Galectin-3 Facilitates Neutrophil Recruitment as an Innate Immune Response to a Parasitic Protozoa Cutaneous Infection

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When infection occurs, neutrophils rapidly migrate to the affected site. Although the neutrophils neutralize microorganisms, they can also cause tissue damage or render invasion pathways to pathogens. Thus, the migration could be either beneficial or unfavorable in the initial control of infection. Studies on neutrophil recruitment revealed its complexity, especially in terms of the regulation of its initiation. Galectin-3 is a member of the galectin family that has an affinity for β-galactoside containing glycoconjugates. In this study, we investigated the role of galectin-3 in neutrophil migration and the biological significance of the rapid migration of neutrophils in an experimental parasitic cutaneous infection with Leishmania major. When the substrain of L. major, LV39, was infected, lack of galectin-3 impaired neutrophil recruitment in the footpads and the draining lymph nodes 1 d following infection. Reduced number of recruited neutrophils correlated with local high parasite burdens. In contrast, neutrophil migration, induced by the other L. major substrain, Friedlin, was unaffected, and the initial parasite burden remained similar in galectin-3 null mice as compared with wild-type mice. Infection with L. major LV39 but not Friedlin induced higher levels of extracellular release of galectin-3. Further, galectin-3 alone was able to initiate neutrophil migration even though galectin-3 is not a chemoattractant for neutrophils. Thus, our data suggest that once extracellularly released, galectin-3 can act as a damage-associated molecular pattern to facilitate early neutrophil migration, which is beneficial in the initial control of the Leishmania infection. The Journal of Immunology, 2013, 190: 000–000.

The most effective defenses against the invasion of disease-causing microorganisms are the ones that can reduce the initial number of the microorganisms right at the point of entry. Upon the recognition of host damages introduced by a pathogen, neutrophils are recruited to the affected site within a few hours (1, 2). It is generally considered that neutrophils are the most effective leukocytes for the neutralization of microorganisms because of their multiple layers of microbicidal factors, high ability to engulf microorganisms, in addition to the fact that massive numbers can migrate in a short period of time to the affected site (3, 4). Even after their death, neutrophils continue to kill microbes through neutrophil extracellular traps (5–7). Dying neutrophils release their DNA into the extracellular space, and it becomes a scaffold of both granule microbicidal contents and microbes, leading to the elimination of the latter, including protozoa parasites, such as Leishmania (6, 7). Simultaneously, however, neutrophil-induced tissue breakdown can be detrimental to the host (8, 9). Thus, the innate defense system requires a spatiotemporal regulation of early neutrophil recruitment (10). In the classical model, neutrophils mainly use two types of membrane-anchored adhesion molecules, selectins and β2 integrins, for their migration (10–17). The immune response is equipped with additional systems that do not use these classical adhesion molecules to optimize migration in the lungs, kidney, liver, cornea, and peritoneum (10, 14–16, 18–29). However, the molecules involved in this additional migration system remain to be fully explored (10, 14–16, 18–29).

Galectin-3 belongs to the galectin family, which is characterized by conserved peptide sequence elements in the carbohydrate recognition domain responsible for glycan binding. Galectin-3’s carbohydrate recognition domain is connected to a N-terminal nonlectin domain, which is involved in the oligomerization of galectin-3 upon binding to glycan ligands on cell surfaces. Galectin-3 is relatively abundant in the tissues that face the external environment, such as mucosal membranes and dermal tissues including footpad tissues (P. Bhaumik and S. Sato, unpublished observations) (30, 31). Alveolar or inflammatory activated macrophages also express high levels of galectin-3, whereas murine neutrophils and quiescent lymphocytes contain very little, if any, galectin-3 (32–35). Our previous reports suggest that galectin-3 is involved in neutrophil migration in alveolar spaces infected with Streptococcus pneumoniae despite the fact that galectin-3 is not a chemoattractant for neutrophils (32, 34). However, it remains elusive whether galectin-3–modified innate responses, especially the rapid neutrophil migration, are a unique phenomenon of the lungs infected with this pathogen or rather play critical roles in other types of infection at the different tissues (36).

Experimental cutaneous leishmaniasis with the protozoan parasite Leishmania major in C57BL/6 mice exhibits strong early...
local dermal inflammation and represents a resourceful model to understand the innate immune response toward infections. Upon dermal infection in C57BL/6, in which L. major infection remains mainly at the site of infection (37), the initial innate immune response triggers massive yet transient neutrophil migration that contributes to the pathogenesis of leishmaniasis. Recently, the immunological significance of this neutrophil recruitment has become controversial (38–45). Some reports suggest the capacity of neutrophils to render the early establishment of L. major infection (41, 46). In those studies, neutrophils were acutely depleted before the infection with one substrain of L. major, Friedlin. The acute depletion may induce unanticipated effects in the innate immunity because neutrophils constitute >50% of circulating leukocytes. Further, it has been recently noticed that there are some differences in the impact of different substrains of L. major on the pathogenesis of leishmaniasis (47). Thus, the biological significance of neutrophils in leishmaniasis deserves further studies that do not depend on the massive depletion of circulating neutrophils. In our study, we investigated whether lack of galectin-3 in C57BL/6 mice has an impact on the innate immune response against two substrains of L. major with an emphasis on early neutrophil migration. In addition, the contribution of locally recruited neutrophils in the initial parasite clearance at the affected sites was also studied. The results presented in this report show that lack of galectin-3 led to reduction in early neutrophil migration (12–24 h postinfection) to L. major LV39–infected sites. Due to the temporal reduction in the number of recruited neutrophils on the first day of infection, significantly higher parasite loads were observed. In contrast to LV39, the infection with the other substrain, Friedlin, induced neutrophil migration to the infection site regardless of the presence or absence of galectin-3, and the initial parasite burden was well controlled. Together, these data suggest a critical role for neutrophils as well as a function for galectin-3 in the migration of neutrophils as part of the host defense system against L. major in this experimental model.

Materials and Methods

Mice
Galectin-3 null (G3KO) mice were obtained from Core F of the National Institute of General Medical Sciences–supported Consortium of Functional Glycomics. Wild-type (WT) C57BL/6 and G3KO mice, C57BL/6 background were reproduced and maintained in our conventional animal facility. All studies were performed using adult, sex-matched mice 8–12 wk old. An approximate number of five to eight mice were used per group of study. Animal breeding and experiments were conducted according to the Canadian Council on Animal Care Guidelines, as administered by the Laval University Animal Care Committee.

Parasites and cells
L. major substrains LV39 and Friedlin V1 promastigotes were maintained in modified Schneider’s Drosophila medium (SDM)-79 (supplemented with 20% heat-inactivated FBS, 5 µg/ml hemin, and penicillin-streptomycin-glutamine) or medium 199 (supplemented with 10% heat-inactivated FBS, 5 µg/ml hemin, and penicillin-streptomycin-glutamine), respectively, as previously described (48). For the preparation of amastigotes, stationary-phase Leishmania promastigotes were injected into the footpads of BALB/c mice, and 6 wk after the challenge, mice were sacrificed, and footpads were collected to recover amastigotes. The amastigotes were gently washed several times and then cultured at 24°C to obtain freshly transformed promastigotes. These transformed promastigotes were cultured for one to two passages, and then the stationary-phase promastigotes were used for infection for optimal virulence. For macrophages, bone marrow cells were collected by flushing the bone marrow cavities of murine femurs and tibias with PBS. Leukocytes were separated using a Ficoll-Paque (Amersham Biosciences) gradient and then washed and cultured in 10% FBS-supplemented DMEM with 750 U/ml GM-CSF (PeproTech). After 24 h incubation, nonadherent cells were removed and cultured in appropriate wells for a week in 10% FBS-supplemented DMEM and GM-CSF to further differentiation. Neutrophils were isolated from bone marrow cells by a Histopaque (Sigma-Aldrich) density gradient centrifugation. Neutrophils were then washed and suspended in 10% FBS-supplemented M199 medium.

Galectin-3
Recombinant galectin-3 was purified as previously published (32, 49). Briefly, galectin-3 was purified by using an affinity chromatography with lactose-agarose (Sigma-Aldrich). Bound galectin-3 was released by lactose followed by repurification through a Hiprep desalting PD10 column to remove lactose. The eluate containing galectin-3 was passed through an ActiClean Etox (Sterogen) column to ensure that the endotoxin level is <1 pg/µg protein.

Leukocyte recruitment to air pouches
Air pouches were raised on the dorsal surface of mice (WT and G3KO), as previously described (50–52). Briefly, an air pouch was raised on the dorsal site by s.c. injection of 3 ml sterile air on days 0 and 3. On day 7, 2 × 10^6 L. major (LV39 or Friedlin) stationary-phase promastigotes in 1 ml PBS or recombinant galectin-3 (4 nmol/mouse) were injected into the air pouch. One or 6 h after the injection, mice were sacrificed, and the pouch was washed with a total of 3 ml (three times × 1 ml) PBS to recover the exudates, which contained released cytokines and recruited leukocytes. In this model, it has been well established that the peak of leukocyte accumulation in the air pouches is between 4 and 6 h, whereas chemokines and proinflammatory factors are readily detectable at 1 h (50–52). The supernatant of the first 1-ml wash was used for cytokine analysis. Total leukocyte populations in the pouches were evaluated by direct counting on a hemocytometer using acetic-blue staining, and differential counts were estimated by microscope using cytospin preparations stained with a Diff-Quik Stain Set (Dade Behring) as previously described (32, 34).

Footpad infections
Mice were s.c. infected in the left hind footpad with 0.5–2 × 10^6 L. major stationary-phase promastigotes suspended in 50 µl PBS. Footpad swelling was recorded at regular intervals using electronic digital calipers. The lesion size (volumes) was estimated by comparing with the size of the noninfected right hind footpad.

Myeloperoxidase assay
To estimate the level of neutrophil recruitment in tissues, activity of myeloperoxidase (MPO), which is a specific marker of neutrophils, was used as previously described (32, 53). Briefly, infected footpads were collected from mice sacrificed at various time points postinfection and homogenized in 50 mM hexadecyltrimethylammonium bromide buffer (pH 6). The footpad section was first exposed to freeze-thawing and then homogenized, followed by sonication. The extract was then centrifuged to remove the remaining insoluble cell debris. MPO in the supernatant was estimated by a reaction with a phosphate buffer (pH 6) containing o-dianisidine and hydrogen peroxide.

Western blot for protein detection
Infected footpads and lymph nodes were homogenized with 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), and 0.12 M NaCl containing protease inhibitors. Proteins of tissue lysates or exudates were fractionated by SDS-PAGE and transferred to nitrocellulose membrane as previously described (34). Galectin-3 was detected with biotinylated anti–galectin-3 (Mac-2) Ab followed by streptavidin-peroxidase.

Parasite burden
At different time points postinfection, mice were sacrificed, and the infected footpads were surgically removed. Cell suspensions were prepared from tissue homogenates in 20% FBS–SDM-79, and 4-fold serial dilutions were carried out in 96-well cell-culture plates. The highest dilutions at which promastigotes were observed under microscope were recorded after 6 to 7 d of culture at 24°C.

FACS analysis of leukocytes of lymph nodes
Mice infected with 2–5 × 10^6 L. major were sacrificed at different time points postinfection, and the draining popliteal lymph nodes were surgically removed. After a brief digestion with 0.1% w/v Collagenase D solution, cell suspensions were prepared and washed in PBS several times. Cells were then fixed in 3% paraformaldehyde and stained with fluorescent Abs as follows: FITC-labeled rat anti-mouse Ly6G/6C (Gr-1) for neu-
trophils, rat anti-mouse F4/80 Ag for macrophages, PE-labeled anti-mouse NK-1.1 for NK cells, anti-mouse CD11c for dendritic cells (DCs), and anti-mouse CD86 as activation markers of DCs. All Abs were matched with respective isotype controls, and staining was performed after blocking with purified rat anti-mouse CD16/CD32 Ab (Fc blocker). All Abs were purchased from BD Biosciences with the exception of F4/80, which was obtained from Serotec.

**Neutrophil depletion with anti-Ly6G Ab**

Mice were injected i.p. with 0.5 mg/ml anti-Ly6G mAb 1A8 and control rat IgG2a (isotype-matched) Ab (BioXCell) in 200 μl PBS. One and a half days after the injection when ~75–90% of neutrophils were depleted (54), mice were infected with a s.c. injection of *L. major* in the footpad as above. Footpad parasite burdens 24 h after the infection were estimated as explained above.

**Macrophage phagocytosis assay**

Macrophages in eight-well LabTek chamber slides (1 × 10^5 cells/well) were coincubated with serum-opsonized (CS-deficient serum from Sigma-Aldrich) *Leishmania* promastigotes (1:5). In some cases, cells were also pretreated with rIFN-γ for stimulation. After incubation for 2 h, the supernatants were removed, cells were carefully washed with medium, and the chamber slides stained with Diff-Quik (Dade Behring). The number of macrophages with phagocytosed parasites was detected by direct counting under microscope. The phagocytosis index was estimated as: (number of macrophages with internalized parasites/total number of macrophages counted) × 100.

**Neutrophil killing assay and phagocytosis**

Neutrophils were coincubated (1:5) for different times with *Leishmania* (serum-opsonized and nonopsonized stationary-phase promastigotes) at 37°C. At the end of coincubation, the neutrophil–*Leishmania* suspensions were directly transferred to serial dilution plates containing 10% FBS-supplemented DMEM. The highest dilutions at which promastigotes were observed under microscope were recorded after 2 to 3 d of culture at 24°C. For phagocytosis behavior analysis, neutrophils from WT and G3KO mice were coincubated with *Leishmania* at 37°C under 5% CO_2_ in a Lab-Tek II chambered coverslip on the microscope stage, and differential interference contrast images were captured every 1 min for 2 h through a HCX PL APO 40× objective (numerical aperture 1.25) by using Halogen lamp as a light source with a Quorum WaveFX spinning Disc Confocal System (Quorum Technologies).

**Cytokine assay**

Draining popliteal lymph nodes of infected mice (*n* = 5) were removed 1 d following infection. Cell suspensions were prepared after thorough washing with 10% FBS-RPMI 1640. Cells (10^6 cell/well) were then incubated in 96-well plates (triplicates for each preparation) in the presence or absence of freshly prepared 50 μg/ml soluble *Leishmania* Ag (SLA). SLA was obtained by centrifuging the parasite suspension, which was prepared by repeated freeze-thawing followed by sonication (55). Cell-free supernatants were collected after 72 h. Cytokine analysis of the supernatant was done using standard sandwich ELISA in duplicate for each sample. IL-12, IFN-γ, IL-10, IL-4, IL-6, IL-17, TNF-α, TGF-β, MIP-2, and keratinocyte chemoattractant (KC) ELISAs were performed using standard kits from BD Biosciences and R&D Systems, following manufacturing company–specified protocols.

**Statistical analysis**

Statistical analyses were performed by Student *t* test (GraphPad Prism software version 4; GraphPad). The *p* values <0.05 were deemed statistically significant.

**Results**

**Reduced neutrophil recruitment in the air pouch of G3KO mice injected with *L. major* substrain LV39**

Because cutaneous parasitic protozoa skin infection induces acute neutrophil recruitment (39–45), we first investigated whether lack of galectin-3 had any impact on the early neutrophil recruitment triggered by *L. major*, using the skin air-pouch model system. It has been previously demonstrated that introduction of *L. major* in the air pouch leads to a rapid and transient accumulation of neutrophils within the pouch with a peak at 6 h after inoculation (52). Thus, this air-pouch model appears to simulate a physiologically comparable environment with respect to an acute stage of cutaneous infection with the added advantage of allowing retrieval of the cells recruited to the infection site. When *L. major* LV39 parasites were injected to the air pouches of WT and G3KO mice, the total number of leukocytes retrieved 6 h after inoculation from G3KO mice was 6-fold lower than that from WT (Fig. 1A). A differential count of the recruited leukocytes in WT mice showed an influx of a mixed population of cells in response to *L. major* with the majority (73.7%) being neutrophils, consistent with a

**FIGURE 1.** Reduction of neutrophil recruitment in the air pouches of G3KO mice injected with *L. major* LV39. (A and B) Recruitment of leukocytes in the air pouch of WT and G3KO mice (*n* = 5) 6 h after inoculation of 2 × 10^6 stationary phase of *L. major* LV39 parasites or LPS (1 μg/ml) (shaded bars). Total cell numbers retrieved from the air-pouch washes of WT were counted. Further, cytospin slides were prepared with the total leukocytes and stained with Diff-Quik (Dade Behring) to obtain a differential count. (C) CXC chemokine secretion in air pouches inoculated with *L. major* LV39. Data shown represent the means ± SD (*n* = 5). The results shown are represented as the mean ± SD (*n* = 5). Similar results were obtained in two independent experiments. The representative results from two different experiments are shown. *p* values were created by comparison with WT. *p* <0.05, **p* <0.01, ***p* <0.001. KC, Keratinocyte chemoattractant.
previous report (Fig. 1B) (52). In contrast, very few neutrophils were recruited into the air pouches of G3KO mice (Fig. 1B). The number of other leukocyte populations, including eosinophils, mononuclear cells (monocytes and lymphocytes), and macrophages, remained low but comparable or not statistically different between WT and G3KO mice, suggesting that lack of galectin-3 significantly compromises the initial neutrophil migration in response to L. major LV39. As shown in Fig. 1C, lack of galectin-3 rather increased the L. major–initiated early CXC chemokine synthesis for neutrophils (MIP-2 and KC), suggesting that this reduced neutrophil migration was not attributable to a low initial chemokine response (32). The levels of proinflammatory cytokines TNF-α and IL-6 in the air-pouch exudates were similar between G3KO and WT mice (data not shown). In contrast, when LPS was similarly injected in the air pouch, no significant reduction of neutrophil recruitment in G3KO mice was observed (Fig. 1A, inset), indicating that there was not any significant defect in LPS-induced neutrophil recruitment in G3KO mice. Thus, there is a defect in neutrophil recruitment in G3KO mice, and this defect is dependent on stimuli.

Impaired neutrophil recruitment in the footpads and draining lymph nodes of G3KO mice infected with L. major

Having detected a considerable impairment of neutrophil recruitment in parasite-injected air pouches of G3KO mice, we next used the experimental cutaneous infection model of C57BL/6 mice with L. major. Parasites were s.c. injected in the hind footpads of mice, and neutrophil migration to the footpads was estimated by the level of a neutrophil-specific marker, MPO. Consistent with previous reports (39, 40, 56), neutrophil recruitment to the infected footpad of WT mice became detectable within 12 h postinfection and appeared to be transient. At 6 h postinfection with L. major LV39, the MPO levels in the tissue homogenates remained under the detection limits. A significant reduction in the number of neutrophils in G3KO mice was observed at both 12 and 24 h postinfection compared with the WT mice (Fig. 2A). In contrast, the number of migrated neutrophils in the footpads of G3KO mice was similar to the one in WT mice at 48 h postinfection (data not shown), suggesting that the impairment in neutrophil recruitment was a transient event. Recent works suggest that dermally recruited neutrophils rapidly migrate to the draining lymph nodes (57, 58). Thus, to determine whether the migration of neutrophils to the site of infection correlated with the presence of neutrophils in the draining lymph nodes, a flow cytometry analysis of leukocytes in draining (popliteal) lymph nodes was also performed. As shown in Fig. 2B, similar to local infection sites, a significant reduction in the number of neutrophils (Gr-1+) in the lymph nodes of G3KO mice compared with WT was observed at 12 and 24 h postinfection. The numbers of macrophages (F4/80+) in the lymph nodes remained similar between the WT and G3KO populations (data not shown). The numbers of CD11c+ DCs corresponded to 0.5–1.5% of the leukocyte population and were similar in both types of mice, whereas the number of CD11c+ CD86+ activated DCs was 2-fold higher in WT as compared with G3KO mice (Fig. 2C). A previous report suggests that lack of galectin-3 in DCs does not affect their differentiation and maturation process (59). In contrast, inflammatory activated neutrophils are known to mature and activate DCs through direct interaction (60). Thus, reduction in the number of CD11c+CD86+ cells in draining lymph nodes could be partly explained by the reduction in recruitment of neutrophils in G3KO mice. The number of NK cells, which are a potential early source of IFN-γ-producing cells, was not significantly different in G3KO and WT mice (data not shown). The leukocyte population analysis of lymph nodes was also consistent with the defects observed in both the air pouches and the footpads, showing a transient deficiency of early neutrophil recruitment in G3KO mice following infection with L. major LV39.

Cytokine analysis in G3KO mice

In addition to early neutrophil recruitment, the initial cytokine milieu in response to L. major infection could also have some impact on the differential responses toward Leishmania infection between WT and G3KO mice. Thus, the cytokine response (IL-12, IFN-γ, IL-4, IL-10, TGF-β, IL-6, IL-17, and TNF-α) in the draining lymph nodes of early infection was next studied. In both mouse strains, the TNF-α, IL-4, and IL-6 levels remained below the detection limits in draining lymph node cell culture supernatants (data not shown). As shown in Fig. 3, the production of IFN-γ, which could promote the leishmanicidal activity of local macrophages, did not show statistically significant difference between the two mouse strains. The levels of IL-10, IL-12, and TGF-β released by cells in draining lymph nodes were not significantly different between the two mouse strains regardless of the presence or absence of SLA. IL-17 production in the presence of SLA was higher in G3KO mice than in WT, which is possibly attributable to the reciprocal response to the reduced recruitment of neutrophils (60–62). Together, despite of the significant reduction in local neutrophil migration at the parasite-infected sites in G3KO mice, these data suggest that lack of galectin-3 did not induce any other significant defect in immediate cytokine responses.

Higher parasite load in G3KO mice

To estimate the impact of the reduced early recruitment of neutrophils on the residual parasite burden in G3KO mice during the first 24 h following infection with L. major LV39, we determined the numbers of live parasites in the footpads of both mouse strains.
As early as 6 h postinfection, a 4-fold increase in the number of parasites in the infected footpads of G3KO mice as compared with WT mice was detected (Fig. 4A). A significant increase in the parasitic burdens of G3KO mice was also observed 24 h postinfection, suggesting a decreased parasite killing at the beginning of infection in these mice.

We also verified whether lack of galectin-3 had any impact on the leishmanicidal activity of neutrophils and macrophages, which are the major cells encountered by the parasites at the onset of infection. Macrophages were infected with opsonized L. major LV39 for 2 h in the absence or presence of IFN-γ. Both types of mice exhibited a similar ability to phagocytose parasites, regardless of the presence or absence of IFN-γ (Fig. 4B and data not shown, respectively). Although a report suggests a defect in phagocytosis of zymosan by G3KO mice macrophages (63), our data suggest that there was no significant defect in the ability of macrophages from G3KO mice to phagocytose Leishmania compared with ones from WT mice. Next we determined whether the neutrophils of G3KO mice had different capacities for parasite killing (Fig. 4C). Neutrophils were coincubated with either nonopsonized or opsonized L. major LV39 parasites at 37˚C for 3 h. The leishmanicidal activity of neutrophils was determined by a serial dilution assay to estimate the presence of remaining live parasites. In the case of nonopsonized parasites, neutrophils killed ~80% of Leishmania within 3 h. Highly efficient microbicidal activity by WT and G3KO neutrophils was observed when opsonized parasites were used, suggesting no major defect in parasite killing by neutrophils from either mouse type. Motility and phagocytosis were also monitored using live-cell time-lapsed imaging (data not shown). The analyses also suggest that neutrophils from both strains are equally mobile and actively phagocytose parasites after a transient quiescent period of ~15 min after the introduction of parasites to neutrophils. Given the similar killing capacity of the phagocytoses of both mouse strains, these data suggest that the increased parasite burdens in G3KO mice in the initial phase of infection were due to the transient reduction in neutrophil recruitment at the early acute phase of infection with L. major LV39.

To verify whether the reduction in the number of neutrophils at the affected site indeed contributes to the increase of local parasitic load, the number of circulating neutrophils was artificially reduced, using an anti-neutrophil Ab treatment (54), thereby creating a physiological disorder similar to what is seen in G3KO mice. An i.p. injection of an Ly6G-specific mAb (1A8) showed a 60–80% reduction in circulating neutrophils with the peak depletion occurring at 1.5 d following injection (data not shown) as previously reported (54). At this point, mice were infected s.c. in the footpads with L. major LV39 and 24 h postinfection, and the numbers of parasites in footpads were determined. As shown in Fig. 4D, between 4- and 16-fold more parasites were detected in footpads of 1A8-treated mice as compared with untreated or control mice injected with isotype-matched Ab. This observation, together with the increased parasite burden in G3KO mice in which the local neutrophil recruitment was impaired (Figs. 1A, 1B, 2A, 2B, 4A), suggests the importance of early neutrophil recruitment for the regulation of the initial burden of L. major substrate LV39 in C57BL/6 mice.

### Different patterns of footpad infection progression in WT and G3KO mice

Having shown transient reduction in neutrophil recruitment and increased parasite load in infected sites of G3KO mice, we further determined whether lack of galectin-3 would have an effect on the pathogenesis of infection with L. major LV39. Mice were injected s.c. with two different doses of L. major LV39, and the development of the cutaneous disease was estimated by measuring lesion sizes. As shown in Fig. 5, a significant difference in lesions was observed between G3KO and WT mice as early as 2 wk postinfection, with G3KO mice developing larger aggravated lesions.
compared with WT. Lesion sizes reached a maximum ~3 to 4 wk postinfection and resolved within the following weeks, as is typically seen in C57BL/6 mice. The pattern of disease development remained similar between the high-dose (Fig. 5A) and low-dose (Fig. 5B) infections; however, the lesions were greater in size for the higher dose. The rapidity with which G3KO mice healed their infection appeared greater than that of WT mice despite the initial aggravated infection in G3KO mice.

Correlation between early neutrophil migration and progression of cutaneous leishmaniasis

To investigate whether the different cell recruitment pattern in WT and G3KO mice, in response to *L. major*, remains similar irrespective of the strain of parasite used, another strain of *L. major*, Friedlin, was used for stimulation of neutrophil migration into the air pouches of mice. Interestingly, the total number of leukocytes (data not shown) as well as that of neutrophils (Fig. 6A) recruited in both mouse strains in response to Friedlin showed no significant difference. Moreover, no detectable difference in the number of Gr-1+ cells present in the draining lymph nodes isolated from mice infected with the Friedlin strain was observed at either 12 or 24 h following infection (data not shown), suggesting that transient defect of neutrophil migration in G3KO mice is specific to *L. major* LV39 but not Friedlin. Furthermore, G3KO mice infected with Friedlin carried equal parasite numbers in the footpads with respect to WT mice (Fig. 6B). Thus, lack of difference in parasite burdens between G3KO and WT mice correlated with similar level of neutrophil migration to the Friedlin-infected site of G3KO and WT mice. Together, the results indicate that reduction in the initial local parasite burden highly depends on the swift local migration of neutrophils, which neutralize *L. major*. The *L. major* Friedlin-induced footpad inflammation pattern in G3KO mice was slightly milder but not worse like *L. major* LV39 infection than WT (Fig. 6C).

Extracellular release of cytosolic galectin-3 into air pouches injected with *L. major*

We next addressed whether the difference in extracellular galectin-3 release triggered by parasite infection in dermal tissues was one of the parameters that contribute to galectin-3–facilitated neutrophil migration. Because dermal cells, such as fibroblasts, tissue macrophages, and adipocytes contain high levels of galectin-3 (35,
Keywords: Neutrophils, Leishmania major, Galectin-3, Migration, Inflammation

Exacerbation of leishmaniasis in G3KO mice. Change in footpad lesion sizes of WT and G3KO mice following infection with 2×10⁶ (A) and 5×10⁵ (B) L. major LV39 parasites. Lesion size index is given by the difference in the size of infected and uninfected footpad lesions. Data shown are represented as the means ± SD (n = 7). The p values were created by comparison with WT mice. Similar results were obtained in two independent experiments. *p < 0.05, **p < 0.01.

PARASITIC burdens of the footpads of mice infected with L. major can induce neutrophil recruitment at the site. which can differ from selectins and integrins, to facilitate migration. As presented in Fig. 7C, together these data suggest that once released extracellularly upon the local infection with L. major, galectin-3 can induce neutrophil recruitment at the site.

Discussion
Precise perception of microorganism invasion by the innate defense system is required to be closely linked to the initiation of neutrophil migration as part of the inflammatory response, because rapid local clearance of microorganisms by neutrophils often reduces the risk of systemic infection. Recent advances have revealed the unexpected complexity of neutrophil migration regulation, which had previously been considered as one of the most well-established paradigms of leukocyte extravasation. These studies confirm the necessity of critical regulation for the initiation and resolution of the migration. For example, in the last few years, reports on neutrophil migration underscored the roles of sentinel cells, which could be classified in at least three groups, blood patrolling nonclassical monocytes that crawl along the luminal side of endothelium, classical tissue macrophages, and stromal cells. In addition, depending on the affected tissue sites or the nature of the insult, neutrophils use different sets of molecules, which can differ from selectins and integrins, to facilitate migration from circulation to the affected sites. However, nonclassical molecules that can facilitate the migration have not been fully explored, and VAP-1 and galectin-3 are some of the few listed as candidates.

Our previous reports and other studies raise the possibility that galectin-3 facilitates neutrophil migration into alveolar spaces infected with S. pneumoniae and into the chronically inflamed peritoneal cavity. Interestingly, this function of galectin-3 is limited and dependent on the nature of the insult. Indeed, in contrast to pneumococcal infection, lack of galectin-3 did not induce any defect in neutrophil migration into alveoli in the case of Escherichia coli infection. In the current study, our data showed that lack of galectin-3 significantly reduced the acute phase of neutrophil migration in a dermal infection model with a protozoa parasite, L. major strain LV39. This defect in the migration in G3KO mice was microorganism-specific, because the recruitment of neutrophils remains unaltered when the dermal inflammation was induced by the other strain of L. major, Friedlin, or by LPS. Because the intensity of neutrophil migration induced by Friedlin in G3KO mice was similar with WT mice, our data suggest that spontaneous apoptosis in migrated neutrophils is not altered significantly by the lack of galectin-3. Importantly, the dermal infection with L. major strain LV39, but not Friedlin, induced significantly higher levels of galectin-3 release into the extracellular space. Moreover, galectin-3 itself can induce significant neutrophil migration in vivo. Together, our data suggest that extracellular release of galectin-3 following the local invasion of L. major strain LV39 triggers galectin-3-dependent local neutrophil migration.

After the invasion of pathogenic microorganisms takes place, some of those pathogens destroy local tissues, and damaged cells release cytosolic components into the extracellular space. This insult condition is associated with the initiation of immune response through its ability to sense the presence of pathogenic

FIGURE 5. Exacerbation of leishmaniasis in G3KO mice. Change in footpad lesion sizes of WT and G3KO mice following infection with 2×10⁶ (A) and 5×10⁵ (B) L. major LV39 parasites. Lesion size index is given by the difference in the size of infected and uninfected footpad lesions. Data shown are represented as the means ± SD (n = 7). The p values were created by comparison with WT mice. Similar results were obtained in two independent experiments. *p < 0.05, **p < 0.01.

FIGURE 6. L. major Friedlin infection. (A) Recruitment of neutrophils in the air pouch of WT and G3KO mice 6 h after inoculation of 2×10⁶ stationary phase of L. major Friedlin. Data shown are represented as the means ± SD (n = 6). Similar results were obtained in two independent experiments. (B) Parasitic burdens of the footpads of mice infected with L. major Friedlin for 24 h. Data shown are represented as the means ± SD (n = 7). (C) Change in footpad lesion sizes of WT and G3KO mice following infection with 2×10⁶ L. major Friedlin. Lesion size index is given by the difference in the size of infected and uninfected footpad lesions. Data shown are represented as the means ± SD (n = 7). The p values were created by comparison with WT mice. *p < 0.05, **p < 0.01.
FIGURE 7. Extracellular release of galectin-3 into the air pouches infected with *L. major*. (A) *L. major* Friedlin or LV39 (2×10⁶ parasites) were injected to the air pouches, and 3 h after the infection, the exudates were collected. Samples were subjected to 15% SDS-PAGE and transferred onto nitrocellulose filters. Galectin-3 on the filters was detected by biotinylated anti–galectin-3 Ab (Mac-2) followed by streptavidin-peroxidase. The data were quantified by densitometry and are represented as the means ± SD (n = 4). p values were created by comparison with WT mice or Friedlin-infected mice. Similar results were obtained in two independent experiments. (B and C). Induction of neutrophil recruitment by galectin-3. Galectin-3 was injected to the air pouches, and the exudates were collected 1, 3, 6, 12, and 24 h after the injection. (B) shows the recruitment of neutrophils in the air pouch 1 h after the injection. (C) shows the kinetics of the recruitment of neutrophils. The data shown are represented as the means ± SD (n = 6). The p values were created by comparison with PBS injection. Similar results were obtained in three independent experiments. *p < 0.05, **p < 0.01.

conditions (76). Based on this model, the definition of damage-associated molecular patterns (DAMPs) has been introduced for those released intracellular components that can activate innate immune response and modulate the initiation of adaptive immunity (73–75, 77). Galectin-3 is synthesized as a cytosolic protein and stored in the cytosol, segregated from its glycan ligands, which exist in the extracellular space (31, 33, 78–82). If galectin-3 is indeed a DAMP, immediate discharge of galectin-3 postinfection would be expected. However, very few, if any, in vivo data put forward to indicate such extracellular discharge of cytosolic galectin-3 in the early stage of infections, because there are very few in vivo systems to distinguish the molecules present in the extracellular space from the one in the cytosol. Although immune responses significantly vary depending on the type of infection, inflammation, and affected tissues (36), we used the bronchoalveolar lavage fluid in the lungs and the exudates in the air pouches to detect the acute discharge of cytosolic galectin-3 into the extracellular space. Through these approaches, we demonstrated that during the initial phase of infections, some pathogen (*S. pneumoniae* in the lungs and *L. major* LV39 in dermal tissue) induce the translocation of cytosolic galectin-3 into the extracellular space (32, 34). In these infection settings, lack of galectin-3 severely impairs neutrophil recruitment. Once released, our data now suggest that galectin-3 itself can trigger the neutrophil migration even though galectin-3 is not a chemotactic agent for neutrophils (32). In addition, galectin-3 facilitates the adhesion of neutrophils, interacts with primed neutrophils, and induces both superoxide productions and secretion of CXCL8 (32, 34, 68, 69, 83, 84). Together, it is likely that galectin-3 promotes early neutrophil recruitment as a DAMP once infection or insult triggers the release of intracellular galectin-3 from cells.

The essential role of neutrophils in experimental leishmaniasis has currently attracted much attention because recent reports unexpectedly suggest that neutrophils aggravate leishmaniasis rather than eliminate the parasites in the initial stage (38, 42, 44, 46, 85). Several reports and the detailed analyses, however, indicate that the impact of neutrophils on leishmaniasis may vary depending on the substrains of *L. major*, in addition to the genetic background of mice (44, 47). The studies to understand the possible exacerbating roles of neutrophils in leishmaniasis used one substrain of *L. major* (Friedlin) (41), although it remained unexplored whether such unexpected adverse function of neutrophils occurs with the infection with other substrains of *L. major*. Indeed, in the infection with the other substrain, LV39, in C57BL/6 mice, in which a high level of neutrophil recruitment is only transient and lasts up to 48 h (39, 40), artificial depletion of circulating neutrophils by anti-Gr-1 Ab in mice is associated with higher parasite loads in skin and draining lymph nodes (45, 56). Our data show that a more specific depletion of neutrophils by a neutrophil-specific anti-Ly6G (1A8) Ab also led to a significantly higher *Leishmania* burden at the footpads (Fig. 4D). The Ab-induced removal of the major circulating leukocytes is a relatively harsh approach to study the role of neutrophils because this treatment may alter the local innate immune response against *Leishmania* other than creating lack of neutrophils in those mice. In this study, the usage of G3KO mice represents one of the alternative approaches to address the role of neutrophils because the majority of innate immune responses, including cytokine responses, remain similar with WT mice despite the fact that lack of galectin-3 can induce transient reduction of local neutrophil recruitment in LV39-induced inflammation. The data from G3KO mice support the notion that acutely recruited neutrophils contribute to the reduction of local parasite loads. Indeed, transient reduction in local neutrophil migration in G3KO mice at the first day of infection was sufficient to increase local parasitic loads and to alter the development of leishmaniasis. In contrast, when neutrophil migration is not compromised in G3KO mice infected with other substrain (Friedlin) of *L. major*, the pattern of the parasite loads of the footpads remains unaffected. Thus, the present data indicate that local neutrophil recruitment at the early stage of the infection is critical to control *L. major* infection. Chtanova et al. (57) showed that the persistent formation of a large neutrophil swarm, which is triggered by cooperative action of neutrophils and parasites, leads to the removal of local macrophages, possibly due to the collateral damage induced by inflammatory-activated neutrophils. Tissue macrophages provide a safe haven for *L. major* to proliferate. Thus, in addition to local parasite killing by neutrophils, it is also possible that neutrophil-induced temporal depletion of macrophages proximal to parasites may also be beneficial to reduce the initial parasitic load in draining lymph nodes. Further investigation is necessary to delineate the difference in the impact of *L. major* substrains on neutrophil recruitment, although swift neutrophil mobilization appears to be beneficial for the regulation of initial parasitic load of *L. major* LV39.

Although our data presented suggest the role of galectin-3 as a DAMP, the separate lines of our previous investigations on in vitro interaction between *L. major* and galectin-3 suggest that galectin-3 specifically binds to the *L. major*–specific glycan epitope (Galβ1-3Gal), attached as the side chains of the phosphoglycan repeating units in lipophosphoglycans or proteophosphoglycans (48, 86). The
binding of galectin-3 to the L. major–specific epitope results in the cleavage of galectin-3 (86). Because this (Galβ1-3)₅₉ epitope is not found in host and is unique to L. major but not other species (like L. donovani or L. maxicana), the epitope is proposed as a pathogen-associated molecular pattern (PAMP) (31, 81, 87, 88). Until now, only limited members of host lectins are known as the host recognition molecules for this L. major–specific PAMP. They are galectin-3 and -9 and a sandfly galectin (31, 48, 81, 86–89). A recent report on the detailed structural analysis of L. major phosphoglycan repeat suggests the substrain-specific differences in the frequency and the length of (Galβ1-3)₅₉ (90). The length of this (Galβ1-3) unit differs significantly between LV39 and Friedlin. The majority (78%) of the phosphoglycan repeats of LV39 are modified with more than two (Galβ1-3) residues, and the average length of the side chain is 3.1. In contrast, 59% of the phosphoglycan repeats of Friedlin contain only one (Galβ1-3) residue (the average length is 0.8). Thus, significantly fewer L. major PAMPs are presented on the surface of Friedlin. Our previous biochemical analysis indicates that the affinity of galectin-3 is enhanced in proportion to the number of (Galβ1-3) units (48). Thus, it is possible that the interactions of galectin-3 with L. major also differ among substrains. Indeed, our unpublished in vitro results indicate that at 37˚C, galectin-3 preferentially interacted with L. major LV39 more than Friedlin (G. St-Pierre and S. Sato, unpublished observations). These results raise the possibility that in vivo, galectin-3 more readily recognizes L. major LV39 than Friedlin. Thus, further investigation is necessary to understand the physiological significance of this substrain-specific difference of the L. major PAMP in the context of the affinity for galectin-3. Nevertheless, galectin-3 can exert its functions as a lectin only when released to the extracellular place. Because only LV39 but not Friedlin infection resulted in the increased release of galectin-3 at the initial stage of the Leishmania infection, for the current study, the contribution of the difference in the binding of galectin-3 with the different substrains of the parasites is expected to be minimal. In contrast, if an infection begins inducing local translocation of galectin-3, galectin-3 could act as a PAMP-recognition molecule as well as a DAMP at the crossroads of DAMP- and PAMP-recognition systems in innate immune responses against the infection.

In addition to its immunomodulative roles in innate immunity, galectin-3 also modulates some of the adaptive immune responses (30, 59, 91–93). Lack of galectin-3 induces a higher Th1-polarized response in atopic dermatitis models, infections with Schistosoma mansoni, Toxoplasma gondii, and as a part of tumor immunity (59, 92, 94, 95). In addition, lack of galectin-3 also restrains IL-4–induced alternative macrophage (M2) activation (93). Importantly, M2-activated macrophages preferentially support the proliferation of L. major (96, 97). Thus, in G3KO mice, in which both Th2-skewed response and M2-like activation in macrophages are suppressed, it can be speculated that L. major infection should be well controlled by adaptive immunity (37). Indeed, in the case of L. major Friedlin infection in which there is no difference in neutrophil recruitment between G3KO and WT mice, the pathogenesis of leishmaniasis in G3KO mice appeared to be slightly yet significantly better controlled than in WT mice (Fig. 6C), which is consistent with the previous reports on the role of galectin-3 in adaptive immunity. However, in the case of LV39, the exacerbation of pathogenesis was observed despite of the immunological background of G3KO mice, also underlining the impact of the higher parasitic loads resulting from the defect in rapid neutrophil migration.

Emergence of previously unanticipated reciprocal relationship between neutrophil migration and monocyte/macrophage recruitment underlines the importance of understanding the molecular mechanisms of the initiation of this cascade (61). Further, although both PAMPs and DAMPs participate to the reciprocal cycles of phagocyte migration, the identification of DAMPs that regulate neutrophil migration in various settings has just begun to be explored (60, 61). The current study may thrust galectin-3 forward as a member of DAMPs that can facilitate initial neutrophil emigration in a microorganism-dependent manner. Unlike other DAMPs that are shown to have distinctive receptors, galectin-3, being a glycan-binding protein, does not necessarily have bona fide specific receptors (30, 31). Indeed, galectin-3 recognizes specific surface glycosylation patterns that are highly rich in lactosamine residues (30, 31, 98). Importantly, those glycan patterns are known to be highly regulated in immunological differentiation/activation and sensitive to the microenvironment and nutrition (99–101). Thus, several layers of regulation could be implicated in galectin-3’s role as a DAMP to facilitate neutrophil migration and have yet to be fully explored.

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