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Recently, proinflammatory activities had been described for S100A8 and S100A9, two proteins found at inflammatory sites and within the neutrophil cytoplasm. In this study, we investigated the role of these proteins in neutrophil migration in vivo in response to LPS. LPS was injected into the murine air pouch, which led to the release of S100A8, S100A9, and S100A8/A9 in the pouch exudates that preceded accumulation of neutrophils. Passive immunization against S100A8 and S100A9 led to a 52% inhibition of neutrophil migration in response to LPS at 3 h postinjection. Injection of LPS was also associated with an increase in peripheral blood neutrophils and the presence in serum of S100A9 and S100A8/A9. Intravenous injection of S100A8, S100A9, or S100A8/A9 augmented the number of circulating neutrophils and diminished the number of neutrophils in the bone marrow, demonstrating that S100A8 and S100A9 induced the mobilization of neutrophils from the bone marrow to the blood. Finally, passive immunization with anti-S100A9 inhibited the neutrophilia associated with LPS injection in the air pouch. These results suggest that S100A8 and S100A9 play a role in the inflammatory response to LPS by inducing the release of neutrophils from the bone marrow and directing their migration to the inflammatory site.

the same vector. Recombinant proteins were produced as previously described (21, 39). Contamination by endotoxins was <1 pg/µg recombinant proteins as assessed using the Limulus amebocyte assay. Recombinant S100A8/A9 was produced by mixing together equimolar quantities of recombinant S100A8 and S100A9 in the presence of HBSS supplemented with 10 mM HEPES, pH 7.4, containing 1.3 mM CaCl2.

Production of polyclonal Abs

New Zealand White rabbits (<2.5 kg) were immunized by intradermal dorsal injections (four sites) with a total of 100 µg of purified murine recombinant S100A8 or S100A9 in 500 µl of endotoxin-free PBS (Sigma-Aldrich, St. Louis, MO) mixed with an equal volume of CFA. Ab responses were enhanced by repeated injections 3 and 6 wk after the initial injection using IFA. Antisera were collected and tested for specificity by ELISA and Western blots against purified recombinant S100A8 and S100A9. IgG from antisera were purified by protein A affinity chromatography (Pierce, Rockford, IL). The anti-S100A8 antisera had titers of 1/100,000 and 1/500 for the detection in ELISA of 10 ng of S100A8 and S100A9, respectively. The anti-S100A9 antisera had titers of 1/250 and 1/100,000 for the detection in ELISA of 100 ng of S100A8 and S100A9, respectively. The absence of cross-reactivity of the purified IgG with the other murine myeloid-related protein or proteins within the air pouch exudates was confirmed by immunoprecipitation assays and Western blots.

CD rats were immunized by i.p. injections with a total of 60 µg of purified murine recombinant S100A8 or S100A9 in 250 µl of endotoxin-free PBS (Sigma-Aldrich) mixed with an equal volume of CFA. Ab response was enhanced by repeated injections 14, 28, and 42 days after the initial injection using IFA. Antisera were collected and tested for specificity by ELISA and immunoblots against purified recombinant S100A8 and S100A9. The anti-S100A8 antisera had titers of 1/10,000 and 1/500 for the detection of 100 ng of S100A8 and S100A9, respectively. The anti-S100A9 had titers of 1/250 and 1/10,000 for the detection of 100 ng of S100A8 and S100A9, respectively.

ELISA

For S100A8 and S100A9, Costar High Binding 96-well plates (Corning, NY) were coated overnight at 4°C with 100 µl/well purified rabbit IgG against S100A8 and S100A9 diluted to a concentration of 1 µg/ml in 0.1 M carbonate buffer, pH 9.6. The wells were blocked with PBS/0.1% Tween 20/2% BSA (150 µl/well) for 30 min at room temperature. The samples and standards (100 µl) were added and incubated for 45 min at room temperature. The plates were washed three times with PBS/0.1% Tween 20, and were incubated with rat IgG (100 µl/well) against S100A8 or S100A9 diluted in PBS/0.1% Tween 20/2% BSA (1/10,000) for 45 min at room temperature. The plates were then washed three times in PBS/0.1% Tween 20. To reveal the immune complex, peroxidase-conjugated goat anti-rat IgG (H+L) (1/10,000 for 45 min at room temperature. After three washes, 100 µl/well anti-S100A8 rat IgG diluted in PBS/0.1% Tween 20/2% BSA (150 µl/well) was added and incubated for 45 min at room temperature. The plates were washed three times with PBS/0.1% Tween 20 then incubated with 100 µl/well anti-S100A9 rat IgG diluted in PBS/0.1% Tween 20/2% BSA (1/10,000) for 45 min at room temperature. The plates were next washed three times in PBS/0.1% Tween 20 and incubated with 100 µl/well horseradish peroxidase-conjugated goat anti-rat IgG at a dilution of 1/10,000 for 45 min at room temperature. After three washes, 100 µl/well TMB-S substrate were added according to the manufacturer’s instructions. The ODs were read on a plate reader at 500 nm. The lower limit of quantification was determined as 4 ng/ml for both S100A8 and S100A9.

For S100A8/A9, 96-well plates were coated overnight at 4°C with purified anti-S100A8 rabbit IgG (1/100 well) diluted 1 µg/ml in 0.1 M carbonate buffer, pH 9.6. The wells were blocked with PBS/0.1% Tween 20/2% BSA (150 µl/well) for 30 min at room temperature. The samples and standards (100 µl) were added and incubated for 45 min at room temperature. The plates were washed three times with PBS/0.1% Tween 20, and were incubated with rat IgG (100 µl/well) against S100A8 or S100A9 diluted in PBS/0.1% Tween 20/2% BSA (1/10,000) for 45 min at room temperature. The plates were then washed three times in PBS/0.1% Tween 20. To reveal the immune complex, peroxidase-conjugated goat anti-rat IgG (H+L) (1/10,000 dilution) was added and incubated for 45 min at room temperature. The plates were washed three times and 100 µl/well TMB-S substrate were added according to the manufacturer’s instructions. The ODs were read on a plate reader at 500 nm. The lower limit of quantification was determined as 4 ng/ml for both S100A8 and S100A9.

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This accumulation was maximal at 6 h postinjection and almost returned to control levels by 12 h. More than 90% of the migrating leukocytes were neutrophils, with few monocytes migrating as well. This accumulation was associated with the release of S100A8, S100A9, and S100A8/A9 in the extracellular milieu following injection of LPS.

To examine the involvement of S100A8, S100A9, and S100A8/A9 in neutrophil migration, we first studied their release in vivo in response to LPS. The air pouch model was selected because this closed environment allows a clear measurement of migrated leukocytes and released proinflammatory factors in the exudates. Few leukocytes were present in the pouch exudates before the injection of either PBS or LPS. Injection of PBS in the air pouch led to a very mild accumulation of neutrophils, probably consecutive to the injury caused by the needle. In contrast, injection of LPS led to an inflammatory reaction associated with redness of the air pouch and the presence of plasma proteins in the air pouch exudates. Injection of LPS also induced the rapid migration of leukocytes to the pouch, first detected 3 h postinjection (Fig. 1A). This accumulation was maximal at 6 h postinjection and almost returned to control levels by 12 h. More than 90% of the migrating leukocytes were neutrophils, with few monocytes migrating as well.

This accumulation was associated with the release of S100A8, S100A9, and S100A8/A9 in the pouch exudates. Low levels of S100A8, S100A9, and S100A8/A9 were detected in the exudates of noninjected or PBS-injected mice (Fig. 1, B–D). In contrast, injection of LPS led to the rapid release of all three S100 proteins. S100A8 was detected as early as 1 h postinjection of LPS (before neutrophil migration, Fig. 1A) and remained significantly above the control levels for the next 23 h. Similarly, the presence of S100A9 was maximal between 3 and 12 h postinjection of LPS, but the levels returned to control values by 24 h postinjection. In contrast, the presence of S100A8/A9 was more transitory, being maximal at 6 h postinjection of LPS and returning to control levels by 9 h postinjection. Although S100A9 and S100A8/A9 concentrations were similar (3–5 µg/ml), S100A8 concentration was lower, reaching only 180 ng/ml. These results suggest that S100A8, S100A9, and S100A8/A9 are released separately during an inflammatory episode and precede neutrophil immigration.
FIGURE 1. Neutrophil accumulation and secretion of S100A8, S100A9, and S100A8/A9 in the air pouch exudates following injection of LPS. Dorsal air pouches were raised in CD-1 mice. On day 7, 1 ml of LPS (1 μg/ml) was injected into the air pouches. At various time points, air pouches were washed and (A) the neutrophils in exudates were counted; (B) S100A8, (C) S100A9, and (D) S100A8/A9 were quantified in the exudates by ELISA. Data represent the mean ± SEM of at least seven mice.

S100A8 and S100A9 are involved in neutrophil accumulation in response to LPS

To evaluate the role played by S100A8 and S100A9 in neutrophil migration, mice were injected i.p. with purified rabbit IgG against S100A8 and S100A9. LPS was then injected in the air pouches and neutrophil accumulation was measured 3 and 6 h later. Anti-S100A8 and anti-S100A9 had no effect on neutrophil accumulation in PBS-injected mice (Fig. 2). Anti-S100A9 slightly reduced neutrophil accumulation 3 h following injection of LPS, but this reduction was not significant (Fig. 2A). In contrast, anti-S100A8 reduced LPS-induced neutrophil accumulation by 52% at 3 h postinjection (p < 0.05, Bonferroni test). This inhibition was not enhanced by the addition of anti-S100A9. By 6 h postinjection, only the combination of anti-S100A8 and anti-S100A9 proved effective in preventing the migration of neutrophils to the air pouch in response to LPS (p < 0.05, Bonferroni test, Fig. 2B). These Abs inhibited neutrophil migration by 82%.

Presence of S100A9 and S100A8/A9 in the serum following injection of LPS in the air pouch

LPS induced the accumulation of >5.4 × 10⁶ cells in the air pouches (Fig. 1A), twice the estimated number of neutrophil content of the blood (~3 × 10⁶ cells) (41). Therefore, LPS stimulated the migration of neutrophils to the air pouch in numbers greater than were present in the blood. This suggests that in mice, LPS could either directly or indirectly induce neutrophilia. To confirm this, LPS was injected in the air pouches of mice and the number of neutrophils was evaluated in the blood. Injection of LPS in the air pouch led to a 4.3-fold increase in the number of neutrophils circulating in the blood 3 h after injection (Fig. 3). This augmentation was transient, returning to control levels at 6 h postinjection. The LPS-induced neutrophilia was associated with an increase in S100A9 and S100A8/A9 serum levels (Fig. 4). Similarly to the number of circulating neutrophils, this increase was maximal at 3 h postinjection and almost returned to control levels by 6 h postinjection, reaching a value of 292.9 ± 66.0 ng/ml S100A9 and 595.3 ± 172.0 ng/ml S100A8/A9 3 h postinjection. Contrary to S100A9 and S100A8/A9 levels, the concentrations of S100A8 remained stable following injection of LPS in the air pouch (data not shown).

Intravenous injection of S100A8, S100A9, and S100A8/A9 results in neutrophilia in mice

The fact that the levels of S100A9 and S100A8/A9 correlated with the LPS-induced neutrophilia suggested that these proteins could participate in the neutrophilia associated with injection of LPS. Therefore, the anti-S100A8 and anti-S100A9 could inhibit neutrophil migration indirectly, following a reduction of the circulating neutrophil caused by an inhibition of LPS-induced neutrophilia. To test this possibility, increasing doses of S100A8, S100A9, and S100A8/A9 were injected i.v. in mice and the peripheral blood was collected 3 h later. As shown in Fig. 5, i.v. injection of S100A8, S100A9, and S100A8/A9 caused an increase in the number of circulating neutrophils. The number of neutrophils after injection reached 6.5, 2.7, and 7.4 × 10⁶ cells/ml in S100A8, S100A9, and S100A8/A9 injected mice, respectively, compared with < 1.5 × 10⁶ cells/ml for the control animals. This increase, detected for injected doses ranging from 5 to 500 μg/kg (0.12–12 μg/mice), was significantly different from control (p < 0.05, Dunnett multiple comparison test) and maximum at a dose of 50–250 μg/kg. Although the total number of circulating leukocytes increased slightly in S100 protein-injected mice, this increase was not significantly different from that in PBS-injected mice. Injection of S100A8, S100A9, and S100A8/A9 did not increase the number of circulating eosinophils, monocytes, or lymphocytes (data not shown). Assuming a total blood content of 79 ml/kg (41), these doses corresponded to serum concentrations ranging from ~600 to 3000 ng/ml at the time of injection. These doses are similar to the ones measured following injection of LPS in the air pouch (Fig. 1, B–D).

S100A8, S100A9, and S100A8/A9 induce the release of bone marrow neutrophils

The kinetic study of S100A8 and S100A9 injection over a 24-h period (Fig. 6, A and B) showed that they induced neutrophilia over a period of 3 to 6 h postinjection. At 3 h, the number of neutrophils was 2.8 ± 0.5 × 10⁶ cells/ml in S100A8-injected mice and 3.5 ± 0.7 × 10⁶ in S100A9-injected mice, compared with 1.0 ± 0.2 × 10⁶ cells/ml for the control mice (p < 0.05, Bonferroni test). The increase in circulating neutrophils returned to the control levels by 12 h postinjection. The increase in the number of neutrophils in the blood induced by S100A8 and S100A9 closely
correlated with a decrease in those of the bone marrow (Fig. 6, C and D). Approximately 22–27% of the bone marrow cells in noninjected mice were segmented and nonsegmented neutrophils. This percentage did not vary significantly in PBS-injected mice. In contrast, the proportion of neutrophils decreased by 50% in bone marrow cells 3 and 6 h postinjection of S100A8 or S100A9 (p < 0.01 and p < 0.05, respectively). This strongly suggests that S100A8 and S100A9 induce the release of neutrophils from the bone marrow to the blood.

Anti-S100A8 and anti-S100A9 inhibit the neutrophilia induced by injection of LPS in the air pouch

To evaluate the role played by S100A8 and S100A9 in LPS-induced neutrophilia, mice were injected i.p. with purified rabbit IgG against S100A8 and S100A9. LPS was then injected in the air pouches and the number of circulating neutrophils was measured 3 h later. As shown in Fig. 7, injection of anti-S100A9 led to an almost complete inhibition of the neutrophilia associated with the local injection of LPS (p < 0.05 Bonferroni test). This inhibition was not increased when anti-S100A8 and anti-S100A9 were injected together. Although the anti-S100A8 also diminished the neutrophilia associated with LPS injection, this inhibition was not significant. As expected, the anti-S100A8, anti-S100A9, and the control IgG had no effect on the number of circulating neutrophils in PBS-injected mice.

Discussion

The presence of S100A8, S100A9, and S100A8/A9 has been associated with bacterial infections in humans and mice (12, 15, 37). However, it is not clear whether this was a consequence of inflammation, or if it contributed actively to the development of the innate immune response. In this study, we demonstrate that S100A8, S100A9, and S100A8/A9 are released in the air pouch exudates and serum during an inflammatory reaction induced by LPS. Their presence in the exudates preceded the migration of neutrophil to the air pouch, while S100A9 and S100A8/A9 presence in serum correlated with LPS-induced neutrophilia. S100A8, S100A9, and S100A8/A9 induced the release of neutrophils from the bone marrow to the blood when injected i.v. and neutrophil accumulation when injected in the air pouch (C. Ryckman and P. A. Tessier, unpublished observations). Finally, passive immunization with purified IgG against S100A8 and S100A9 resulted in the inhibition of neutrophilia and neutrophil migration to the air pouch.

The kinetic of neutrophil accumulation to the air pouch offers a way of deciphering the role of S100A8 and S100A9 in the sequential steps of the mechanism of neutrophil migration from the bone marrow to the inflammatory site. Preliminary results using intravital microscopy demonstrated that neutrophil emigration from the blood vessel to the air pouch tissue begins within the first hour following injection of LPS and that neutrophils do not reach the air pouch lumen before 2 h postinjection. Consequently, exudate neutrophils at 3 h postinjection emigrated from the blood at the most 1 h postinjection. As shown in Fig. 3, the number of circulating neutrophils is not increased at 1 h postinjection. This indicates that exudate neutrophils at 3 h postinjection originate mostly from the preinjection peripheral blood pool of neutrophils. Therefore, blocking molecules at the 3 h time point provide indications about the role of the blocked molecule in neutrophil migration from the blood to the inflammatory site. In contrast, by 6 h postinjection,
neutrophils had enough time to be released from the bone marrow storage pool, circulate in the peripheral blood, and emigrate to the exudates. As a consequence, inhibition by blocking Abs at 6 h postinjection can be due to the inhibition of neutrophil release from the bone marrow or neutrophil migration to the inflammatory site. These two possibilities can be further resolved by analyzing the effect of the blocking Abs on the numbers of circulating blood neutrophils at 3 h postinjection of LPS.

By analyzing the effect of the blocking Abs at the two time points, we conclude that S100A8 and S100A9 play a role at the levels of both neutrophil migration to the air pouch and neutrophil release from the bone marrow, respectively. Proof of this comes from the fact that anti-S100A8 inhibited neutrophil migration to the air pouch at 3 h postinjection of LPS (Fig. 2A), but failed to significantly reduce neutrophil release from the bone marrow (Fig. 7). In addition, by 3 h postinjection, $2.1 \times 10^6$ neutrophils had migrated to the air pouch, which is less than the $\sim 3 \times 10^6$ neutrophils circulating in the blood of a resting mouse. This indicates that by 3 h postinjection, the pouch neutrophils originated from the circulating, but not the bone marrow storage pool of neutrophils. Because no increase in peripheral blood neutrophils was detected in LPS-injected mice before 3 h postinjection, this suggests that the anti-S100A8 IgG directly inhibited neutrophil migration to the air pouch. Therefore, the role of S100A8 would be to assist in neutrophil migration to the inflammatory site. Support for this hypothesis comes from the fact that murine S100A8 was found to be chemotactic for neutrophils (19), and to activate Mac-1 (42), an integrin important in neutrophil transendothelial migration (43–46).

By contrast, the role of S100A9 appeared to be primarily on assisting neutrophil release from bone marrow. For instance, the anti-S100A9 had no significant effect on neutrophil migration at

FIGURE 4. Presence of S100A9 and S100A8/A9 in the serum of mice injected with LPS. One milliliter of LPS (1 ng/ml) was injected into the air pouch raised on CD-1 mice and the serum was collected at various times. A, S100A9 and (B) S100A8/A9 were quantified by ELISA. Data represent the mean ± SEM of at least five mice for each group.

FIGURE 5. Accumulation of neutrophils in blood after i.v. injection of S100A8, S100A9, and S100A8/A9. CD-1 mice were injected or not i.v. in the tail vein with increasing concentrations of (A) S100A8, (B) S100A9, and (C) S100A8/A9 (0.05–500 μg/kg) or PBS (control). After 3 h, neutrophils in blood were counted following Wright-Giemsa staining. Data represent the mean ± SEM of at least seven mice for each group.
3 h postinjection of LPS (Fig. 2A), suggesting that S100A9 is not essential to neutrophil migration in the tissue. However, several facts point toward a role for S100A9 in neutrophil release from bone marrow. For example, S100A9 was found at high concentrations in the serum of mice injected with LPS (Fig. 4) and, when injected i.v., stimulated the release of neutrophils from the bone marrow to the blood (Fig. 6). Moreover, the anti-S100A9 IgGs inhibited the neutrophilia associated with the local injection of LPS (Fig. 7). Therefore, it is likely that one of the functions of S100A9 is to induce the release of neutrophils from the bone marrow.

The role of S100A8/A9 is more difficult to evaluate. At 6 h postinjection of LPS, only the combination of anti-S100A8 and anti-S100A9 inhibited neutrophil migration to the air pouch (Fig. 2B). This probably reflects an effect of the Abs on both neutrophil release from the bone marrow (S100A9) and on neutrophil migration to the air pouch (S100A8). Alternatively, these Abs could have inhibited the activity of S100A8/A9. Few things are known about the biological activity of S100A8/A9. We recently demonstrated that human S100A8/A9 is chemotactic and stimulates neutrophil adhesion (21). Human S100A9 and S100A8/A9, but not S100A8, also facilitate monocyte transendothelial migration in vitro (22). Therefore, it is possible that S100A8/A9 plays a role in neutrophil migration at 6 h postinjection. Nonetheless, the absence of Abs specifically directed against murine S100A8/A9 makes its function more difficult to assess. Further studies will be necessary to decipher its role in neutrophil migration.

Although the present study shed light on the functions of the S100 proteins during inflammatory reactions, it also raises questions about the source of the S100 proteins released at the inflammatory site. There were approximately four times less S100A8/A9 and 60 times less S100A8 in the pouch exudates in response to LPS injection compared with S100A9 (Fig. 1). This result suggests that S100A9 is preferentially secreted at inflammatory sites. Intriguingly, S100A8 and S100A9 were detected in the pouch in relative absence of S100A8/A9. It is possible that S100A8/A9 is more readily eliminated from the exudates following binding to receptors such as the scavenger receptor CD36. Alternatively, factors present in the exudates might stabilize the homodimeric S100 proteins or destabilize the heterodimeric proteins. There was also no correlation between the presence of S100 proteins in the serum and at inflammatory sites (Figs. 1 and 4). In the serum, S100A9 and S100A8/A9 levels were transitory, whereas their presence in the exudates was more sustained. In addition, S100A8 was detected in exudates, but not in serum following injection of LPS. These results suggest that the presence of S100 proteins in serum is not due to leakage from the inflammatory site (air pouch). Interestingly, S100A8 and S100A9 are also found on the endothelium near inflammatory sites.

**FIGURE 6.** Mobilization of neutrophils from the bone marrow to the blood after injection of S100A8 and S100A9. CD1 mice were injected i.v. in the tail vein with (A and C) S100A8, (B and D) S100A9 (250 μg/kg), or PBS (control). At various times, blood (A and B) and bone marrow cells (C and D) were collected and neutrophils were counted following Wright-Giemsa staining. Data represent the mean ± SEM of at least seven mice for each group. *p < 0.05 compared with PBS-injected mice, Dunnett multiple comparison test.

**FIGURE 7.** Effect of anti-S100A8 and anti-S100A9 on LPS-induced neutrophilia. Dorsal air pouches were raised in 10- to 12-wk-old mice. The mice were injected i.p. with 2 mg of purified rabbit IgG against S100A8 and/or S100A9 or nonimmune animals 16 h before the injection of 1 ml of LPS (1 μg/ml) into the air pouch. Neutrophils in blood were counted 3 h later following Wright-Giemsa stainings. Data represent the mean ± SEM of at least five mice for each group. *p < 0.05 compared with control IgG-injected mice, Bonferroni test.
sites (47, 48). Therefore, it is possible that the presence of S100 proteins in serum indicates preferential release by neutrophils of S100A9 and S100A8/A9 during their migration. This observation correlates with the stimulating activity of S100A9 and S100A8/A9 on monocyte transendothelial migration (22), and with the binding of S100A9 and S100A8/A9, but not of S100A8, to glycosaminoglycans on endothelial cells (48). Alternatively, the absence of S100A8 in the serum might be due to the presence of specific proteases not found in the exudates.

Results from this study demonstrate that the presence of S100A8, S100A9, and S100A8/A9 is not merely a consequence of inflammation. These proteins rather play an active role in the generation of the inflammatory reaction. High concentrations of S100A8/A9 in serum occur in pathologies associated with increased numbers of circulating neutrophils or their activity. Elevated levels of S100A8/A9 (>1 μg/ml) are observed in the serum of patients suffering from various infections and inflammatory pathologies such as cystic fibrosis, tuberculosis, and juvenile rheumatoid arthritis (11–13). They are also expressed at very high levels in the synovial fluid and plasma of patients suffering from rheumatoid arthritis and gout (C. Ryckman and P. A. Tessier, unpublished observations). Local secretion of the proteins has also been detected in periodontal infections and in experimental murine abscesses (14, 15). Recent results obtained in our laboratory demonstrate that S100A8 and S100A9 are also important for neutrophil migration in a murine model of acute gouty arthritis (C. Ryckman and P. A. Tessier, unpublished observations). This suggests that they might play a very important role in pathologies involving neutrophil migration. More importantly, their presence in numerous inflammatory reactions suggests that they could represent a new class of early cytokines involved in innate immune responses.

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