The Class A Macrophage Scavenger Receptor Attenuates CXC Chemokine Production and the Early Infiltration of Neutrophils in Sterile Peritonitis

Alessia Cotena, Siamon Gordon and Nick Platt

*J Immunol* 2004; 173:6427-6432; doi: 10.4049/jimmunol.173.10.6427
http://www.jimmunol.org/content/173/10/6427
The Class A Macrophage Scavenger Receptor Attenuates CXC Chemokine Production and the Early Infiltration of Neutrophils in Sterile Peritonitis

Alessia Cotena,* Siamon Gordon,* and Nick Platt2†

The macrophage scavenger receptor (SR-A) is a multifunctional receptor that is associated with several important pathological conditions, including atherosclerosis. In this study, we show, using a sterile peritonitis model, that it can regulate the inflammatory response. SR-A null mice display an increased initial granulocytic infiltration because of overproduction of the CXC chemokines, MIP-2 and keratinocyte-derived cytokine. This differential response is dependent upon particle internalization and can be mimicked by advanced glycation end product-BSA-conjugated latex beads. Thus SR-A is a nonactivating receptor, which is the first example of a pattern recognition receptor that serves to counter the activities of proinflammatory receptors and attenuates the production of specific chemokines to ensure an inflammatory response of the appropriate magnitude. The Journal of Immunology, 2004, 173: 6427–6432.

The inflammatory response is characterized by infiltration of effector leukocytes and its induction is dependent upon the detection of proinflammatory stimuli by specific receptors of the innate immune system (1). A consequence of ligand activation of these receptors is the production of chemotactic molecules, particularly chemokines, which coordinate the directed migration, activation, and retention of cells at the inflamed site (2). Despite the obvious connection between the recognition of proinflammatory stimuli and the generation of specific chemokines, we have a relatively poor understanding of the nature of receptors involved, their exact contributions in different settings, and how the process is regulated to ensure an adequate, but not excessive response.

Granulocytic infiltration is typically one of the earliest signs of an inflammatory response (3) and in mice, the CXC chemokines, MIP-2 and keratinocyte-derived chemokine (KC) orchestrate their trafficking; MIP-2 and KC are chemotactic for neutrophils in vitro (4) and bind to IL-8R, which is required for thioglycollate broth (TG)-stimulated peritoneal migration (5). Their levels are elevated in severe peritonitis (6) and granulocyte entry into the cavity can be almost completely abolished with Abs against the two chemokines (7). Localized gradients of the two chemokines are sufficient to induce migration into the peritoneum (7, 8). Therefore, the regulation of these chemokines is a critical event in the control of granulocyte extravasation.

Class A macrophage scavenger receptor (SR-A) is a macrophage (Mφ)-restricted multifunctional molecule (9). It can bind an unusually broad range of ligands, a property that underlies its many activities (9, 10). These ligands include modified lipoproteins, LPS of Gram-negative bacteria, and lipoteichoic acid of Gram-positive species, β-amyloid, and advanced glycation end product (AGE). It is best known in the context of vascular disease, but is also associated with Mφ adhesion, host defense, clearance of dying cells, and nervous system disorders (10). Understanding of its biological contributions has been greatly facilitated by studies of mice in which the SR-A gene has been deleted (11).

We have investigated TG peritonitis in SR-A-deficient mice (SR-A−/−). In the absence of SR-A, we found a significant early increase in neutrophil recruitment that correlated with higher levels of MIP-2 and KC and provide evidence that the receptor functions to modulate chemokine levels in specific acute inflammatory conditions.

Materials and Methods

Animals

Mice deficient in SR-A on a 129/ICR genetic background have been described (12). C3H/HeJ and C3H/HeN mice were obtained from Harlan (Oxon, U.K.). Mice were kept under specific pathogen-free conditions in the animal facility at Sir William Dunn School of Pathology (Oxford, U.K.) and used between 6 and 8 wk of age.

Induction of peritonitis

Sterile 3% TG (Difco, Detroit, MI) was prepared by autoclaving and was stored at room temperature in the dark for at least 6 mo before use. Mice received i.p. injection of either 1 ml of TG, 100 ng of LPS from Escherichia coli 0111:B4 (Sigma-Aldrich, St. Louis, MO), 1 ml of 1% oyster glycogen (Sigma-Aldrich), 0.5 ml of zymosan particles (0.5 mg/ml; Sigma-Aldrich), or 500 ng of MIP-2 chemokine (R&D Systems, Minneapolis, MN). At specified times, they were sacrificed, and peritoneal cells were recovered by lavage. When appropriate, this was centrifuged to remove cellular debris and the chemokine content was measured. The absolute number, frequency, and identity of peritoneal cells were determined by hemocytometer and FACS analysis. Cells were stained with anti-Ly6G (3; clone RB6-8C5; BD Biosciences, San Jose, CA), anti-F4/80 (Serotec, Oxford, U.K.), or annexin V (Molecular Probes, Eugene, OR), and analyzed on a FACS Calibur using CellQuest software (BD Biosciences).
Assessment of neutrophil apoptosis in vitro

Neutrophils were recovered by peritoneal lavage following TG injection, placed in culture, and the extent of apoptosis was determined at specific times by annexin V staining.

In vitro chemokine production by resident peritoneal Mφ (RPMφ)

RPMφ were harvested from untreated mice and cultivated at a density of 5 x 10^6/ml in RPMI 1640, 1% FCS, l-glutamine, and penicillin-streptomycin (all Invitrogen Life Technologies, Carlsbad, CA). Cells were stimulated with medium containing either 25% (v/v) of 3% TG, 1% glycogen, zymosan particles (25 particles per cell), or 1 μg/ml LPS. Supernatants were collected at 5 h for chemokine determination, cells were lysed, and protein content was measured by a BCA assay (Pierce, Rockford, IL). For experiments requiring inhibition of actin polymerization, cytochalasin D (Sigma-Aldrich) at a concentration (2 μM) sufficient to inhibit zymosan uptake (not shown) was added to cells 30 min before the addition of a proinflammatory stimulus. These were coincubated for 60 min, supernatants removed, washed, and reincubated with fresh medium without stimulus or cytochalasin D for an additional 4 h. These were subsequently combined to assess chemokine content. In a reciprocal experiment, cells were incubated with a proinflammatory stimulus for 60 min, supernatants removed, washed, and incubated with fresh medium containing cytochalasin D for a further 4 h.

Measurement of chemokines

MIP-2, KC, IL-1β, and TNF-α levels in supernatants, lavages, and blood were determined using specific ELISA (R&D Systems) according to the manufacturer’s protocols.

Preparation of protein-conjugated latex beads

Two-micrometer latex beads (Polysciences, Warrington, PA) were incubated with 0.5 mg/ml AGE-BSA (235 U/mg protein), prepared according to Ref. 24 or BSA (0.8 U/mg) in carbonate buffer at 4°C overnight, washed, and incubated with 0.5 mg/ml AGE-BSA (235 U/mg protein), prepared according to manufacturer’s protocols.

Results

SR-A-deficient mice exhibit enhanced TG-dependent inflammatory cell recruitment

Within 3 h of i.p. injection of TG, both 129 and SR-A^-/-^ mice had mounted inflammatory responses, but we consistently recorded a >2-fold recruitment of inflammatory cells into the peritoneum of SR-A^-/-^ mice relative to 129 (Fig. 1A). This differential infiltration was still present at 5 h postinjection (Fig. 1A) and was maintained through to at least 48 h (data not shown). There was no significant difference in the frequency of cells obtained from untreated 129 and SR-A^-/-^ mice (Fig. 1A), which contained equal numbers of F4/80^-^ RPMφ and no granulocytes (data not shown), confirming the absence of an inflammatory reaction before injection. There were equivalent numbers of granulocytes in the blood of 129 and SR-A^-/-^ animals post-TG inoculation (data not shown). There was no differential recruitment when peritonitis was provoked with glycogen or zymosan (Fig. 1B). Similarly, chemotaxis triggered by MIP-2 was not affected in SR-A^-/-^ animals (Fig. 1D).

To characterize the cellular composition of the infiltrate, we performed FACS analysis of the peritoneal washouts. This confirmed that at 3 and 5 h, it consisted of predominantly Gr-1^-high^ granulocytes (3) that accounted for 44 ± 8%, n = 25 (129) and 49 ± 12%, n = 23 (SR-A^-/-^) of total cells (Fig. 1C). We were able to exclude that the increased numbers of granulocytes in SR-A^-/-^ animals was due to enhanced longevity because their kinetics to undergo apoptosis in culture was unaltered (Table I). We did not observe greater numbers of annexin V^-^ apoptotic neutrophils in the peritoneum, suggesting their clearance or exiting were not affected (Table I).

![FIGURE 1. SR-A^-/-^ mice display enhanced peritonitis stimulated by TG. A, SR-A^-/-^ mice recruit greater numbers of inflammatory cells in response to i.p. TG than 129 animals (□). Data shown are representative of at least five independent experiments and reveal significant differences in cell recruitment between 129 and SR-A^-/-^ mice at 3 h (**, p < 0.01) and 5 h (***, p < 0.001). B, In contrast, SR-A^-/-^ and 129 mice respond equivalently to i.p. injection of glycogen or zymosan. C, SR-A^-/-^ mice recruit significantly greater numbers of Gr-1^-high^ granulocytes than 129 animals at 3 h (**, p < 0.05) and 5 h (***, p < 0.05) post-TG injection. D, Granulocytic extravasation into the peritoneum triggered by MIP-2 is the same in 129 and SR-A^-/-^ mice. Error bars represent SEM.

Differential production of CXC chemokines MIP2 and KC by 129 and SR-A^-/-^ RPMφ, but not TNFα and IL-1β following exposure to TG in vitro and in vivo

The most intensively studied molecules that can direct granulocyte recruitment are CXC chemokines (1). We hypothesized that the
enhanced recruitment of granulocytes in SR-A−/− animals reflected aberrant production of MIP-2 and KC by RPMφ and chose to determine their levels in the cavity and their secretion by RPMφ. ELISA measurements confirmed that RPMφ from SR-A−/− mice secreted [>2-fold higher amounts of MIP-2 and KC when exposed to TG in vitro for 5 h compared with 129 (Fig. 2, A and B). We measured the chemokine content of washouts of peritoneal cavities of mice 3 h post-TG injection and detected a difference in their relative amounts comparable to that in vitro (Fig. 2, C and D). The absolute quantities of chemokines differed in vitro and in vivo, which likely reflects loss due to catabolism, binding, uptake, adsorption in the cavity, or combinations of the above. Inflammation stimulated with glycogen or zymosan in vitro did not induce differential secretion (Fig. 2, E–H), consistent with their inability to provoke differential recruitment in vivo (Fig. 1B).

We detected different concentrations of the KC chemokine in the circulation of TG-treated mice (at 3 h: 129, 450 ± 63 ng/ml, n = 18; SR-A−/−, 1468 ± 150 ng/ml, n = 20, p < 0.05). MIP-2 was below detection (4). Although production of MIP-2 and KC by Mφ occurs early after exposure to a proinflammatory stimulus (13), specific cytokines can directly induce their secretion (4). To determine whether the differential levels of the two chemokines reflected prior cytokine generation, we measured the concentrations of TNF-α and IL-1β produced by RPMφ in vitro (Fig. 2). TNF-α concentrations were below detection, while levels of IL-1β were not different (undetectable at 1 h; at 3 h: 129, 1049 ± 95 pg/ml, n = 17; SR-A−/−, 1139 ± 145 pg/ml, n = 19). This is in agreement with a previous study of TG-induced peritonitis that also failed to detect TNF-α secretion (4).

**Enhanced granulocyte infiltration in SR-A−/− mice is not triggered by LPS**

LPS is a powerful proinflammatory stimulus (14) and a ligand for SR-A (9). We chose three approaches to exclude the possibility that endotoxin was responsible for the inflammatory response elicited by TG. First, we tested whether LPS could trigger differential chemokine production in vitro. SR-A−/− and 129 RPMφ responded identically (Fig. 3, A and B). Second, we injected mice with LPS and saw that even at high doses (>0.1 mg), we could not detect granulocytes within the period in which there was significant recruitment with TG (3–5 h; Fig. 3C). At the earliest point at which neutrophils cells did appear after LPS (7 h), there was no difference in recruitment (Fig. 3C). Third, we compared TG-stimulated peritonitis in C3H/HeJ mice, a LPS-hyposensitive strain because of a mutation in TLR4 (15), and C3H/HeN mice, a LPS-sensitive strain. There was no difference in inflammatory cell recruitment to TG (Fig. 3D). We deduced that neither is LPS the proinflammatory moiety in TG, nor is TLR4 likely to be required.
for the activation of peritoneal cells by ligand(s) in TG that result(s) in leukocyte recruitment.

**TG-stimulated chemokine production requires actin polymerization and is mimicked with AGE-BSA-conjugated latex beads**

Because TG is of heterogeneous composition, we made initial attempts to mimic the peritoneal responses of 129 and SR-A<sup>−/−</sup>/H11002 mice using identified ligands for SR-A. However, we were unable to stimulate either inflammatory cell recruitment into the peritoneum (data not shown) or significant chemokine secretion by RPM<sub>9278</sub> with maleylated BSA or AGE-BSA (Fig. 4A), both of which inhibited SR-A-dependent endocytosis (data not shown). Because this could be due to their soluble nature, we examined the consequences of pharmacological inhibition of actin polymerization with cytochalasin D. In the presence of cytochalasin D, almost all TG-stimulated chemokine production was suppressed (Fig. 4B), whereas secretion triggered by soluble LPS was unaffected (Fig. 4C). This suggested that TG-induced chemokine production required internalisation of particulate material. To present SR-A ligands as particles, we made latex beads conjugated with AGE-BSA or BSA, incubated them with RPM<sub>9278</sub>, and measured the secretion of MIP-2. We observed efficient ingestion by 129 and SR-A<sup>−/−</sup>/H11002 RPM<sub>9278</sub> (data not shown). There was significantly greater chemokine secretion by SR-A<sup>−/−</sup>/H11002 RPM<sub>9278</sub> that had ingested AGE-BSA beads. This differential response was inhibited with cytochalasin D when given at the same time as AGE-BSA beads, but not when added subsequent to the stimulus (Fig. 4D). In contrast, inhibition of actin polymerization did not influence significantly the chemokine secretion stimulated by soluble LPS (Fig. 4E). BSA-beads induced equal amounts of chemokine, and unconjugated latex particles were less active (Fig. 4D).

**FIGURE 4.** Particulate SR-A ligand can mimic TG-induced differential CXC chemokine production. MIP-2 secretion (picograms per milliliter) by 129 (□) and SR-A<sup>−/−</sup> (■). A, RPM<sub>9278</sub> exposed to soluble SR-A ligands in vitro. Only TG is capable of triggering differential chemokine production (*, p < 0.05). B, MIP-2 secretion by RPM<sub>9278</sub> triggered by TG in the presence or absence of cytochalasin D. Differential chemokine secretion is abolished by the coadministration of cytochalasin D with TG (**, p < 0.05). C, MIP-2 secretion by RPM<sub>9278</sub> triggered by LPS in the presence or absence of cytochalasin D. There is no significant difference in MIP-2 secretion triggered with LPS in either the presence or absence of cytochalasin D. D, MIP-2 secretion by RPM<sub>9278</sub> incubated with different particulate ligands. Only AGE-BSA beads induce differential chemokine secretion (*, p < 0.05). E, Differential MIP-2 secretion (*, p < 0.05) is blocked by the coinoculation of cytochalasin D with AGE-BSA latex beads (***, p < 0.05), but not when it is added subsequent (AGE-BSA + Cyt D) to the inflammatory stimulus. F, Chemokine secretion is not inhibited by cytochalasin D when added concurrently (Cyt D + LPS) or subsequent to LPS (LPS + Cyt D). Error bars represent SEM. Data representative of four independent experiments are shown.
Discussion
A hallmark of inflammation is the infiltration of specific leukocytes into tissues affected by infection, foreign material, or damage. We found in the absence of SR-A, there was enhanced granulocyte recruitment in sterile peritonitis that correlated with increased secretion of the proinflammatory mediators, MIP-2 and KC. The phenotype of SR-A−/− mice supports the concept that SR-A functions to limit the initial magnitude of this inflammatory pathway.

Inflammatory responses result from the detection of proinflammatory stimuli by receptors on cells of the innate immune system and an early consequence is the expression of chemoattractants that induce migration of effector cells (1, 16). Regulated secretion is critical to control leukocyte extravasation appropriate to ensure effective host defense without exacerbating tissue damage, and is presumably achieved through integration of signals originating from innate receptors. The magnitude of the initial period of inflammatory cell recruitment was influenced significantly by the absence of SR-A, and was associated with localized increases of MIP-2 and KC. RPMΦ would seem to be an important source of MIP-2 and KC in vivo because SR-A expression is restricted to these cells within the normal peritoneal cavity (17). This accords with the demonstration that depletion of rat RPMΦ reduced subsequent TG-induced neutrophil infiltration by >50% (18). We interpret the failure of glycogen and zymosan to elicit differential chemokine secretion and infiltration as due to the absence of SR-A ligands in these phlogistic agents, which presumably interact with other receptors to elicit sterile peritonitis.

We focused on the earliest phase of the inflammatory response to study recruitment only and avoid later stages when the concurrent processes of phagocytic removal and exiting from the cavity also determine cell numbers. The phenotype of SR-A−/− mice did not reflect altered properties of neutrophils or reduced phagocytic clearance by RPMΦ because the chemotactic movement of granulocytes to rMIP-2 was not affected, their ability to undergo apoptosis was not altered, nor was there an increased frequency of apoptotic cells within the inflamed peritoneum (19). Therefore, the peritonitis phenotype solely reflects enhanced recruitment. Interestingly, Thomas et al. (20) demonstrated that SR-A−/− mice were more susceptible to infectious peritonitis caused by Staphylococcus aureus because of the inability of MΦ to phagocytose, but the recruitment of granulocytes was normal. We suggest that the exact contribution of SR-A to neutrophil chemotaxis must in some way be influenced by the nature of the ligand for the receptor.

An important conclusion of the hypersensitive peritonitis phenotype is that SR-A is nonactivating because it is not required for chemokine production (i.e., in its absence, secretion of MIP2 and KC is actually enhanced). Hampton et al. (21) made a similar suggestion in a study of hepatic clearance of LPS. We can hypothesize a number of mechanisms through which SR-A may influence significantly the secretion of proinflammatory mediators. For example, the receptor may normally direct an intracellular fate for the bound inflammatory stimulus that results in either the noninflammatory detoxification of the molecule or the engagement of anti-inflammatory mechanisms. This fate is changed in SR-A−/− cells. The absence of SR-A may delay intracellular destruction of the particle, and hence, prolong persistence of the inflammatory stimulus. It may be that lack of the receptor reduces uptake significantly so that extracellular levels of the inflammatory stimulus remain high, but this was not the case for LPS clearance in SR-A−/− mice (22). Secretion of anti-inflammatory mediators may be reduced in the absence of the receptor. Through some direct biochemical interaction with other receptors, SR-A may modulate the secretory activities of MΦ, and this compensatory mechanism is affected in receptor-deficient cells. For example, MΦ that lack another scavenger receptor, SR-BI, spontaneously secrete significantly greater amounts of superoxide (23) However, at least with respect to CXC chemokine secretion, we could not measure production by resting cells, and their differential secretion was dependent upon the presence of an appropriate ligand. The enhanced level of MIP-2 and KC in SR-A−/− mice is consistent with the receptor either not being involved directly in their secretion, or that it negatively regulates their generation. We are currently investigating these possibilities.

TG-induced differential secretion of MIP-2 required phagocytic ingestion. Only particulate AGE-BSA could induce differential production, whereas binding or endocytosis of the soluble ligand failed to generate a similar response or cell infiltration. Therefore, the mechanism by which SR-A influences chemokine generation is dependent upon receptor clustering, cytoskeletal changes, and signaling mechanisms associated with phagocytosis (24). The ineffectiveness of cytochalasin D to block specific chemokine production when added 60 min subsequent to exposure to the particulate proinflammatory stimulus would suggest that the triggering of secretion is dependent upon phagocytosis or an event closely associated with it. This is probably why endotoxin, which binds to SR-A and other receptors, did not recapitulate the effects of TG, although it may be that SR-A plays only a minor role in the response of RPMΦ to LPS. SR-A is best recognized as an endocytic receptor, but is involved in the phagocytosis of at least three other particles, apoptotic thymocytes (19), S. aureus (20), and Neisseria meningitidis (25). Unfortunately, at present, very little is known of the specific intracellular signaling events that follow ligation of the receptor (10), particularly in the context of phagocytosis. It is pertinent to point out that there would appear to be major differences in the signaling pathways and the initiation of inflammatory responses between different scavenger receptors. For example, deletion of the class B receptor, CD36, diminishes the proinflammatory response of MΦ to β-amyloid (26).

We found that we could induce differential chemokine production with latex particles bearing the known SR-A ligand, AGE-BSA (9, 10). However, we do not know whether activity is specific to this particle and are currently investigating whether other SR-A ligands can elicit similar effects. Although TG has been used extensively to induce sterile inflammation, the identity of its proinflammatory moiety and the mechanism that underlies its activity are still obscure. Although TG activity has been shown to correlate with the presence of AGE modification because inhibition of its formation significantly impaired its ability to elicit inflammatory MΦ (27), it is not clear that AGE alone is responsible for the proinflammatory effects. Indeed, soluble AGE-BSA alone was ineffective when injected into the peritoneum, which suggests existence within TG of additional material(s) required for full activity (27). Agar accumulates in RPMΦ elicited with TG (28) and may represent a particulate ligand. Because TG is chemically poorly defined, it may contain ligands for SR-A other than AGE.

As SR-A is nonactivating, there must be a counter receptor(s) necessary for chemokine induction. We have not as yet identified this proinflammatory receptor, and although our data suggest it is unlikely to be TLR4, we cannot exclude other TLRs. We are not aware whether the characteristics of TG peritonitis have been described in mice in which candidate receptors have been deleted or blocked. This includes receptors that are known to bind AGE, which include the scavenger receptors, CD36, Lox-1, and FEEL-1 (29), the AGE receptors (30), and Galectin-3 (31). Our prediction would be that deletion of the counterpart receptor should impair granulocyte extravasation.
In summary, we have shown that SR-A attenuates the initial phase of granulocytic infiltration in sterile peritonitis by limiting the production of specific CXC chemokines and suggest that because of its broad ligand-binding properties and the promiscuity of its ligands, this receptor is likely to play a similar role in other inflammatory situations.

Acknowledgments

We thank Helen Vlaseara for the generous gift of AGE-BSA and helpful comments on the manuscript.

References