



Integrin $\alpha_D\beta_2$ (CD11d/CD18) Modulates Leukocyte Accumulation, Pathogen Clearance, and Pyroptosis in Experimental *Salmonella* Typhimurium Infection

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Specialty section:

This article was submitted to
Microbial Immunology,
a section of the journal
Frontiers in Immunology

Received: 07 March 2018

Accepted: 04 May 2018

Published: 24 May 2018

Citation:

Nascimento DdO, Vieira-de-Abreu A, Arcanjo AF, Bozza PT, Zimmerman GA and Castro-Faria-Neto HC (2018) Integrin $\alpha_D\beta_2$ (CD11d/CD18) Modulates Leukocyte Accumulation, Pathogen Clearance, and Pyroptosis in Experimental *Salmonella* Typhimurium Infection. *Front. Immunol.* 9:1128. doi: 10.3389/fimmu.2018.01128

β_2 integrins are critical in host defense responses to invading pathogens and inflammation. Previously, we reported that genetic deficiency of integrin $\alpha_D\beta_2$ in mice altered outcomes in experimental systemic infections including accelerated mortality in animals infected with *Salmonella enterica* serovar Typhimurium. Here, we show that deficiency of $\alpha_D\beta_2$ results in impaired accumulation of leukocytes in response to peritoneal infection by *S. Typhimurium*, impaired pathogen clearance *in vivo*, defective bacterial elimination by cultured peritoneal macrophages, and enhanced pyroptosis, a cell death process triggered by *Salmonella*. *Salmonella*-infected animals deficient in $\alpha_D\beta_2$ had increased levels of peritoneal cytokines in addition to other markers of pyroptosis, which may contribute to inflammatory injury and increased mortality in the context of impaired bacterial killing. These observations indicate important contributions of leukocyte integrins to the host response in experimental *Salmonella* infection and reveal previous activities of $\alpha_D\beta_2$ in bacterial infection.

Keywords: *Salmonella enterica* serovar Typhimurium pathogenesis, non-typhoidal *Salmonella*, myeloid leukocytes, macrophage, integrin, pyroptosis

INTRODUCTION

Trafficking of leukocytes in tissues and organs, and cell–cell interactions between immune effector cells, are essential for defense against pathogens, innate and acquired immunity, and physiologic and pathologic inflammation (1, 2). Integrins are critical in these processes. Integrins are transmembrane heterodimers that are broadly expressed in varying patterns on metazoan cells (3, 4). In addition to cellular trafficking, integrins mediate adhesion-dependent cellular localization, signaling, intercellular communication, interactions with matrix, and regulation of apoptosis (3, 5). A subfamily of integrins called the β_2 or CD11/CD18 integrins, also termed leukocyte integrins or leukointegrins, consists of four members: $\alpha_L\beta_2$ (6), $\alpha_M\beta_2$ (CD11b/CD18, MAC-1, CR3), $\alpha_X\beta_2$ (CD11c/CD18), and $\alpha_D\beta_2$ (CD11d/CD18) (3, 7). As in all integrins, the α subunits are not expressed on the cell surface without non-covalent heterodimerization with the β subunit, and *vice versa* (3–5). Leukocyte integrin heterodimers are expressed in varying patterns on circulating myeloid leukocytes and lymphocytes and on tissue leukocytes, including macrophages and dendritic cells, and have

important activities in infection, inflammation, and immunity (8–10). Critical roles of leukocyte integrins in defense against bacterial infection, innate immune responses, wound surveillance, and tissue repair are clearly demonstrated by heritable human leukocyte adhesion deficiency syndromes in which the expression of β_2 integrins is absent or dramatically reduced, or inside-out signaling of integrin heterodimers is impaired (10). Experimental models in which β_2 integrins are genetically deleted or blocked with specific antibodies confirm essential physiologic contributions of leukocyte integrins in host defense and inflammation (5, 7–10). Additional integrins of the β_1 and other integrin subclasses are also found on individual leukocyte types depending on their lineage and activation state (5, 7, 9, 10).

The most recently discovered member of the leukocyte, or β_2 , integrin subfamily is $\alpha_D\beta_2$ (CD11d/CD18), which is expressed on circulating leukocytes and tissue macrophages in humans and on a subpopulation of blood leukocytes and distinct subsets of macrophages in uninfected mice (6, 10–14). Experiments with primary leukocytes from humans or mice or with transfected cell lines indicate that $\alpha_D\beta_2$ recognizes ICAM-3, ICAM-1, VCAM-1, and various matrix proteins (6, 11, 13–15). *In vivo* studies of wild type (WT) and α_D^- demonstrate that $\alpha_D\beta_2$ expression can be dynamically modulated in response to infection and that it has complex activities in immune regulation and systemic inflammatory responses (12, 13). Nevertheless, little is known about the functions of $\alpha_D\beta_2$ in infectious and inflammatory syndromes (10).

We previously reported that $\alpha_D\beta_2$ expression is altered on splenic and hepatic macrophages in response to the rodent malarial pathogen, *Plasmodium berghei* Anka, and that targeted deletion of α_D conferred a survival advantage in this model of severe malaria (13). In parallel, we examined mice infected with *Salmonella enterica* serovar Typhimurium (16) to determine if genetic deletion of $\alpha_D\beta_2$ has stereotyped or, conversely, differential effects in lethal systemic infections. *S. enterica* is an intracellular facultative anaerobe that is one of the leading causes of enteric diseases in the United States. *Salmonella* can cause diseases such as typhoid, gastroenteritis, bacteremia, and chronic asymptomatic carriage (17). *S. enterica* serovar Typhimurium, the causative agent of gastroenteritis, has developed different strategies to evade host defense mechanisms (18). In contrast to increased survival at early time points in the experimental malaria model, mortality was accelerated in α_D^- infected with *S. Typhimurium* (13). Here, we further explore functions of $\alpha_D\beta_2$ in animals challenged with *S. Typhimurium*.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0127:b8) and from *S. enterica* serotype Typhimurium were purchased from Sigma-Aldrich. Live/Dead BacLight bacterial viability kit and latex beads were obtained from Molecular Probes. Alexa 488-conjugated mAb 205c (13) was used to label α_D for flow cytometry analysis. Anti-TLR4-PE was purchased from eBioscience, San Diego, CA, USA. YVAD (N-Ac-Tyr-Val-Ala-Asp-CMK), a caspase-1 inhibitor, was purchased from

Cayman-Chemical. Fluorochrome inhibitor of caspase (FLICA) was purchased from Immunochemistry Technologies (Bloomington, MN, USA). Necrostatin-1 (Nec-1) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Nec-1 is an inhibitor of the receptor interacting protein 1 kinase and acts as an inhibitor of necroptosis (19).

Mice

C57Bl/6 WT mice and mice with targeted deletion of α_D (α_D^-) to yield deficiency of $\alpha_D\beta_2$ weighing 20–25 g of both sexes were obtained from the Oswaldo Cruz Foundation breeding unit. We have previously characterized these α_D^- mice in detail. The animals were kept at constant temperature (25°C) with free access to food and water in a room with a 12-h light/dark cycle. The protocols employed in this work were approved by the Oswaldo Cruz Foundation Animal Welfare Committee under the license number 0011-00.

Bacterial Strains

Salmonella enterica serovar Typhimurium (16) ISC 5302/2004 was a generous gift from the Department of Microbiology of the Oswaldo Cruz Foundation. The bacteria were cultured in Luria-Bertani broth (Guria Broth Miller; Sigma-Aldrich) for 16–18 h at 37°C to obtain stationary growth phase cultures and were then centrifuged (1,000 × g) for 10 min at 4°C. The pellets were resuspended in PBS to an OD of 0.1 at 660 nm, corresponding to 10⁸ CFU/mL. WT and α_D^- mice were infected by intraperitoneal (i.p.) injection of 200 μ L of the bacteria suspension (10⁵ CFU *Salmonella*/mouse). Control WT mice were sham injected (saline alone) in parallel.

As YVAD (N-Ac-Tyr-Val-Ala-Asp-CMK) is a selective and irreversible inhibitor of interleukin-1 beta converting enzyme (Caspase-1), the effect of caspase-1 action on the absence of α_D^- during *Salmonella* Typhimurium infection was analyzed *in vivo*. For this, in some experiments, 15 min after *S. Typhimurium* infection, WT and α_D^- mice were injected i.p. with YVAD (200 μ g/animal) or Nec-1 (1.65 mg Nec-1/kg body weight). Animals injected with sterile saline or DMSO served as controls.

Leukocytes Counts

Total leukocyte counts were performed in Neubauer chambers by means of an optical microscope after diluting the peritoneal wash in Turk's solution (2% acetic acid). Differential leukocyte analysis was performed under an oil immersion objective on cytocentrifuged cells stained with May-Grunwald-Giemsa dye.

Cytokine Measurements

Levels of IL-6, TNF- α , MIP-1 α , and IL-1 β in peritoneal fluid were evaluated by enzyme-linked immunosorbent assay measurements using specific monoclonal antibodies, according to manufacturer instructions (Duo Set Kit from R&D Systems). Mice were sacrificed in a CO₂ chamber at designated time points and the peritoneal cavities were opened and rinsed with PBS. The particulate matter was removed by centrifugation (252 × g) for 10 min, and the supernatant fractions were used for immunoassays.

Intraperitoneal LPS Challenge

The administration of LPS, an endotoxin present on the cell wall of Gram-negative bacteria, has been extensively used in the study of sepsis, since it promotes the development of clinical signs observed in patients with this syndrome (20). Thus, as *S. Typhimurium* is a Gram-negative bacteria, we have determined whether LPS inoculation would alter the response pattern in $\alpha_D^{-/-}$ mice. For this, mice were injected intraperitoneally with 500 ng per cavity of LPS diluted in sterile saline. Control animals received equal volumes of saline.

Toll-Like Receptor 4 (TLR4) Molecule Labeling

Toll-like receptor 4 is an important PRR receptor (pattern recognition receptor) for Gram-negative bacteria recognition. We use TLR4 molecule labeling for investigation of a possible activation pathway modulated by $\alpha_D\beta_2$ integrin. TLR4 molecule from peritoneal cells of WT and $\alpha_D^{-/-}$ mice was labeled by Flow cytometry analysis, according to manufacturer instructions (eBioscience, San Diego, CA, USA).

Measurement of Caspase-1 Activity

Caspase-1 activity was measured with FAM-YVAD FMK (5-carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethylketone, FLICA) in peritoneal cells of WT and $\alpha_D^{-/-}$ mice, infected or not. The addition of FLICA was done according to the manufacturer's instructions (Immunochemistry Technologies, Bloomington, MN, USA). FLICA⁺ cells were detected by FACS analysis.

Macrophage Culture and *In Vitro* Infection

Peritoneal cells from WT (16) C57Bl/6 and $\alpha_D^{-/-}$ mice were collected by lavage with sterile RPMI 1640 cell culture medium. Macrophages (1×10^6 cells/mL) were allowed to adhere for 2 h at 37°C in a 5% CO₂ atmosphere with RPMI 1640 cell culture medium containing 2% FCS. The non-adherent cells were removed after vigorous PBS wash. Macrophages were incubated with *S. Typhimurium* (10^5 CFU/well) (21) or with fluorescent beads (FluoSpheres[®] polystyrene microspheres), 1.0 μ m, yellow-green fluorescent (Invitrogen), or IDC Surfactant-free Latex Beads (Invitrogen) (1:1), for 1 h at 37°C in 5% CO₂ atmosphere. After this incubation period, the cultures were washed three times with PBS to remove non-internalized bacteria or beads. Then, the cells were incubated in RPMI 1640 cell culture medium for 1 h at 37°C in a 5% CO₂ atmosphere in the absence or presence of 5 μ g/mL gentamicin to kill the remaining extracellular bacteria. Sterile saline was used as control. After incubation, macrophages were lysed with 0.1% saponin for 20 min at room temperature. Aliquots of the cell lysates were plated on tryptic soy agar medium and incubated at 37°C for CFU determinations. The cell-free supernatants were recovered and stored at -20°C.

Analysis of Nitric Oxide

Analysis of the presence of nitric oxide was performed using the DAF-FM probe (D-23842, Molecular Probes), according to manufacturer instructions. As the maximum length of excitation and emission fluorescence are 495 and 515 nm, respectively, and therefore similar to fluorescein (FITC), a flow cytometer was used.

Flow Cytometry

After adjusting the concentration of cell samples to $1-2 \times 10^6$ cells/100 μ L specific antibodies and their respective control antibodies were incubated with the leukocytes for 30 min on ice. Samples were washed with FACS medium (HBSS without Ca²⁺ and Mg²⁺/0.1% NaN₃/0.5% human serum albumin) and fixed in 3.7% formalin. The samples were stored at 4°C and protected from light until analysis.

For cell size analysis, we utilized a single parameter histogram along the X-axis (FCS—Forward Scatter), gating on the peritoneal macrophage region. This parameter is a measurement of the amount of the laser beam that passes around the cell, yielding a relative size for the cell. Cells were analyzed by flow cytometry using a FACSCalibur[®] device (Becton Dickinson, San Jose, CA, USA) equipped with CellQuest software. A gate excluding cell debris and non-viable cells was utilized. Analyses were done after recording 10,000 events for each sample.

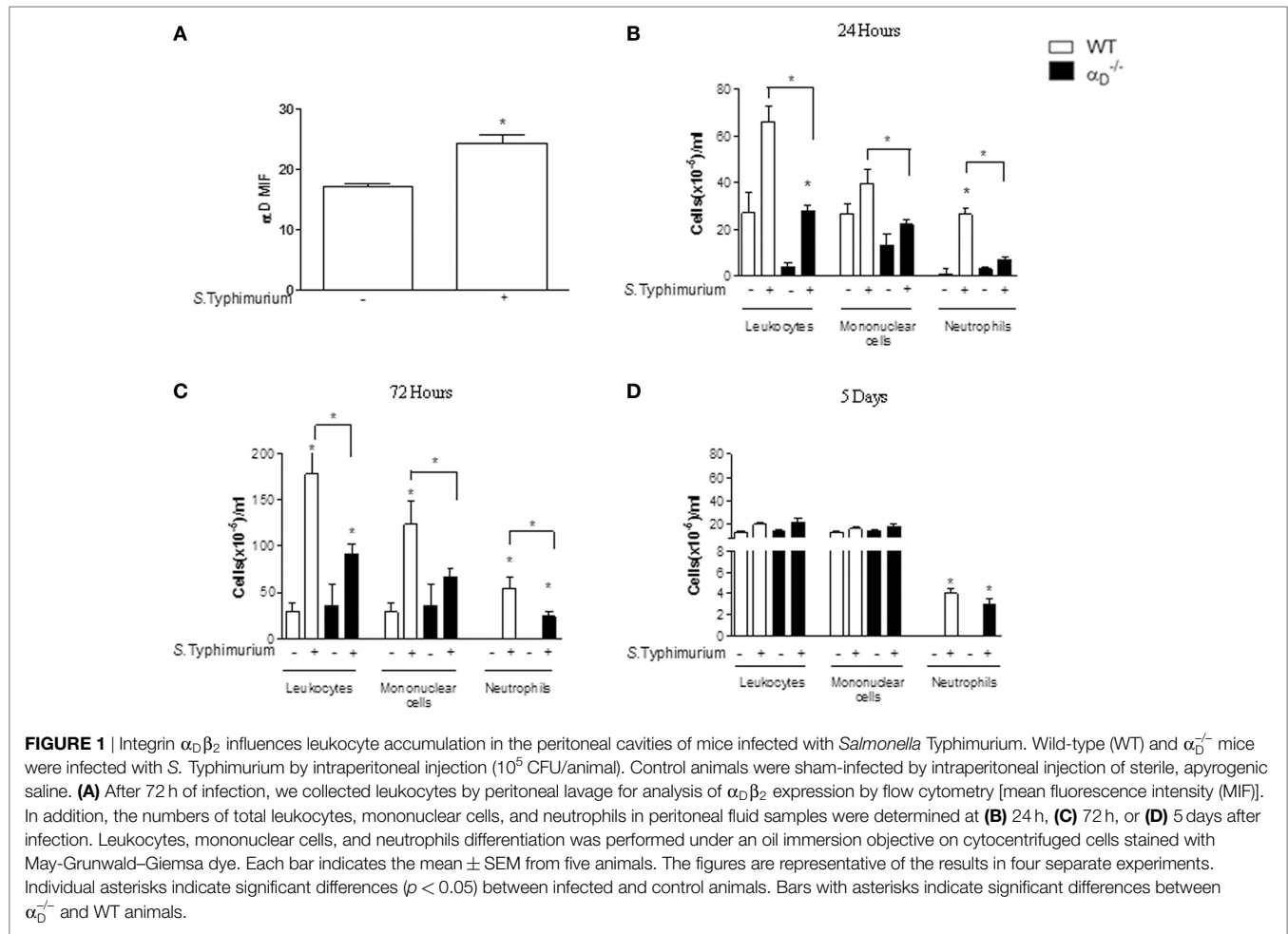
Statistical Analysis

All data are expressed as mean \pm SEM and compared using a two-tailed Student's *t*-test and one-way ANOVA. Data were considered statistically significant with a $p \leq 0.05$.

RESULTS

Cellular Host Defense Responses to *S. Typhimurium* Are Differentially Altered in Mice Deficient in $\alpha_D\beta_2$

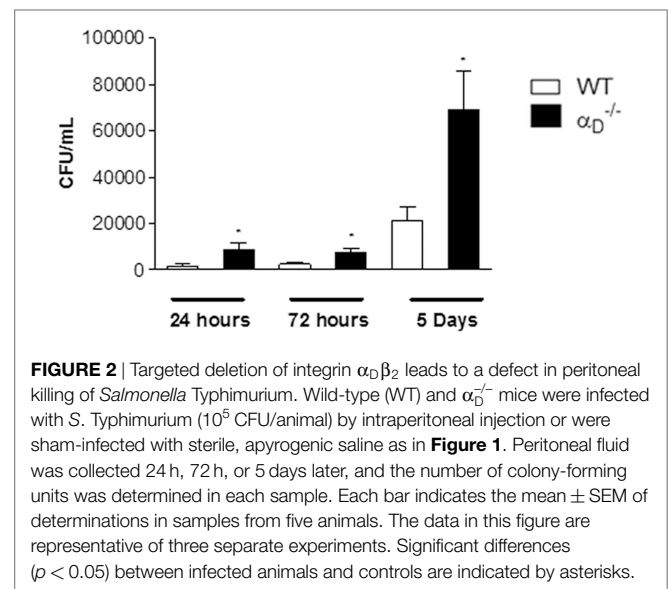
We utilized an i.p. challenge model of *Salmonella* infection to mimic intraperitoneal and systemic infectious complications, which occur clinically (22), and to relate the findings to previous observations (13, 23). Infection of WT C57BL/6 mice with *S. Typhimurium* increased expression of $\alpha_D\beta_2$ on peritoneal macrophages when analyzed by flow cytometry (Figure 1A; Figure S1 in Supplementary Material), indicating that this integrin is involved in the host response to *Salmonella*. Because myeloid leukocyte accumulation is a feature of *S. Typhimurium* infection (23, 24), we examined inflammatory cell numbers in WT and $\alpha_D^{-/-}$ mice subjected to i.p. challenge with this pathogen. There was impaired leukocyte accumulation in the peritoneal cavities of $\alpha_D^{-/-}$ mice mainly with 72 h after infection, with substantially decreased numbers of total leukocytes, mononuclear cells, and neutrophils recovered in peritoneal fluid when compared with WT-infected animals (Figure 1C). The cause of the difference in cellularity observed in Figure 1B is unknown, but it is possible that it is related to the intrinsic conditions of the groups tested. This result demonstrates that $\alpha_D\beta_2$ influences acute inflammatory cellular responses to intraperitoneal infection by *Salmonella*. After 5 days of infection, however, there were no significant differences in the numbers of mononuclear leukocytes or neutrophils or in total leukocyte numbers in peritoneal fluid samples from WT or $\alpha_D^{-/-}$ animals (Figure 1D). This indicates that alternative mechanisms, likely involving other β_2 integrins and/or other members of the integrin family expressed by leukocytes (7, 9, 10), contribute to peritoneal leukocyte accumulation at later time points. Consistent with this, we found that α_M was increased on peritoneal



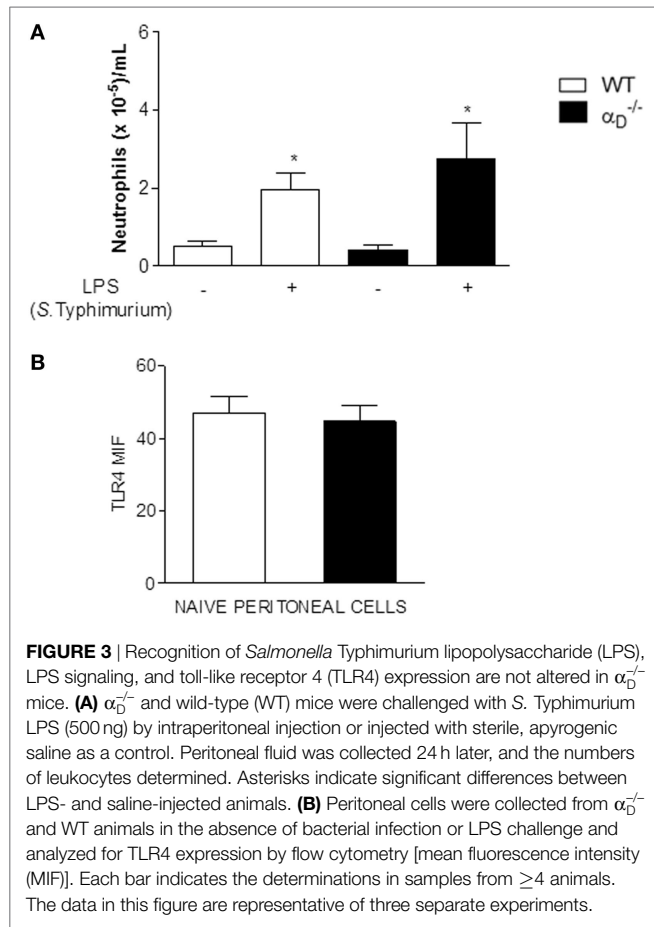
leukocytes from WT and $\alpha_D^{-/-}$ animals at 72 h after i.p. infection when examined by flow cytometric analysis, indicating increased expression of $\alpha_M\beta_2$ (Figure S2 in Supplementary Material).

We also determined if genetic deficiency of $\alpha_D\beta_2$ influences bacterial clearance in this model. The number of colony-forming units per milliliter (CFU/mL) of *Salmonella* in peritoneal fluid (Figure 2), in spleen and blood (Figure S3 in Supplementary Material) was higher in $\alpha_D^{-/-}$ mice compared with WT animals at 24 h, 72 h, and 5 days of infection. Together with the analysis of cell numbers (Figure 1), these findings indicate that $\alpha_D\beta_2$ mediates leukocyte responses and mechanisms involved in clearance and/or multiplication of *Salmonella* in the infected peritoneal cavity.

While β_2 integrins have been reported to participate in *S. Typhimurium* recognition and dissemination based on knock-out of all β_2 (CD18) heterodimers (25), little is known about the contributions of individual leukocyte integrins to host defense against this bacterium and to the pathogenesis of *Salmonella* infections. Therefore, we examined other features of *Salmonella* challenge in $\alpha_D^{-/-}$ mice. We first excluded the possibility that TLR4 expression and recognition of *Salmonella* LPS are altered in $\alpha_D^{-/-}$. There were no differences in peritoneal neutrophil accumulation in WT and $\alpha_D^{-/-}$ mice 24 h after instillation of *S. Typhimurium* LPS (Figure 3A), although the cell numbers in both WT and $\alpha_D^{-/-}$ animals were lower than in mice challenged with live *Salmonella*



bacteria (Figure 1). Consistent with intact recognition of LPS, the expression of TLR4 was not different on leukocytes from WT and $\alpha_D^{-/-}$ animals (Figure 3B).

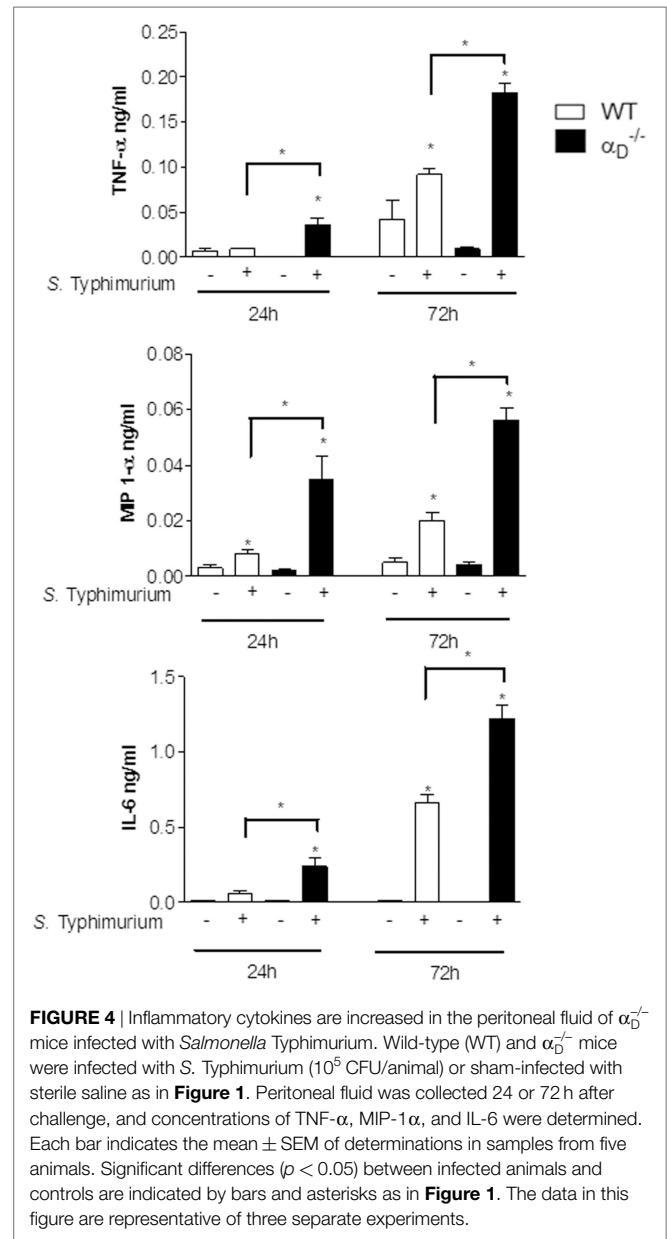


The Cytokine Response Is Altered in $\alpha_D^{-/-}$ Mice Infected With *Salmonella*

In parallel to analysis of peritoneal leukocyte accumulation in $\alpha_D^{-/-}$ mice challenged with *S. Typhimurium*, we also examined release of cytokines, which are central mediators in the host response to microbial pathogen invasion. Unexpectedly, there were higher levels of TNF α , MIP-1 α , and IL-6 in the peritoneal fluid of infected $\alpha_D^{-/-}$ mice compared with levels in samples from WT controls (**Figure 4**). Thus, although total peritoneal leukocyte numbers and the numbers of myeloid leukocytes were decreased in $\alpha_D^{-/-}$ mice at early time points (**Figure 1**), $\alpha_D\beta_2$ -deficient animals had amplified peritoneal accumulation of pro-inflammatory cytokines.

Integrin $\alpha_D\beta_2$ Modulates *Salmonella* Killing and Release of Cytokines by Peritoneal Macrophages *In Vitro*

We then examined bacterial elimination and cytokine release by isolated peritoneal macrophages to mechanistically correlate these activities with *in vivo* determinations. We found that peritoneal macrophages from $\alpha_D^{-/-}$ mice cultured with *S. Typhimurium in vitro* contained increased numbers of viable bacteria compared with numbers of *Salmonella* in WT peritoneal macrophages (**Figure 5A**). Impaired elimination of *Salmonella*



by $\alpha_D^{-/-}$ macrophages *in vitro* (**Figure 5A**) parallels defective *in vivo* elimination of the pathogen by $\alpha_D^{-/-}$ mice that we observed (**Figure 2**). Ingestion of microbeads by $\alpha_D^{-/-}$ and WT macrophages was similar (**Figure 5B**), indicating that global phagocytosis is intact in $\alpha_D^{-/-}$ macrophages and consistent with increased intracellular CFU in macrophages cultured with the pathogen (**Figure 5A**). In addition, we found that peritoneal macrophages from $\alpha_D^{-/-}$ mice released increased levels of IL-1 β , TNF α , and MIP-1 α compared with WT macrophages when challenged with *S. Typhimurium in vitro* (**Figure 5C**). This pattern was again similar to that in animals challenged with the bacterium *in vivo* (**Figure 4**) and indicates that peritoneal macrophages are a potential source of elevated cytokine levels in the peritoneal exudate in infected $\alpha_D^{-/-}$ mice.

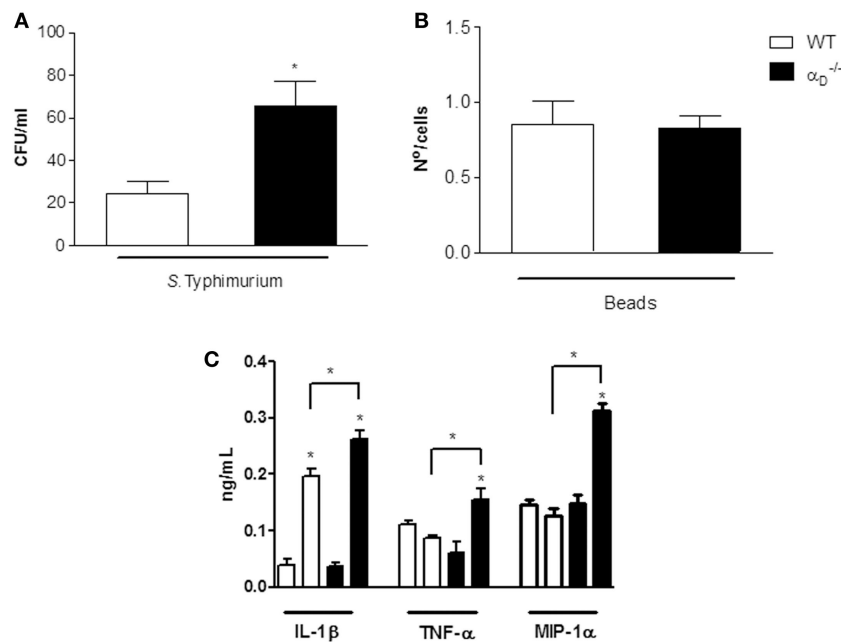


FIGURE 5 | Integrin $\alpha_D\beta_2$ regulates *Salmonella* Typhimurium killing and release of inflammatory cytokines by peritoneal macrophages challenged *in vitro*.

(A) Peritoneal macrophages (10^6 cells/mL) were collected from wild-type (WT) or $\alpha_D^{-/-}$ mice and challenged *in vitro* with *S. Typhimurium* (10^5 CFU/mL) or incubated in medium alone for 1 h at 37°C . The medium was removed, and the cells were incubated for an additional hour with fresh medium without antibiotics. After 1 h of incubation at 37°C , the macrophages were lysed with saponin (0.1%), and bacterial CFU were determined in each sample. **(B)** WT and $\alpha_D^{-/-}$ peritoneal macrophages were incubated with latex particles (10^7 /well). The media were removed, and fresh media added as in panel **(A)**, and the number of intracellular beads counted by light microscopy. Each bar indicates the mean \pm SEM of beads in 100 consecutively counted cells. **(C)** Supernatants were collected from peritoneal macrophages infected with *S. Typhimurium* or from uninfected peritoneal macrophages cultured as described in panel **(A)** after 1 h of incubation and assayed for IL-1 β , TNF- α , and MIP-1 α . Three to five wells were analyzed in each experiment. The data in this figure are representative of three experiments with similar results. Significant differences ($p < 0.05$) between infected and control cells are indicated by asterisks and between cells from $\alpha_D^{-/-}$ and WT animals by bars with asterisks.

Salmonella Typhimurium Infection of $\alpha_D^{-/-}$ Mice Triggers Enhanced Pyroptosis

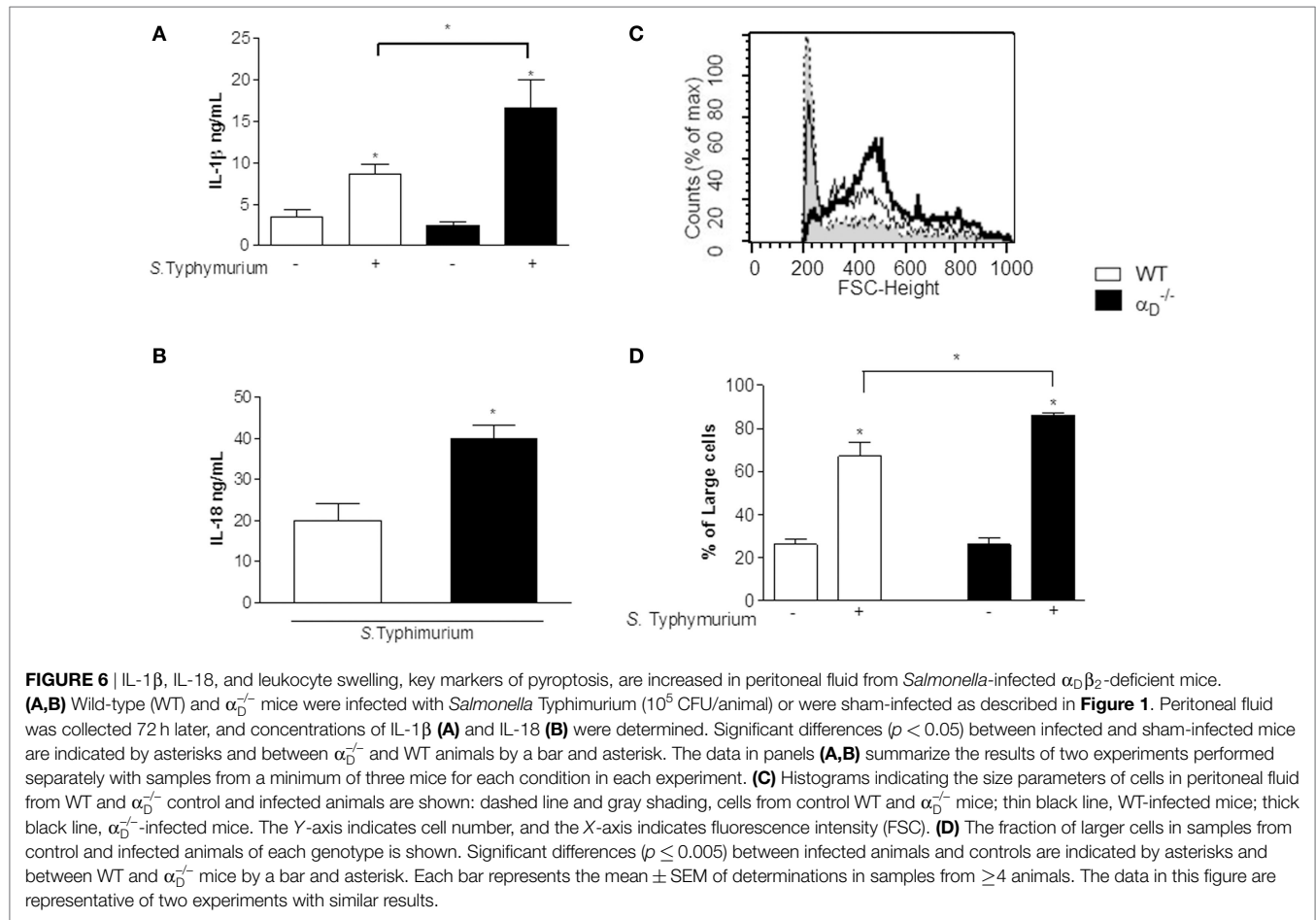
Pyroptosis is a host response to *Salmonella* that involves a cell death program and resultant release of pro-inflammatory cytokines by infected macrophages (26–30). Caspase-1 is the inflammatory caspase crucial to canonical inflammasome-mediated pyroptosis and cytokine maturation (31). Enhanced release of IL-1 β and IL-18, resulting from caspase-1-mediated cleavage of the pro-forms of these inflammatory proteins to yield mature, bioactive cytokines, is a defining feature of pyroptosis although other cytokines including IL-6 and TNF α are also released in an enhanced fashion (28, 29). Therefore, we measured IL-1 β and IL-18 in peritoneal fluid from mice infected 72 h previously with *S. Typhimurium* and found that these interleukins were increased in samples from $\alpha_D^{-/-}$ mice when compared with levels in peritoneal fluid from WT animals (Figures 6A,B). Thus, these key markers of pyroptosis are increased in the peritoneal inflammatory response of $\alpha_D^{-/-}$ mice infected by *Salmonella*.

Analysis of peritoneal cells demonstrated increased cell size in samples from $\alpha_D^{-/-}$ animals compared with cells in the peritoneal fluids of WT mice after infection with *Salmonella* (Figures 6C,D). Cell swelling, eventual osmotic cell lysis, and release of cytokines are central features of pyroptosis (26, 28, 29). In parallel, we found increased caspase-1 activity in peritoneal macrophages

from *S. Typhimurium*-infected $\alpha_D^{-/-}$ mice (Figures 7B,C) using a cell-permeant fluorescent probe that specifically binds to activated caspase-1 (16). Caspase-1 activation is a defining mechanism in pyroptosis and is upstream of cell swelling, cell lysis, and cytokine release (26, 28–30, 32). Preliminary results after administration of Nec-1 *in vivo* showed no change in the leukocyte migration pattern between infected WT and $\alpha_D^{-/-}$ mice, suggesting that there is no necroptosis in our model (Figure S4 in Supplementary Material). Thus, each of the key cellular and biochemical features of pyroptosis is enhanced in $\alpha_D^{-/-}$ mice infected with *S. Typhimurium* (Figures 4, 6, and 7). There was impaired killing of *Salmonella* by $\alpha_D^{-/-}$ macrophages with increased caspase-1 activity (Figure 7A), consistent with *in vitro* experiments (Figure 5A) and increased numbers of bacteria in the peritoneal fluid of infected $\alpha_D^{-/-}$ mice (Figure 2).

DISCUSSION

This study identifies three previously unrecognized features of the biology of integrin $\alpha_D\beta_2$ directly relevant to infection and inflammation: this specialized heterodimer influences early leukocyte accumulation at extravascular sites in mice infected with a bacterial pathogen, regulates *in vivo* and *in vitro* elimination of *S. Typhimurium*, and indirectly or directly influences pyroptosis, a complex leukocyte response triggered by infection with



Salmonella and other microbes. In previous studies, antibodies raised against α_D (3, 4, 6), altered accumulation of macrophages and neutrophils at sites of experimental spinal cord injury (33, 34) or in non-infectious peritonitis caused by thioglycollate (35). Here, we found that the accumulation of mononuclear cells and neutrophils was depressed in the peritoneal cavities of mice genetically deficient in $\alpha_D\beta_2$ when the animals were infected by *S. Typhimurium*, although the impairment in leukocyte accumulation was not sustained at 5 days after infection (**Figure 1**). A similar early—but temporally limited—influence of $\alpha_D\beta_2$ on leukocyte accumulation in spinal cord injury in rats was previously reported (34). Integrin $\alpha_D\beta_2$ mediates adhesiveness of primary murine macrophages (13, 15), mouse cell lines (15), and human leukocyte subsets and cell lines (6, 11, 14), and is also reported to alter monocyte/macrophage migration *in vitro* (35). Expression of $\alpha_D\beta_2$ is increased on peripheral blood monocytes after peritoneal inflammation in mice (35). Thus, the defect in early myeloid leukocyte accumulation in the peritoneal cavities of *Salmonella*-infected $\alpha_D^{-/-}$ mice is likely in part due to impaired adhesion and/or migration during inflammatory trafficking. The residual ability of myeloid leukocytes to accumulate in the inflamed peritoneal cavities of $\alpha_D^{-/-}$ mice (**Figure 1**) may be due to other β_2 integrins such as $\alpha_M\beta_2$, which also contribute to adhesion, migration, and pathogen recognition by myeloid leukocytes (7, 35, 36).

Previous studies indicate that impairment of myeloid leukocyte accumulation in mice challenged with *S. Typhimurium* may be sufficient to disrupt killing and clearance of the pathogen by the infected host (24). Thus, early impairment of leukocyte accumulation in the infected peritoneal cavity (**Figure 1**) might account for the faster demise of $\alpha_D\beta_2$ -deficient animals challenged with *Salmonella* that we saw in previous experiments (13). Nevertheless, we also found additional alterations in host responses of infected $\alpha_D^{-/-}$ mice that may alter the natural history of the infection and contribute to increased mortality. There were greater numbers of colony-forming *Salmonella* in peritoneal fluid samples from $\alpha_D^{-/-}$ animals. The increased pathogen burden was seen not only at early time points but also at 5 days, after peritoneal leukocyte numbers had equalized in $\alpha_D^{-/-}$ and WT mice (**Figures 1** and **2**). We observed a similar impairment in bacterial elimination in $\alpha_D^{-/-}$ macrophages challenged with bacteria *in vitro* (**Figure 5**), suggesting one or more defects in intracellular killing of the *Salmonella* pathogen. The impaired elimination of *Salmonella* by $\alpha_D^{-/-}$ macrophages in our experiments was not due to a generalized defect in phagocytosis (**Figure 5**), which is mediated by specific β_2 integrins (8).

Because integrins, including members of the leukocyte integrin subfamily, mediate outside-in signaling in addition to cellular adhesion and surface binding of ligands and microorganisms (3–5, 7, 9, 10), $\alpha_D\beta_2$ may signal to critical pathways

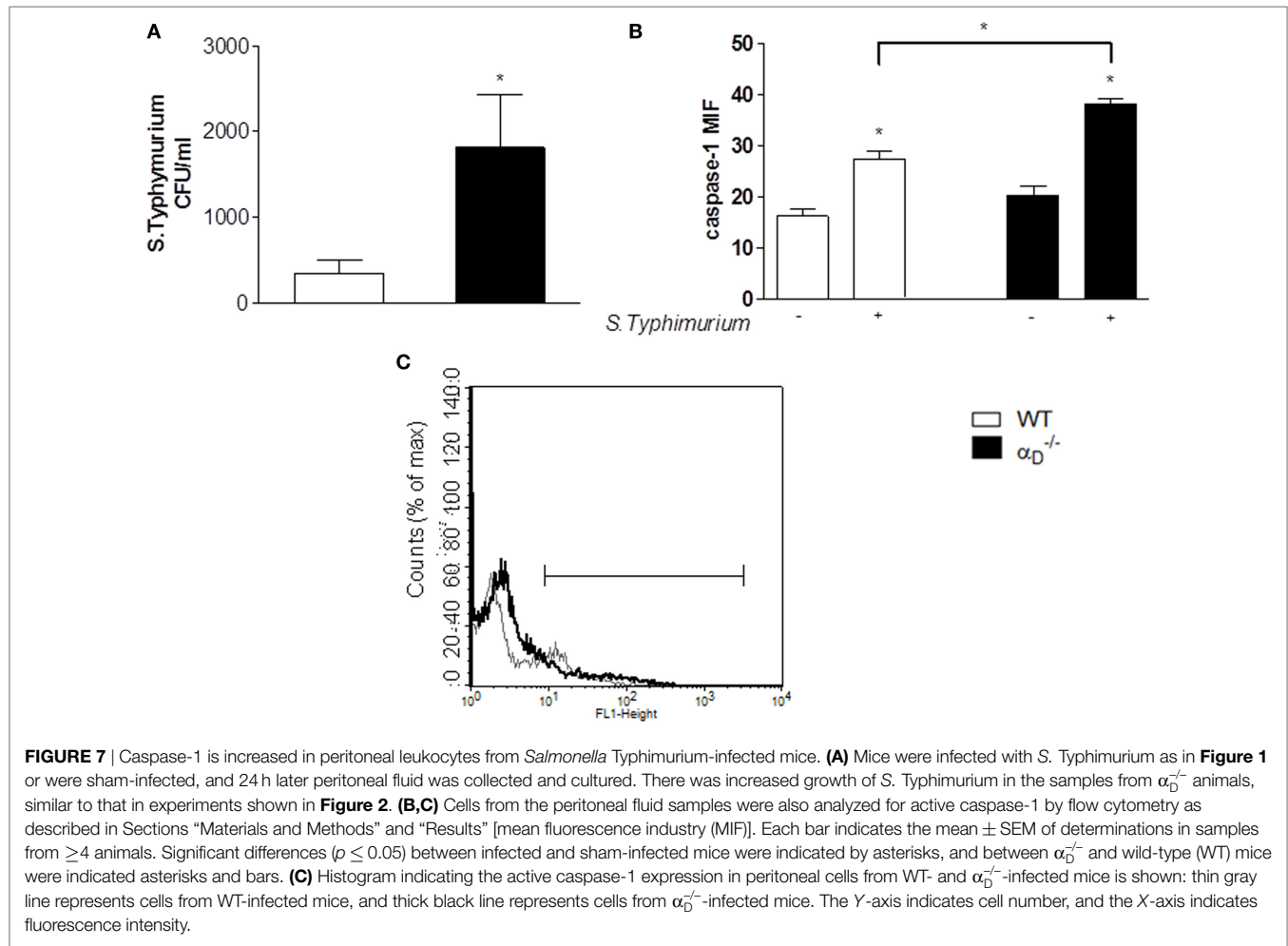


FIGURE 7 | Caspase-1 is increased in peritoneal leukocytes from *Salmonella* Typhimurium-infected mice. **(A)** Mice were infected with *S. Typhimurium* as in **Figure 1** or were sham-infected, and 24 h later peritoneal fluid was collected and cultured. There was increased growth of *S. Typhimurium* in the samples from $\alpha_D^{-/-}$ animals, similar to that in experiments shown in **Figure 2**. **(B,C)** Cells from the peritoneal fluid samples were also analyzed for active caspase-1 by flow cytometry as described in Sections “Materials and Methods” and “Results” [mean fluorescence intensity (MIF)]. Each bar indicates the mean \pm SEM of determinations in samples from ≥ 4 animals. Significant differences ($p < 0.05$) between infected and sham-infected mice were indicated by asterisks, and between $\alpha_D^{-/-}$ and wild-type (WT) mice were indicated asterisks and bars. **(C)** Histogram indicating the active caspase-1 expression in peritoneal cells from WT- and $\alpha_D^{-/-}$ -infected mice is shown: thin gray line represents cells from WT-infected mice, and thick black line represents cells from $\alpha_D^{-/-}$ -infected mice. The Y-axis indicates cell number, and the X-axis indicates fluorescence intensity.

that determine *Salmonella* killing versus intracellular survival (37). In preliminary experiments, we found intact production of nitric oxide by macrophages from $\alpha_D^{-/-}$ mice (Figure S5 in Supplementary Material), suggesting that this is not the molecular explanation for defective elimination. While it is clear that β_2 integrins regulate multiple leukocyte functions (5, 8, 10, 36), specific activities of individual β_2 heterodimers—such as $\alpha_D\beta_2$ —in pathogen recognition, killing, and clearance are largely unexplored.

We also found evidence for increased pyroptosis in $\alpha_D^{-/-}$ mice challenged with i.p. *Salmonella*. *Salmonella* infection of macrophages induces complex cellular responses (37), including autophagy (38) and pyroptosis (27–30). Pyroptosis, originally characterized as caspase-1-dependent death of *Shigella*- or *Salmonella*-infected macrophages, occurs *in vitro* and *in vivo* (29, 30, 39–41), and may also be triggered by other bacteria including *Yersinia*, *Pseudomonas*, *Bacillus anthracis*, and *Burkholderia* (28, 41). Pyroptosis is mechanistically distinct from other forms of programmed cell death (26, 28, 30). Despite intensive studies of inflammasomes, the regulating mechanisms of IL-1 β /IL-18 production and pyroptosis after caspase-1 (canonical pathway) or caspase-11 (non-canonical pathway) activation are unknown. Recent work has shown the participation of Gasdermin D as a new

component involved in these processes (42–45). As there were no differences in peritoneal neutrophil accumulation in WT and $\alpha_D^{-/-}$ mice after LPS instillation (**Figure 3A**), our data suggest that in our model there is the involvement of the canonical pathway of inflammasome activation. In the non-canonical inflammasome, the inflammatory protease, caspase-11 acts as a receptor for LPS that gains access to the cytosol (46).

Caspase-1 activation and inflammasome-mediated production of mature IL-1 β and IL-18, cellular swelling due to plasma membrane permeabilization, and release of pro-inflammatory cytokines are defining and/or critical features of pyroptosis (28–30, 47). We found that each of these essential markers of pyroptosis is enhanced in $\alpha_D^{-/-}$ mice infected with *S. Typhimurium* (**Figures 5–7**). Pyroptotic cell death of macrophages may contribute to reduced total numbers of leukocytes in the peritoneal fluid of $\alpha_D^{-/-}$ animals early after *Salmonella* infection (**Figure 1**).

Enhancement of pyroptosis in $\alpha_D\beta_2^{-/-}$ mice was an unexpected finding. Integrin signaling regulates apoptosis in some cell types (5, 48) and can alter activities of caspases 3 and 8 (36, 49, 50). Nevertheless, β_2 integrins are not known to influence macrophage death or pyroptosis, or to specifically signal to caspase-1 or to events downstream of caspase-1 in the pyroptosis pathway

(27–29). Our observation that pyroptosis is enhanced in $\alpha_D^{-/-}$ mice may indicate that outside-in signaling by $\alpha_D\beta_2$ regulates caspase-1 activation and/or downstream events in the pyroptosis pathway such as inflammasome assembly or processing of pro-IL-1 β and pro-IL-18 (28, 51). This novel possibility and the Gasdermin D participation remains to be further explored. Alternatively, increased numbers of bacteria in $\alpha_{D2}^{-/-}$ mice (Figures 2, 5A, and 7A; Figure S1 in Supplementary Material) may drive pyroptosis in this model. Pyroptosis is thought to limit bacterial replication and to be protective in infection by *Salmonella* and, potentially, other intracellular pathogens (26, 28–30, 52). Consistent with this interpretation, caspase-1-deficient mice are more susceptible to infection and to death when challenged with *Salmonella* (26, 30, 53, 54). Here again, a potential involvement of Gasdermin D needs to be better studied in our model, since Kambara et al. (55) showed that Gasdermin D deficiency unexpectedly and paradoxically augments host defenses against extracellular *E. coli* by delaying neutrophil death and by the increase in the host bactericidal activity to *E. coli* infection.

Nevertheless, while enhanced pyroptosis is predicted to have a protective effect (28–30, 52), this component appears to be outweighed by parallel defects in *Salmonella* elimination, with mechanisms yet to be established, in $\alpha_D^{-/-}$ animals. In this context, the enhanced release of cytokines by infected macrophages (Figures 4–6)—a key feature of pyroptosis (26, 28, 29)—may contribute to systemic inflammatory injury and to accelerated mortality in $\alpha_D^{-/-}$ animals, as we reported previously (13). Of note, we should say that increased cytokine production in $\alpha_D^{-/-}$ mice infected with *S. Typhimurium* could also be a consequence of the increased bacteria burden observed in those animals. Excessive, unregulated generation of pro-inflammatory cytokines is a central pathophysiologic mechanism in sepsis and other lethal systemic infectious and non-infectious syndromes (56, 57).

The potential for $\alpha_D\beta_2$ to deliver outside-in signals to pathways that regulate cytokine synthesis in multiple leukocyte types adds to the complexity of the phenotype of $\alpha_D^{-/-}$ animals infected by *S. Typhimurium* or other pathogens.

Our observations indicate previously unrecognized activities of integrin $\alpha_D\beta_2$ and reveal additional contributions of leukocyte integrins to the host response to experimental *S. Typhimurium* challenge. Infection with *S. Typhimurium* and other nontyphoidal *Salmonella* strains are major causes of invasive infection and bacteremia in humans (22, 58, 59). Thus, our identification of previously unrecognized activities of integrin $\alpha_D\beta_2$

in experimental *S. Typhimurium* infection may have clinical relevance and lead to new mechanistic insights in human disease. In addition, we have found evidence for differential activities of $\alpha_D\beta_2$ in experimental infections by *Salmonella* (this study) and malarial parasites (13), indicating that this leukocyte integrin has complex roles in host defense against invading pathogens. Further studies of these features of integrin $\alpha_D\beta_2$ in models of infection may yield mechanistic information with translational relevance.

ETHICS STATEMENT

The animals were kept at constant temperature (25°C) with free access to food and water in a room with a 12-h light/dark cycle. The protocols employed in this work were approved by the Oswaldo Cruz Foundation Animal Welfare Committee under the license number 0011-00.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DN, GZ, and HC-F-N. Performed the experiments: DN, AV-d-A, and AA. Analyzed the data: DN, HC-F-N, PB, and GZ. Contributed reagents/materials/analysis tools: HC-F-N, PB, and GZ. Wrote the paper: DN, GZ, and HC-F-N.

ACKNOWLEDGMENTS

This work was supported by PROEP-IOC, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) of Brazil. GZ's work is supported in part by an MERIT award from the National Institutes of Health (R37HL044525) and a Ciência Sem Fronteiras Special Visiting Professorship from the Brazilian Ministry of Science, Technology and Innovation. The authors thank Alexandra Greer for excellent contributions to preparation of the manuscript, and our colleagues and students for helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01128/full#supplementary-material>.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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