100A9−/− bone marrow cells (BMCs) with TNF-α induced a recovery of S100A8 homodimers on the protein level, as observed before for S100A9−/− keratinocytes (Supplemental Figure 4B). In addition, S100A8 was released into the supernatant (Supplemental Figure 4B). In the absence of prolonged TNF-α stimulation, S100A8 homodimers were rapidly degraded in the proteasome (Supplemental Figure 4C). Taken together, these data demonstrate a strong induction of the S100A8 protein after prolonged TNF-α expression in the absence of S100A9 in vivo. As shown above, S100A8 cannot form tetramers in the absence of S100A9. Lack of this autoinhibitory mechanism results in expression of dominant active S100A8 dimers, which drive uncontrolled and, in the case of TTP KO, even fatal inflammation. Healthy S100A9−/− mice are protected from fatal inflammatory reactions.
due to appropriate proteasome degradation over time, while this mechanism is inadequate during chronic TNF-α conditions.

Identification of the S100A9-binding site on TLR4/MD2. Structural analysis revealed several aa hidden in the (S100A8/S100A9)2 tetramer interface (Figure 5A), but freely accessible in the heterodimer (Figure 5B) (28), most of which were associated with the second calcium-binding EF-hands of both S100 subunits. We changed selected aa of S100A9 (E64, D65, K72, Q73, E77, and R85) to alanine as a model of active S100 dimers (29). These aa were not involved in the coordination of the calcium ion, showed little contact with neighboring aa, and were freely accessible in the dimer (Figure 5C). Four of the six exchanges in S100A9 (E64A, D65A, Q73A, and E77A) already showed a trend in binding reduction of about 20% compared with WT S100A9 homodimers in a quantitative TLR4/MD2-binding assay (Figure 5D). However, by changing 2 of the selected aa, S100A9 dimers lost their affinity to the TLR4/MD2 complex almost completely (Figure 5E). Changes in the overall structure of the mutated
proteins were excluded by fluorescence emission spectrometry (Supplemental Figure 7A).

After almost complete trypsin digestion of S100A9 (Figure 5F, insert), half of the inflammatory activity on monocytes was still present, indicating that a peptidic fragment or fragments of S100A9 are sufficient to activate monocytes (Figure 5F). Using this preparation, 1 specific peptide (peptide no. 15 of 17 possible peptides without missed cleavages) was detected by a TLR4/MD2 pull-down assay and identified by mass spectrometry analysis, revealing the sequence of S100A9 from aa 73 to 85 (Figure 5G), which is almost identical to that of the peptide identified by structural analysis and site-directed mutagenesis described above. We next synthesized a peptide comprising the whole EF-hand II (EF) showed reductions in binding properties similar to (S100A8/S100A9)2 tetramers. Tetramers are unable to bind to those observed for homologues mutations in the S100A9 full-length protein (Supplemental Figure 7B).

Table 1. Putative contact residues of S100A9 homodimer and TLR4/MD2

<table>
<thead>
<tr>
<th>Residues in S100A9</th>
<th>Residues in S100A9</th>
<th>Residues in TLR4</th>
<th>Residues in MD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAL 24</td>
<td>HIS 431</td>
<td>LYS 89</td>
<td></td>
</tr>
<tr>
<td>ASP 30</td>
<td></td>
<td>LYS 435</td>
<td></td>
</tr>
<tr>
<td>GLU 39</td>
<td></td>
<td>LYS 50 ALA 610, GLN 616</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LYS 54 SER 613, ASP 614</td>
<td></td>
</tr>
<tr>
<td>ASP 65</td>
<td></td>
<td>LYS 125</td>
<td></td>
</tr>
<tr>
<td>THR 68</td>
<td></td>
<td>TYR 131</td>
<td></td>
</tr>
<tr>
<td>THR 68</td>
<td></td>
<td>THR 84</td>
<td></td>
</tr>
<tr>
<td>ASP 71</td>
<td></td>
<td>ARG 90</td>
<td></td>
</tr>
<tr>
<td>LYS 72</td>
<td>ARG 90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLN 73</td>
<td>GLY 123</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLU 77</td>
<td>ASN 86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARG85</td>
<td></td>
<td>THR 84</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The concept of sterile inflammation driven by endogenous danger signals has markedly increased our understanding of numerous inflammatory diseases. S100A8/S100A9 are the most abundant alarmins in many clinically relevant disorders, such as rheumatoid arthritis or other autoimmune diseases, inflammatory lung or bowel diseases, allergies, or cardiovascular complications (3, 6). Although the alarmin concept is supported by different gene KO models in mice, clear mechanistic models of their local regulation are lacking. We now demonstrate an autoinhibitory mechanism in which calcium-induced tetramer formation abolishes the proinflammatory activity of S100A8/S100A9 dimers, which has several implications for the field of alarmin-driven inflammation in general and S100 biology in particular. Our data unravel a model of a local self-control mechanism of S100 alarmin–driven inflammation. In the intracellular low-calcium milieu, S100A8 and S100A9 are present as heterodimers that are secreted by activated phagocytes at the site of inflammation (30–32). These dimers interact with and activate TLR4/MD2-expressing cells via a specific binding site mainly located in the second EF-hand, thus promoting inflammatory processes. Due to high extracellular calcium concentrations, heterodimers subsequently associate to (S100A8/S100A9) tetramers. Tetramers are unable to bind to TLR4/MD2, thereby restricting the biological activity of S100 proteins locally in order to avoid undesirable systemic inflammatory side effects. The binding of other TLR4/MD2 agonists, including LPS and S100 homodimers, is unaffected by tetramer formation. Our findings explain many contradictory reports regarding inflammatory S100 effects, since most studies did not consider the fact that different complex forms may show different inflammatory activities (33). The biological relevance of our hypothesis was confirmed in 2 independent TNF-α–driven mouse models of inflammation. In both of these models, S100A9 deficiency promoted uncontrolled inflammation due to S100A8 dimer recovery. The presence of dominant active S100A8 dimers prevented calcium-induced tetramer formation and controlled the inflammatory effects by autoinhibition due to the absence of the S100A9 subunit. Analysis of protein samples directly obtained from phagocytes, serum, or other biological samples indicated that S100A8/S100A9 heterocomplexes are the predominant form of these molecules in vivo (33). This assumption is supported by experimental evidence analyzing S100A8/S100A9 interactions in vivo (e. g., yeast 2-hybrid system) (34) and comparative biophysical analysis of homo- and heterocomplexes (35) as well as structural data (28, 29, 36), indicating that homodimers are a useful
Figure 5. Identification of the S100A9-binding site on TLR4/MD2. (A–C) The PDB files of the S100A8/S100A9 tetramer (1XK4, A) and S100A9 dimer (1IRJ, C) were retrieved from the RSCB PDB website. (B) 1XK4 was modified containing only the E (S100A8, gray) and G (S100A9, green) chains resembling 1 heterodimer. Masked aa in the tetramer were analyzed by Cluspro. (B, yellow- or cyan-labeled aa for S100A8 or S100A9). Labeled S100A9 aa were chosen for mutation studies (C). (D) Single mutated S100A9 was analyzed for TLR4/MD2 binding compared with WT S100A9. All S100A9 mutants showed reduced binding to TLR4/MD2. (E) Double-mutated S100A9 was analyzed for binding to TLR4/MD2 as shown in D and showed significantly reduced binding properties compared with WT S100A9. Data represent mean ± SD of 5 independent experiments. *P < 0.05; **P < 0.01, 1-way ANOVA. (F) Monocytes were stimulated for 4 hours with intact S100A9 (0 h = no trypsinization) or the fragment mixture, and TNF-α levels were quantified by ELISA. Western blot shows remaining intact S100A9 (insert). One representative of 3 independent experiments is shown. (G) Tryptic fragments of hS100A9 (F) were analyzed for TLR4/MD2 binding, and peptide 15 corresponding to aa 73–85 showed specific interaction with TLR4/MD2, as detected by nanoUPLC/ESI-Q-TOF MS/MS (m/z 1614). Peptide 6 (NIETIINTFHQYSVK) was also detected in the control setting without TLR4/MD2 and reflects unspecific binding. (H) Peptides comprising aa 63–79 of S100A9 (MEDLDTNADKQLSFEF, MW: 2032 g/mol), control peptides 63–79 5A (MAALDTNADAALSFAEF, MW: 1758 g/mol) or a scrambled peptide (DSELMEENLADQFKDF, MW: 2032 g/mol) were investigated for TLR4/MD2 binding as shown in G. Unspecific binding was analyzed by using peptides 63–79 without TLR4/MD2. Only S100A9 peptides 63–79 showed binding to TLR4/MD2 (3 independent experiments). Asterisks indicate polyethylene glycol impurities.
relevant target for therapeutic approaches. The heterocomplex, also known as calprotectin, is widely used as a biomarker for detection and monitoring the disease activity of inflammatory bowel disease, psoriasis, or rheumatoid arthritis (15). S100A8/S100A9 expression predicts therapeutic efficiency and disease flares of arthritis, further emphasizing the functional significance of this heterodimer in disease pathology.

The identification of a specific molecular structure responsible for S100 TLR4/MD2 interaction presented here markedly increases the chances of addressing S100A8/S100A9 as a highly relevant target for therapeutic approaches. The heterocomplex, also known as calprotectin, is widely used as a biomarker for detection and monitoring the disease activity of inflammatory bowel disease, psoriasis, or rheumatoid arthritis (15). S100A8/S100A9 expression predicts therapeutic efficiency and disease flares of arthritis, further emphasizing the functional significance of this heterodimer in disease pathology.

Figure 6. Proposed structural modeling of S100A9 homodimer interaction with TLR4/MD2. (A) Modeling of the S100A9 peptides 63–79 hTLR4/MD2 complex in ribbon representation showing a close-up view with the putative interaction aa (labeled in gray for S100A9 aa and red for MD2-specific aa) as indicated. The model is based on the crystal structure of the TLR4/MD complex (PDB ID 3FXI), with LPS being omitted and the coordinates of peptides 63–79 being taken from PDB ID: 1IRJ. (B) Model of the potential binding site of S100A9 homodimer and TLR4/MD2 showing a number of contact aa summarized in Table 1. (C) Close-up view of B focused on the S100A9 EF-hand II, MD2 binding region. (D) Model is based on the crystal structures of S100A9 (PDB ID: 1IRJ, chains G and H) and of the human TLR4/MD2 complex (A chain of TLR4 and C chain of MD2) with LPS being omitted. The side chains of S100A9 aa are labeled in gray or brown and those of TLR4/MD2 in red. (D) Structural modeling of S100A8/S100A9 heterodimer (PDB ID 1XK4, chains E and G) interaction with TLR4/MD2 confirms identical binding regions for the homodimer S100A9 as well as for the heterodimer. Molecular modeling and graphics preparation were performed using Cluspro and PyMOL software packages.
of these proteins in clinically relevant diseases (38). The extraor-
dinarily high expression of S100A8/S100A9 at local sites of active
inflammation compared with clinically noninvolved tissue or
systemic levels further highlights the relevance of the molecular
mechanism described here for local inflammatory activity (30,
39). The frequently local and discontinuous patterns of inflamma-
tory symptoms in many autoimmunity diseases may be explained
by the fact that local manifestation of clinical inflammation due
to underlying systemic autoimmunity needs an additional tissue-
derived stress trigger such as locally active S100 alarms. The
high local expression of these molecules during inflammation is
also demonstrated in in vivo imaging studies of different inflam-
matory disease models in mice (27). General KO of the S100A8/
S100A9 system significantly blocks inflammation, and biological
relevance of this system has been confirmed in various preclinical
models of inflammatory processes, such as LPS-induced septic
shock, arthritis, psoriasis, and SLE-like disease, all of which reflect
the expression pattern of S100A8/S100A9 in human diseases
very well (8, 11, 12, 40). Finally, several sophisticated models of
metastasis development during tumor progression indicate a role
of S100 TLR4–driven inflammation in the establishment of pre-
metastatic niches and the potential use of targeted blockade of
S100A8/S100A9 to prevent dissemination of tumor cells (41, 42).

TLR4 has already been shown to play a role in sterile inflam-
mation in the absence of any microbial ligands (1–4), and S100A8
and S100A9 are the major endogenous TLR4 ligands in many
inflammatory diseases, especially rheumatoid arthritis, psoria-
sis, and inflammatory bowel disease (38, 40, 43). TLR4-targeted
therapies are currently under development for acute and chronic
inflammation. In terms of therapy, targeting S100 proteins instead
of TLR4 has an obvious advantage, as specific blocking of active
S100A8/S100A9 heterodimers would not primarily affect the
TLR4-dependent antimicrobial defense mechanisms. Although
concentrations of S100A8/S100A9 are relatively high during
inflammatory diseases, our data indicate that the pharmacologi-
cally relevant target is only the minor fraction of active heterodi-
mers. There are already 2 lines of evidence that blocking of S100
proteins may be effective. Molecules of the so-called Q-compound
family have been shown to inhibit the interaction of S100A8/
S100A9 with TLR4, show an antiinflammatory effect, and have
been successfully used in phase III trials for treatment of multiple
sclerosis (44, 45). In addition, recombinant peptide bodies have
been shown to block S100-dependent effects in a murine model
of tumor metastasis. However, it is not clear whether Q-compounds
or these peptide bodies specifically address the TLR4–binding
region of S100 proteins described here (46). Along with the mecha-
nism of locally restricted activity of S100A8/S100A9–driven
inflammation shown here, these molecules are promising candid-
ates for a very specific and local antiinflammatory therapy avoid-
ing systemic side effects. Taking our data together, we describe a
molecular mechanism by which alarmins drive sterile inflamma-
tion restricted to local sites of injury by autoinhibitory regulation.

Methods

Animal studies. The WT C57BL/6, S100A9–/–, TFP–/–, TNF–/–, hTNF-tg
(strain Tg197), and ihTNF-tg mouse strains have been described
elsewhere (13, 18, 26). All mouse lines had a C57BL/6 genetic back-
ground except the ihTNF-tg mice, which consisted of C57BL/6 ×
FVB-N hybrids. For analysis of the role of S100A8/S100A9 proteins
in the development of TNF–/– mice, further hybrid mice with
the S100A9 deficiency in combination with other KO genes and
transgenes were generated and are described in Results. Genotyping
was performed by PCR using primers and conditions as described
elsewhere (13, 18, 26).

Scoring criteria of the psoriasis-like skin disease of TTP–/– S100A9–/–
mice. To evaluate the severity of the psoriasis-like phenotype of TTP–/–
S100A9–/– mice, an adapted PASI score was used, as follows: grade
0, 0% involved area; grade 1, less than 10% involved area; grade 2,
10%–29% involved area; grade 3, 30%–49% involved area; grade 4,
50%–69% involved area; grade 5, 70%–89% involved area; and grade
6, 90%–100% involved area.

Scoring criteria of ihTNF-tg/S100A9–/– and ihTNF-tg mice. To
induce expression of the human TNF–/– transgene, 6-week-old
mice were kept on drinking water containing dox (1 ng/ml, Sigma-
Aldrich) and 5% sucrose for the indicated times. The water was
protected from light and exchanged every 3 days. Clinical assess-
ments, such as body weight, paw swelling, and grip strength, were
performed twice a week during the dox stimulation of the mice. Paw
swelling was graded from 0 to 3, and grip strength was graded from
0 to –3 as previously described (47).

Anti-TNF–/– treatment. To neutralize the excess of TNF–/– in
TFP–/– S100A9–/– mice, the animals received either 40 mg/kg cer-
tolizumab (UCB), a PEGylated Fab’ fragment of a humanized TNF–/–
inhibitor monoclonal antibody, or a monoclonal antibody of irre-
levant specificity as control antibody into the back skin on days 4, 6,
and 8 after birth. The antibody is already in clinical use as arthritis
and psoriasis therapy.

Reagents. LPS from E. coli (055:B5) was purchased from Sigma-
Aldrich. SB202190 and PD98059, specific inhibitors for p38 MAPK and
MEK were from Calbiochem. Staurosorpine and GF109203X (inhib-
tors of protein kinase C) were from Alexis Biochemicals and Molecu-
lar Probes (via MoBiTec). The TLR4 antagonist RS-LPS (LPS from
Rhodobacter sphaeroides) was from Invivogen, and the blocking mouse
monoclonal antibody to human TLR4 (HTA125) was from Abcam.

Determination of cytokine concentrations. TNF–/– and IL-1β protein
levels were analyzed in culture supernatants using commercial ELISA
kits (BD Bioscience). Cytokine concentrations in tissues were assayed
using the FACS-based FlowCytomix Multiplex Kit (eBioscience).

Cells and cell-culture conditions. Methods of cell purification and
culture conditions have been described in detail elsewhere (8). Briefly,
for stimulation experiments, human monocytes (105 cells/ml) were
washed with varying concentrations (100 ng/mg to 5 μg/mg) of purified
S100A8, S100A9, or S100A8/S100A9 proteins or mutants thereof,
LD50 (1–10 ng/ml), and/or IFN-γ (500 U/ml) for the times indicated
in figure legends.

Human monocytes were isolated from blood samples after leuka-
pheresis via Pancoll (Pan Biotech) and subsequent Percoll (GE Health-
care) gradient centrifugation (48). Cells were cultivated overnight
in Teflon bags in McCoy’s 5a Medium (Biochrome) supplemented with
15% FCS (Gibco Life Technologies, Thermo Fisher Scientific) prior to
use for stimulation or in functional assays.

Mouse BMCs were flushed from femurs and tibias with Dulbecco’s
medium. Erythrocytes were lysed with lysis buffer (155 mM NH4Cl, 10
mM KHCO3, 0.1 mM EDTA) for 5 minutes at room temperature, and
the remaining cells were washed once with PBS. Murine whole-blood cells were collected from heparin blood after lysis of erythrocytes with lysis buffer. Collected cells were either lysed for Western blot analysis or centrifuged on glass slides for S100A8 and S100A9 staining.

A549 human alveolar epithelial cells (ATCC, CCL-185) were grown in HAM’s F-12 medium supplemented with 2 mM l-glutamine and 10% heat-inactivated FCS. The experiments reported here were performed using confluent cells in serum-rich medium.

HEK293 cells stably transfected with human TLR4, CD14, and MD2 genes or TLR4 only as well as control cells transfected with empty plasmids for murS100A8 or murS100A9 were provided by Marco E. Bianchi (San Raffaele Scientific Institute, San Raffaele University, Milan, Italy). HEK293 cells were grown in DMEM with 4.5 g/l glucose, 10% FBS at 37°C in 5% CO₂.

Primary epidermal keratinocytes were isolated from the skin of 2- to 3-day-old newborn mice as described (49) and cultured in FAD medium containing 50 μM calcium ions. All cell culture media (Merck) were entirely free of endotoxin, as detected by SDS-PAGE, were collected. Samples were stored as stock solutions at -20°C. Correct refolding and complex formation were assessed by SDS-PAGE, MALDI-mass spectrometry (MALDI-MS), and electrospray ionization–MS (ESI-MS) (16, 50). To confirm the structural integrity of double-mutated S100A9 homodimers, fluorescence spectra were recorded at 20°C with a spectrophotometer (Spx, Model FluoroMax II, Instruments SA) using protein concentrations of 5 μg/ml. Changes in the conformation of S100A9 due to the mutation or mutations would be easily detectable by altered intrinsic Trp and Tyr fluorescence properties. All fluorescence spectra were buffer corrected (35). Possible endotoxin contaminations were excluded by LAL assay (BioWhitaker). LPS could not be detected or was lower than 1 pg/μg S100 protein in the different batches. As an additional control, S100A9 protein samples were completely heat inactivated at temperatures at which LPS was still fully active, but no biological response was registered. Moreover, LPS contaminations were further excluded by blocking experiments using polymyxinB sulfate (Sigma-Aldrich) for all preparations.

DNA microarray hybridization. Total cellular RNA (Macherey-Nagel) was isolated from human monocytes that were exposed to 5 μg/ml of S100A8, S100A9, or S100A8/S100A9 or 1 ng/ml LPS or to medium for 4 hours (3 independent experiments) and used for analysis by Illumina human HT-12 Expression BeadChips. The protocol was performed according to the manufacturer’s instructions. Data were imported into Partek Genomics Suite V6.6 (PGS, Partek) using robust multarray average (RMA), which is an algorithm used for background correction, log₂ transformation, and quantile normalization of expression data prior to batch correction. A 1-way ANOVA model was performed to calculate the 1,000 most variable and differentially expressed genes between the different stimulating conditions. Differentially expressed genes were defined by a fold change (FC) greater than 2.5 or less than -2.5 and a false discovery rate–corrected (FDR-corrected) P value of less than 0.05. To visualize the structure within the data, we performed hierarchical clustering (HC) on the 1,000 most variable genes, with default settings in PGS, on FDR-corrected values according to the expression values of the samples across the conditions as described (39).

Quantitative real-time PCR. RNA from HEK cells, human monocytes, or snap-frozen mouse tissue was isolated using the NucleoSpin...
RNA II Kit (Macherey-Nagel), and 1 μg of total RNA was reverse-transcribed to cDNA using RevertAid H Minus Transcriptase (Fermentas). Expression levels of selected genes were confirmed by quantitative reverse-transcriptase PCR (RT-PCR), which was performed using Sybr Green (PeqLab). Data were acquired with the CFX 384 Real-Time System and CFX-manager software, version 3.0 (Bio-Rad). Each measurement was set up in duplicate, and the relative expression was calculated as $2^{ΔΔCt}$ using ribosomal protein L (RPL), GAPDH, or β-actin as the housekeeping control genes. Sample data are presented as fold induction compared with naive cells.

The following primers were used for human PCR analysis: IL-6 forward, 5′-AGAGGACCTGGACAGAAAACACT-3′; IL-6 reverse, 5′-AGGCAAGCTCTCCATATGCTC-3′; SOCS2 forward, 5′-AGCGCTCTCCTCCGATTT-3′; SOCS2 reverse, 5′-CCATGGTTCCAGGCAAGTAATA-3′; SOCS1 forward, 5′-AGCTCTCTCTTCCGCCAGATT-3′; SOCS1 reverse, 5′-CCACTGGTTCCAGGCAAGTAATA-3′; SOCS3 forward, 5′-CCTTGTTGCCTCTCCATATT-3′; SOCS3 reverse, 5′-AGCTCGCTCCAGTAGAAC-3′; TNF-α forward, 5′-AGTGAAGATGGTTGTA-3′; TNF-α reverse, 5′-AGCTTCTTCTCGCTCAGACG-3′; RAGE reverse, 5′-CATCTGCTCAGTTCTGGATA-3′; CD80 forward, 5′-CTGCTTTTGCCCGACAGTGC-3′; CD80 reverse, 5′-CAGATCTTTTTCACGCCCTTGC-3′; S100A8 forward, 5′-CTGCTTTTGCCCGACAGTGC-3′; S100A8 reverse, 5′-AGCTCGCTCCAGTAGAAC-3′; β-actin forward, 5′-ATCCCTGGAAGTGAAATGGA-3′; β-actin reverse, 5′-AGGAGGACCTGGACAGAAAACACT-3′. Each measurement was set up in duplicate, and the relative expression was calculated as $2^{ΔΔCt}$ using ribosomal protein L (RPL), GAPDH, or β-actin as the housekeeping control genes. Sample data are presented as fold induction compared with naive cells.

The following primers were used for murine PCR analysis: TNF-α forward, 5′-AGGAAGACAGATTGCGGGA-3′; TNF-α reverse, 5′-CCTTGTTGCCTCTCCATATT-3′; IL-10 forward, 5′-AGACTTCTGGAACAGACGGATG-3′; IL-10 reverse, 5′-CCAGTTCTGACCTCTGAGA-3′; RAG-1 forward, 5′-ATCACCAGTGCTCGTCTTAC-3′; RAG-1 reverse, 5′-CTCAGGAGGTCTCTCAGAT-3′; β-actin forward, 5′-AGGGGAAGATGGTTGTA-3′; β-actin reverse, 5′-AGCTCGCTCCAGTAGAAC-3′. Each measurement was set up in duplicate, and the relative expression was calculated as $2^{ΔΔCt}$ using ribosomal protein L (RPL), GAPDH, or β-actin as the housekeeping control genes. Sample data are presented as fold induction compared with naive cells.

**Histology and immunohistochemistry.** The paws were fixed overnight in 4% paraformaldehyde, decalified with 0.5 M EDTA solution, and embedded in paraffin. Sections measuring 4 μm in thickness were analyzed. The extent of inflammation and cartilage damage in the paws was determined by morphometry as described elsewhere (47). Usually, 4 different sections per mouse sample were quantified from 5 to 6 mice for each mouse line. Proteoglycan loss was determined as the percentage of detained cartilage area. For skin samples, mice were sacrificed at day 9, and skin from the back was fixed in 4% paraformaldehyde or embedded in Tissue-Tek (Sakura) for cryosectioning. Fixed skin samples were embedded in paraffin according to procedures used for H&E staining and immunohistochemistry. For immunohistochemistry, paraffin skin sections (4 μm) were dewaxed, blocked 30 minutes with 10% normal goat serum (Invitrogen), and incubated with rabbit anti-S100A9 or anti-S100A8 antibodies for 1 hour at room temperature. Vectastain ABC-AP (BA-1000) and Vector Red Substance Kits (SK-5100) (Vector Laboratories) were used for visualization of the stained proteins. For immunofluorescence analyses, sections (5 μm) of frozen tissue were fixed in ice-cold acetone. After blocking with 10% normal goat serum in PBS for 30 minutes, the sections were washed in PBS (3 x 5 minutes). Primary antibodies dissolved in PBS were applied for 1 hour at room temperature or overnight at 4°C. After washing in PBS, sections were incubated with anti-rabbit or anti-rat Alexa Fluor 488-coupled IgG. Sections were then counterstained with propidium iodide or DAPI. Finally, after washing in PBS, sections were fixed with 4% paraformaldehyde and mounted in Mowiol (Sigma-Aldrich). The following primary polyclonal antibodies were used: anti-keratin 14 (1:100; clone poly 19053, BioLegend), anti-K10 (1:100; clone poly 19054, BioLegend), anti-Lor (1:2000; clone poly 19051, BioLegend) (Covance). Anti-Ki67 (1:100; ab15580, Abcam) rat monoclonal antibodies were used on frozen sections as follows: F4/80 (1:100; clone 3C3-A1, Bio-Rad), anti-Gr-1 (1:200; clone RB6-8C5, BD Bioscience), and anti-CD3 (1:1000; clone 17A2, Thermo Fisher). Sections were counterstained with propidium iodide to visualize the nuclei. Fluorescence stainings were analyzed using a Zeiss Axio Vision Microscope.

**Detection of MyD88 translocation by immunofluorescence microscopy.** A549 cells were cultured on fibronectin-coated LabTec chamber slides (Nunc, Thermo Scientific) until confluence. After stimulation, as indicated in the figure legends, cells were fixed with formaldehyde (3.7%, 20°C, 10 minutes) and permeabilized with Triton-X 100 (0.5%, 20°C, 10 minutes). After blocking of unspecified binding sites with FCS (3.7%, 20°C, 10 minutes) and permeabilization with Triton-X 100 (0.5%, 20°C, 10 minutes), primary antibodies dissolved in PBS were applied for 1 hour at room temperature or overnight at 4°C. After washing in PBS, sections were incubated with anti-rabbit or anti-rat Alexa Fluor 488-coupled IgG. Sections were then counterstained with propidium iodide or DAPI. Finally, after washing in PBS, sections were fixed with 4% paraformaldehyde and mounted in Mowiol (Sigma-Aldrich). The following primary polyclonal antibodies were used: anti-keratin 14 (1:100; clone poly 19053, BioLegend), anti-K10 (1:100; clone poly 19054, BioLegend), anti-Lor (1:2000; clone poly 19051, BioLegend) (Covance). Anti-Ki67 (1:100; ab15580, Abcam) rat monoclonal antibodies were used on frozen sections as follows: F4/80 (1:100; clone 3C3-A1, Bio-Rad), anti-Gr-1 (1:200; clone RB6-8C5, BD Bioscience), and anti-CD3 (1:1000; clone 17A2, Thermo Fisher). Sections were counterstained with propidium iodide to visualize the nuclei. Fluorescence stainings were analyzed using a Zeiss Axio Vision Microscope.

**Immunoprecipitation of IRAK-1.** Human monocytes (20 x 10⁶ cells per sample) were preincubated overnight with human recombinant IFN-γ (500 U/ml, Bender MedSystems) and stimulated with medium alone and subsequently stimulated with 10 ng/ml LPS or S100A9 (5 μg/ml) for 45 minutes. Cells were then lysed in 1 ml lysis buffer (0.5% Nonidet P-40, 10% glycerol, 50 mM HEPES, pH 7.9, 250 mM NaCl, 20 mM glycerophosphate, 5 mM p-nitrophenyl phosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 5 mM dithioerythritol, 1× complete protease inhibitors; Roche). IRAK-1 was immunoprecipitated using 2 μg per sample IRAK-1-specific monoclonal antibody (catalog sc-5288, Santa Cruz Biotechnology Inc.) and a mixture of protein G–sephrose and protein A–agarose (Roche). In vitro kinase assays were performed as described previously (52).
were then treated with inhibitors of the proteasome (MG132, UBP-conjugating enzyme E2 (BAY-11-7821, Tocris Bioscience) at the indicated concentrations, ubiquitin-activating enzyme E1 (PYR-41, UBPBio), or ubiquitin-conjugating enzyme E2 (BAY-11-7821, Tocris Bioscience) at the indicated concentrations, or for stimulation of human monocytes for 4 hours. TNF-α, IL-6, and IL-10 were measured in the supernatants in triplicate by using ELISA kits.

Proteasome and ubiquitin enzymes E1 and E2 inhibition assays
BMCs were isolated from S100A9−/− mice. Cell pellet was resuspended in BMC growth medium (DMEM, 10% FCS, 20% L929 cell supernatant, 1% penicillin/streptomycin, 2% L-glutamine), and cells were seeded at 1 × 10^6 cells/well in a 24-well plate at 1 ml per well. Cells were then treated with inhibitors of the proteasome (MG132, UBP-Bio), ubiquitin-activating enzyme E1 (PYR-41, UBPBio), or ubiquitin-conjugating enzyme E2 (BAY-11-7821, Tocris Bioscience) at the indicated concentrations and incubated at 37°C, 5% CO₂. The control cells were treated with DMSO.

Tryptic digestion of human S100A9 homodimers. Immobilized TPCK trypsin (25 μl of settled gel, Pierce) was used to digest 30 μg human S100A9 at 37°C for different time points, as indicated in Figure 5F. Subsequently, samples were centrifuged (5 minutes, 400 g) using a resin separator to remove trypsin-loaded beads. Aliquots of the supernatants were taken either for analyses by SDS-PAGE/Western blotting or for stimulation of human monocytes for 4 hours. TNF-α concentrations in supernatants of stimulated monocytes were then determined by ELISA (OptEIA, BD Biosciences). The fragments generated by tryptic digestion of hS100A9 were incubated with TLR4/MD2-coupled protein A/G agarose beads in the presence of 100 μM calcium. Bound S100 proteins were eluted at pH 2.0 and analyzed via SDS-polyacrylamide gels and transferred to nitrocellulose membranes.

Immunoprecipitation studies to identify TLR4/MD2-binding peptides. Anti-His antibody (catalog 37-2900, Invitrogen) and His-tagged TLR4/MD2 (R&D Systems) were mixed and coupled to protein A/G agarose beads (Pierce, Thermo Scientific). Tryptic peptides of S100A9 were added in the presence of 1 mM calcium. Bound pepticidic fragments were eluted at pH 2.0 and analyzed by nano-ESI-Q-TOF and MALDI-TOF mass spectrometry. Identical experiments were performed to analyze the binding of the chemically synthesized peptides of S100A9 (aa 63–79, MEDLDTNADKQLSFEF, corresponding control peptides (aa 63–79 5A, MAAALDTNAALALSFEF, aa 63–79 2A, MEALDTNAALALSFEF), MALDITNADKQLSFEF; bold letters indicate introduced mutations), or a scrambled peptide of S100A9 (aa DLSMTNEAQLADKPF).

Binding of WT S100A9 and mutated S100A9 to TLR4/MD2. Binding of S100A9 to TLR4/MD2 was analyzed by immuno-staining with cell pellets. Briefly, TLR4/MD2 (R&D Systems) was coupled to the wells of a plate and served as a capturing molecule. S100A9 or mutant S100A9 proteins were added at a concentration of 2 μg/ml each in the presence or absence of 100 μM calcium. Bound S100 proteins were detected by the addition of a primary anti-S100A9 antibody (1 μg/ml, polyclonal rabbit, homemade) and the secondary anti-rabbit IgG antibody coupled to HRP (1 μg/ml, Cell Signalling Technology). Tetramethylbenzidine (TMB) was used as substrate to quantitatively bind primary antibodies through absorbance readings at 450 nm (Anthos Mikrosysteme).

Computer-assisted ligand/receptor interaction studies. PDB files of an S100A8/S100A9 tetramer (PDB ID: 1XK4, chains E,F,G,H), S100A9 tetramer (PDB ID: 1IRJ), and TLR4/MD2 dimer (PDB ID: 3FXD) were retrieved from the RCSB PDB website. The S100A8/S100A9 PDB file was modified so that it contained only the E and G chains resembling the heterodimer. The S100A9 PDB file was modified so that it contained only the G and H chains resembling I homodimer. The TLR4/MD2 PDB file was modified so that it contained only the A chain of TLR4 and C chain of MD2. The modified PDB files of S100 proteins were then docked against TLR4/MD2 using Cluspro (http://cluspro.ub.edu/login.php) (53). PyMOL (https://pymol.org/2/) and SwissPdb viewer (https://spdbv.vital-it.ch/) software packages were used to visualize the docking interactions. We concentrated our analyses on the identification of aa that are not involved in Ca²⁺ binding and are sterically free for binding to TLR4. The aa in S100A9 (positions 64, 65, 72, 73, 77, and 85) were chosen for mutation studies.

SEC. SEC experiments were performed using a Superdex 75 7.5 × 30 cm column connected with an Äkta Purifier 90 FPLC (GE Healthcare) controlled by UNICORN software. The SEC separation runs were performed for 0.5 × 640 mm column, and UV detection was conducted at a wavelength of 280 nm by running a linear flow rate of 0.25 ml/min. The column was calibrated with 8.56 kDa ubiquitin, 12.4 kDa cytochrome c, 23.3 kDa trypsin, 43 kDa ovalbumin, and 66 kDa serum albumin, and the running buffer was either HBS, 1 mM EDTA, 1 mM DTT, pH 7.4. or HBS, 5 mM Ca²⁺, 1 mM DTT, pH 7.4.

Statistics. Values are shown as mean ± SD unless otherwise stated. P values are given in the figure legends, and values of P > 0.05 were considered not to be significant. Statistical analyses were performed by parametric tests (2 tailed t test or 1-way ANOVA) and the Mann-Whitney U test.

Study approval. Experimental procedures were approved by the local ethics committee in accordance with German regulations of the Society for Laboratory Animal Science, the European Health Law of the Federation of Laboratory Animal Science Associations, and the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.

Data availability. All original microarray data were deposited in the NCBI’s Gene Expression Omnibus database (GEO GSE109646). Further information about the data and about the reagents used is available upon request to the corresponding author.

Author contributions
T Vogl, AS, JR, SL, TL, and TP designed and supervised the study and the experiments, performed animal studies, and wrote the manuscript. VW, T Völker, ST, SZ, DC, KL, MF, PP, and CW performed animal studies and experiments and analyzed the data. SH, CG, and M Schäfers performed PET and OI experiments. SKJ, OP, M Stoll, and JLS performed the microarray study and analyzed the data. SK and AD performed mass spectrometry experiments. All authors edited the manuscript.

Acknowledgments
We thank H. Berheide, H. Hater, and D. Lagemann for excellent technical support. This work was supported by grants from
Cluster-of-Excellence EXC 1003 Cells in Motion, Münster, Germany (FF-project-2013-24 to JR), the Interdisciplinary Center of Clinical Research at the University of Muenster (Vo2/004/14, Ro2/003/15, Lo2/004/16 and core unit PIX to T Vogl, JR, and KL), the German Research Foundation (DFG) (CRC 1009 B7, B8, and B9 to KL, T Vogl and JR), CRC Transregio-128 (A2 to JR and A10 to KL, CRC 656 A9 to Vogl and Sh), the Federal Ministry of Education and Research (BMBF), project AID-NET and E-RARE, TREAT-AID (to JR), and the CRC 704 and Excellence Cluster ImmunoSensation (to JLS). T Völler was supported by a DFG postdoctoral fellowship (grant VO 2273/1-1).

Address correspondence to: Thomas Vogl, Institute of Immunology, University of Münster, Röntgenstr. 21, 48149 Münster, Germany. Phone: 0049.2518352943; Email: vogl@uni-muenster.de

T Völler’s present address is: Institute of Microbiology, Eidenössische Technische Hochschule (ETH) Zurich, Zurich, Switzerland.


