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The expression of integrin $\alpha_D\beta_2$ (CD11d/CD18) on neutrophils orchestrates the defense mechanism against endotoxemia and sepsis

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Abstract

Neutrophil-macrophage interplay is a fine-tuning mechanism that regulates the innate immune response during infection and inflammation. Cell surface receptors play an essential role in neutrophil and macrophage functions. The same receptor can provide different outcomes within diverse leukocyte subsets in different inflammatory conditions. Understanding the variety of responses mediated by one receptor is critical for the development of anti-inflammatory treatments. In this study, we evaluated the role of a leukocyte adhesive receptor, integrin $\alpha_D\beta_2$, in the development of acute inflammation. $\alpha_D\beta_2$ is mostly expressed on macrophages and contributes to the development of chronic inflammation. In contrast, we found that α_D-knockout dramatically increases mortality in the cecal ligation and puncture sepsis model and LPS-induced endotoxemia. This pathologic outcome of α_D-deficient mice is associated with a reduced number of monocyte-derived macrophages and an increased number of neutrophils in their lungs. However, the tracking of adoptively transferred fluorescently labeled wild-type (WT) and $\alpha_D^{-/-}$ monocytes in WT mice during endotoxemia demonstrated only a moderate difference between the recruitment of these two subsets. Moreover, the rescue experiment, using i.v. injection of WT monocytes to α_D-deficient mice followed by LPS challenge, showed only slightly reduced mortality. Surprisingly, the injection of WT neutrophils to the bloodstream of $\alpha_D^{-/-}$ mice markedly increased migration of

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

monocyte-derived macrophage to lungs and dramatically improves survival. α_D-deficient neutrophils demonstrate increased necrosis/pyroptosis. $\alpha_D\beta_2$-mediated macrophage accumulation in the lungs promotes efferocytosis that reduced mortality. Hence, integrin $\alpha_D\beta_2$ implements a complex defense mechanism during endotoxemia, which is mediated by macrophages via a neutrophil-dependent pathway.

Keywords

CD11d/CD18; endotoxemia; inflammation; integrin $\alpha D\beta 2$; macrophages; neutrophils; sepsis

1 | INTRODUCTION

The regulation of the inflammatory response is an essential mechanism for maintaining homeostasis, host defense, and survival.[1,2] Neutrophils and monocytes/macrophages are the major subsets of leukocytes that are involved in innate immune regulation.[3,4] Proinflammatory and anti-inflammatory stimuli, which are generated by neutrophils and macrophages, may have protective as well as pathologic outcomes, depending on the source of inflammation, stage of the disease, and its severity.[5,6] The critical molecules, that determine all major immune cell functions and the direction of inflammatory response, are various surface receptors expressed on neutrophils and macrophages.[7] Stimulation or inhibition of particular receptors is a promising approach in the development of new and novel treatments.[8,9]

Our research is focused on the evaluation of leukocyte receptor integrin $\alpha_D\beta_2$ and its role in the development of inflammation. $\alpha_D\beta_2$ (CD11d/CD18) belongs to the family of integrins, which are ubiquitous α/β heterodimeric adhesion receptors that mediate cell-cell and cell-extracellular matrix (ECM) interactions and transduce signals in and out of the cell.[10] Integrin $\alpha_D\beta_2$ is the most recently discovered leukocyte integrin and our understanding of its function is limited. $\alpha_D\beta_2$ demonstrates a low/moderate expression on circulatory neutrophils and monocytes,[11,12] but significantly up-regulates on proinflammatory (M1-like) macrophages in the inflamed tissue.[13,14] The unique expression of $\alpha_D\beta_2$ on myeloid leukocytes make it a promising target in the regulation of innate inflammatory response.

We recently demonstrated that integrin $\alpha_D\beta_2$ contributes to the development of chronic inflammation, particularly during atherosclerosis and diabetes.[13,14] Its major function is related to accumulation of M1 macrophages in the inflamed tissue, preventing the resolution of inflammation. The outcome is based on the significant up-regulation of $\alpha_D\beta_2$ on proinflammatory macrophages that dramatically increases macrophage adhesion and inhibits migration.[14] These data suggest a proinflammatory role for $\alpha_D\beta_2$ during chronic inflammatory diseases. In addition, the pathologic role of $\alpha_D\beta_2$ was demonstrated during spinal cord injury,[15,16] brain injury,[17] and *P. berghei* malaria infection[18,19] .

In our current paper, we examined the contribution of $\alpha_D\beta_2$ to LPS-induced endotoxemia and sepsis. Sepsis combines systemic inflammatory response with immune dysregulation, leading to tissue damage and multiple organ failure.[20,21] Cytokine storm is thought to be one of the major events that promote endotoxemia/sepsis severity.[22] Accordingly, the recruitment

and activation of neutrophils and monocytes/macrophages, the major sources of cytokines in the damaged organs, plays a critical role in pathologic outcomes during sepsis. Several groups, including ours, have demonstrated that $\alpha_D\beta_2$ signaling may generate the expression of proinflammatory cytokines during inflammatory diseases.[13,18,23] Taken together with the contribution of $\alpha_D\beta_2$ to macrophage retention,[14] these observations would suggest a pathologic role of $\alpha_D\beta_2$ during endotoxemia and sepsis. However, this is not the case. Surprisingly, we discovered that $\alpha_D\beta_2$ has a significant protective effect during these acute inflammatory diseases. Moreover, we found that $\alpha_D\beta_2$ expression on neutrophils, not macrophages, is the most critical for survival. Therefore, we propose that neutrophils have a protective component during sepsis and endotoxemia and this protective contribution is or chestrated via integrin $\alpha_D\beta_2$.

2 | MATERIAL AND METHODS

2.1 | Reagents and antibodies

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). LPS from *Escherichia coli* O55:B5 was purchased from Sigma-Aldrich. Antibodies against Ly6G (clone 1A8), Ly6C (clone HK1.4), CD11b (clone M1/70), F4/80 (clone BM8), and Siglec F (clone 1RNM44N) were purchased from eBioscience (currently part of Thermo Fisher Scientific). Polyclonal antibody against the α_D I-domain was described previously.[24,25] The antibody recognizes both human and mouse α_D I-domains and has no crossreactivity with recombinant human and mouse α_M, α_X, and α_L I-domains.The antibody was isolated from rabbit serum by affinity chromatography using α_D I-domain-Sepharose.

2.2 | Animals

Wild-type (WT; C57BL/6J, stock #000664) and integrin α_D-deficient (B6.129S7-*Itgad*^{tm1Bll}/J, stock #005258) mice were bought from Jackson Laboratory (Bar Harbor, ME, USA). α_D-deficient mice have been backcrossed to C57BL/6 for at least 10 generations. All procedures were performed according to animal protocols approved by East Tennessee State University IACUC.

2.3 | Cecal ligation and puncture (CLP)

Male mice that were 16 wk old underwent CLP to induce polymicrobial sepsis as previously described.[26] The cecum was exteriorized, the contents were massaged distally, and half of cecum was ligated distal to the ileocecal junction. The cecum was punctured once with a 25 gauge needle in an avascular region near the distal end and a bleb of cecal contents was extruded from the puncture. Mice were recovered with 1 ml warmed saline. Mice were followed for survival and analyzed by Kaplan-Meier method. Sham surgery (laparotomy alone) mice were used as a control for surgery and anesthesia.

2.4 | LPS-induced endotoxemia

Male WT and $\alpha_D^{-/-}$ mice were injected with sublethal dose of LPS (10–15 μg/g of body weight depending on LPS batch). The survival rate was determined by Kaplan-Meier method. The severity of pathologic conditions in LPS-induced endotoxemia correlates with

the significant drop of temperature during the first 24 h after the treatment, which was more significant (23–25°C in sublethal conditions) when compared to the CLP model (32–33°C). We examined temperature as an additional clinical parameter during nonlethal experiments.

2.5 | Isolation of peritoneal neutrophils

Peritoneal neutrophils from 8 to 12 wk old mice (WT and α_D deficient) were harvested by lavage of the peritoneal cavity with 5 ml of sterile PBS 4 h after i.p. injection of 1 ml 4% thioglycollate. The cells were counted and analyzed by flow cytometry.

2.6 | Isolation of monocyte and neutrophils from the bone marrow of mice

Monocytes and neutrophils were isolated from the bone marrow of WT and α_D-deficient mice using magnetic bead separation kits for the negative selection of neutrophils and monocytes according to manufacture manual (Miltenyi Biotec, Gaithersburg, MD, USA). Purified cells were analyzed by flow cytometry using antibodies to CD11b, Ly6G, and Ly6C. The purity for both populations was 90–93%.

2.7 | Adoptive transfer of monocytes and neutrophils in LPS-induced endotoxemia

This approach is based on a previously published method[13,14,27] with some modifications. Monocytes and neutrophils were isolated from the bone marrow of WT and α_D-deficient mice and labeled with red, PKH26 (WT), or green, PKH67 ($\alpha_D{}^{-/-}$) fluorescent dyes. A total of 1×10^6 red and 1×10^6 green monocytes (or neutrophils) were mixed together in the equal numbers and injected into the tail veins of WT C57BL6 mice, which were injected with sublethal concentration of LPS immediately after adoptive transfer. After different time points, mice were sacrificed and perfused with PBS. Spleen, liver, and lung were isolated and digested with Collagenase IV 2 mg/ml (Worthington) and DNase I 50 units/ml (Sigma-Aldrich). Obtained cell solution was precleaned by filtration via cell strainer (70 μm mesh size) and analyzed using FACS (Fortessa X-20, BD, Becton Dickinson, Franklin Lakes, NJ, USA) and imaging flow cytometry (ImageStream Mark II, Amnis, Seattle, WA, USA).

For the rescue experiments WT or α_D-deficient neutrophils or monocytes were isolated from bone marrow as described earlier. Freshly isolated cells (only WT or $\alpha_D{}^{-/-}$) were injected in tail vein of WT or $\alpha_D{}^{-/-}$ mice immediately before the injection of sublethal dose of LPS. The survival rate was monitored by Kaplan-Meier method.

2.8 | Flow cytometry and imaging flow cytometry analyses

Flow cytometry analysis was performed to assess the expression of integrin α_D on mouse immune cells to detect neutrophils and macrophages in tissue, to evaluate the level of neutrophil apoptosis, and to determine PKH26 and PKH67-fluorescently labeled cells in digested liver, spleen, and lungs.

For $\alpha_D\beta_2$ expression, cells were harvested and pre-incubated with Fc-Blocking solution (eBioscience) for 30 min at 4°C, then 2×10^6 cells were incubated with anti-Ly6G/PE, anti-CD11b/FITC and anti-α_D polyclonal antibody for 30 min at 4°C, followed incubation with Alexa 633 or anti-mouse IgG (at a 1:1000 dilution) for 30 min at 4°C. Finally, the cells were washed and analyzed using a Fortessa X-20 (Becton Dickinson). For the apoptosis assay,

cells were incubated with Annexin V and propidium iodide according to manual (Annexin apoptosis kit, Invitrogen, Carlsbad, CA, USA). Pyroptosis was detected using FAM-FLICA-caspase-1 kit according to protocol (Bio-Rad, Hercules, CA, USA).

To detect neutrophils and macrophages in the tissue, lungs were digested with Collagenase IV 2 mg/ml (Worthington Biochemical corporation, Lakewood, NJ, USA) and DNase I 50 units/ml (Sigma-Aldrich). Obtained cell solution was precleaned by filtration via cell strainer (70 μm mesh size). Cells were pre-incubated with Fc-Blocking solution (eBioscience), stained with live/dead reagent, and incubated with a mix of antibodies: anti-Ly6G/PE, anti-CD11b/PE-Cy7, anti-F4/80/APC, and Seglec F/FITC (for some experiments). In another design, mice were injected with PKH67-labeled neutrophils before the LPS treatment. In these experiments, the analysis was performed on PKH67-positive subpopulation. To track neutrophils and monocytes migration, the digested cell solution from the lung, spleen, and liver, which were obtained after the adoptive transfer of PKH26-, PKH67-labeled neutrophils or monocytes, were analyzed by flow cytometry (Fortessa X-20) and imaging flow cytometry (ImageStream Mark II, Amnis). To determine macrophage differentiation, the samples were incubated with anti-F4/80 antibody conjugated to Alexa 633.

Imaging flow cytometry was performed using ImageStream Mark II instrument (Amnis). The results were analyzed applying IDEAS 6.2 software. The PKH26 and PKH67 labeled cells were detected on channels 2 and 3, correspondingly. The phagocytosis of PKH67-labeled cells in lungs was determined using Internalization wizard of IDEAS 6.2 software.

2.9 | Cytokine expression in blood

The blood was collected from WT and α_D-deficient mice 2 or 24 h after LPS injection. Plasma was isolated by centrifugation. The pooled plasma samples from five mice per group were evaluated using a mouse Inflammation antibody array (RayBiothech, Inc. Peachtree Corners, GA, USA) according to the kit manual. The membrane identification chart is shown in manual: https://doc.raybiotech.com/pdf/Manual/AAM-INF-1.pdf

2.10 | Phagocytosis and efferocytosis

Phagocytosis of *E. coli* by neutrophils and macrophages was evaluated using pHrodo-conjugated Red *E.coli* bio particles according to the manual. Briefly, WT and $\alpha_D^{-/-}$ neutrophils or macrophages were plated in 96-plates (1×10^5 cells/well). pHrodo-conjugated *E. coli* were added to the wells (100 μm/well) for 2–4 h. The phagocytosis was detected by fluorometer or fluorescent microscope and visualized by time-lapse video recording.

In vitro efferocytosis of PKH67-labeled neutrophils by PKH26 labeled macrophages was assessed by fluorescent microscopy as described previously.[28] Briefly, peritoneal macrophages were isolated from the peritoneal cavity at 72 h after the administration of thioglycollate. Macrophages were plated on cover slips in 12-well palates in RPMI 1640 media supplemented with penicillin and streptomycin. Nonadherent cells were washed out after 2 h. Peritoneal neutrophils were activated with 30 ng/ml PMA and 0.1 mM H_2O_2 to stimulate apoptosis/necrosis as described previously.[29] A total of 1×10^6/well neutrophils were added to macrophages and incubated overnight. The efferocytosis was detected by

fluorescent microscopy using EVOS Cell Imaging system and analyzed by Image Analysis Software (EVOS, Thermo Fisher Scientific).

In vivo efferocytosis of PKH67-labeled neutrophils in lungs after LPS treatment was evaluated using Imaging flow cytometry (Amnis) and analyzed using IDEA 6.2 software, internalization wizard.

2.11 | Statistical analysis

Statistical analyses were performed using Student's *t*-test, Student's paired *t*-tests, or Kaplan-Meier test using SigmaPlot 13. A value of $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | Integrin α_D-deficient mice have increased mortality in both CLP-induced sepsis and LPS-induced endotoxemia models

The contribution of $\alpha_D\beta_2$ to acute inflammation was tested in the CLP model of sepsis and LPS-induced endotoxemia, which are two clinically relevant models of polymicrobial sepsis.[30] Both $\alpha_D^{-/-}$ and WT mice underwent CLP surgery and the survival rate was monitored for 7 d (Fig. 1A). The mortality of $\alpha_D^{-/-}$ mice (vs. WT control) was significantly increased (survival 66.67% [WT] vs. 20% [$\alpha_D^{-/-}$]). Integrin $\alpha_D\beta_2$ is expressed exclusively on leukocytes, particularly on myeloid subsets; therefore $\alpha_D\beta_2$-dependent *E. coli* phagocytosis may contribute to the protective effect of $\alpha_D\beta_2$. However, we did not find any difference in *E. coli* phagocytosis between WT and α_D-deficient neutrophils and macrophages (Supporting Information Fig. S1). Accordingly, we hypothesized that the mechanism of the protective effect of $\alpha_D\beta_2$ is *E. coli* phagocytosis independent. To verify our results, we tested α_D-deficient mice in a model of sterile infection, LPS-induced endotoxemia (Fig. 1B). The injection of a sublethal dose of LPS demonstrated a similar pattern, as α_D deficiency significantly reduced survival (73% [WT] vs. 27% [$\alpha_D^{-/-}$]). The mortality rate in this model strongly correlates with the drop in body temperature. The reduction in temperature to 24–25°C at 18 h after LPS injection leads to lethality in 95% of mice. Therefore, the drop of body temperature during endotoxemia serves as an additional pathologic parameter. When taken together, these data indicate that $\alpha_D\beta_2$ integrin plays a protective role in CLP sepsis and LPS induced endotoxemia.

3.2 | Cytokine levels in the blood of $\alpha_D^{-/-}$ mice are not changed in response to endotoxemia

Cytokine storm is one of the major events associated with mortality during endotoxemia. We tested how α_D deficiency affects proinflammatory cytokine secretion during the first day after the injection of a sublethal dose of LPS. Blood was isolated from WT and $\alpha_D^{-/-}$ mice 2 and 24 h after treatment. The development of endotoxemia was verified by the drop of body temperature in both groups. The level of cytokines was evaluated using the mouse inflammation antibody array (RayBiotech, Inc.) Cytokine expression was not significantly changed in α_D-deficient mice (Supporting Information Fig. S2). The most marked change was detected in the expression of IL-12, which was increased in $\alpha_D^{-/-}$ mice 1.7-fold at 24 h.

Based on these data we concluded that cytokine expression during the first 24 h is not significantly modified by α_D deficiency.

3.3 | The number of macrophages in lungs of α_D-deficient mice is dramatically decreased during endotoxemia

Our previous results demonstrated that integrin $\alpha_D\beta_2$ is involved in regulation of macrophage migration and accumulation at the site of chronic inflammation.[13] The recruitment of neutrophils and monocytes to the internal organs, particularly lungs, is a hallmark of the inflammatory response during LPS-induced endotoxemia and sepsis. Using flow cytometry analysis, we evaluated neutrophils and macrophage distribution in lungs at 48 h after LPS injection. We detected neutrophils as $CD11b^+/Ly6G^+/F4/80^-$ cells, monocyte-derived macrophages were identified as $CD11b^+/Ly6G^-/F4/80^+$ cells, and resident alveolar macrophages as $CD11b^-/SeglecF^+/Ly6G^-/F4/80^+$ cells. According to the previously published results, the number of resident alveolar macrophages is reduced during inflammation[31] and monocyte-derived macrophages represent the majority of the macrophage population in lungs. We found that at 48 h after LPS-injection the number of monocyte-derived macrophages in lungs of α_D-deficient mice is dramatically reduced (5-fold), whereas the number of neutrophils was slightly increased (~1.8-fold) in comparison to WT mice (Fig. 2). Based on these results, we hypothesized that $\alpha_D\beta_2$ expression on macrophages is required for macrophage migration/accumulation and related defense mechanisms.

However, the direct tracking of monocyte recruitment from blood to the lungs did not confirm this hypothesis. We tested the accumulation of WT and $\alpha_D^{-/-}$ macrophages in lungs and other organs using the adoptive transfer approach. For this purpose, fluorescently labeled WT (PKH26, red dye) and $\alpha_D^{-/-}$ (PKH67, green dye) monocytes were mixed in equal amounts and injected in tail veins of WT mice 20 min before the injection of LPS (Fig. 3A). The recruitment of adoptively transferred cells was evaluated in lungs, livers, and spleens after different time points using flow cytometry. Our result demonstrated 1.5–2-fold decrease in the number of α_D-deficient monocyte-derived macrophages in lungs and other organs after LPS treatment (Fig. 3B and C). We verified our results using imaging flow cytometry (Fig. 3D). Moreover, we showed that i.v. injected fluorescently labeled monocytes differentiate to macrophages after the recruitment to the tissue, because tissue isolated fluorescently labeled cells have increased expression of macrophage marker, F4/80.[32] These data demonstrate that $\alpha_D\beta_2$ have some regulatory mechanism on macrophage migration after LPS treatment. However, because the direct analysis of lungs isolated from WT and $\alpha_D^{-/-}$ mice demonstrated a 5-fold difference in monocyte-derived macrophage numbers (Fig. 2), we concluded that $\alpha_D\beta_2$ expression on monocyte/macrophages does not represent a critical mechanism that regulates macrophage accumulation in lungs during endotoxemia.

3.4 | α_D deficiency does not affect neutrophil migration to the lungs during endotoxemia

The analysis of leukocyte distribution in the lungs (Fig. 2) demonstrates a slight increase in the number of neutrophils in α_D-deficient mice. It has been reported previously that integrin $\alpha_D\beta_2$ has some level of expression on neutrophils.[11] Because $\alpha_D\beta_2$ interacts with VCAM-1,[33] which is a principal adhesive receptor on endothelial cells, we tested whether

$\alpha_D\beta_2$ deficiency can modify neutrophil recruitment to the tissue during endotoxemia. Neutrophils were isolated from WT and $\alpha_D^{-/-}$ mice and labeled with PKH26 and PKH67 fluorescent dyes, correspondingly. WT and $\alpha_D^{-/-}$ neutrophils were mixed in equal amount and injected i.v. to WT mice followed by injection of sublethal doses of LPS. We found, that α_D deficiency resulted in a tendency for reduced numbers of neutrophils in lung and spleen at 44 h after LPS injection (Fig. 4A and B). These results do not concur with neutrophil distribution in lungs of α_D-deficient mice (Fig. 2) and demonstrate that neutrophil accumulation in lungs is regulated not only by direct migration, but also via some additional mechanism.

Therefore, we found a controversy in the analysis of $\alpha_D^{-/-}$ monocyte and neutrophil recruitment from circulation and their distribution in lungs during endotoxemia.

3.5 | The adoptive transfer of WT neutrophils dramatically improves the survival of $\alpha_D^{-/-}$ mice during endotoxemia

To clarify the role of $\alpha_D\beta_2$ on leukocyte subsets during endotoxemia, we performed rescue experiments using the adoptively transferred monocytes (Fig. 5) and neutrophils (Fig. 6). The experimental design is shown in Figure 5A. WT and $\alpha_D^{-/-}$ monocytes were isolated by Miltenyi Biotec kit and purity was assessed as 90–93% using flow cytometry with anti-CD11b and anti-Ly6C antibodies (Fig. 5B, left panel). Because Ly6C is also expressed on a subset of neutrophils, we assessed neutrophil contamination using anti-Ly6G antibody and found less than 5% $Ly6G^+$ cells (Fig. 5B, right panel). WT and α_D-deficient mice were injected with either WT or $\alpha_D^{-/-}$ monocytes (10^6 cells/mice) followed by injection of LPS. The body temperature of mice and survival rate were monitored. We found that injection of WT or $\alpha_D^{-/-}$ monocytes did not significantly change the temperature or mortality of WT recipient mice (Fig. 5C and D). In contrast, the injection of WT monocytes to $\alpha_D^{-/-}$ mice increased body temperature after LPS treatment and slightly improved survival (Fig. 5E and F; survival 70% [injected-WT] vs. 50% [injected $\alpha_D^{-/-}$]). According to these results, $\alpha_D\beta_2$ expression on macrophages has some protective effect. However, based on the survival rate for WT and $\alpha_D^{-/-}$ mice (Fig. 1), we concluded that $\alpha_D\beta_2$ expression on macrophages is not a critical factor for $\alpha_D\beta_2$-mediated defense mechanism during sepsis development.

To test a potential role of $\alpha_D\beta_2$ in neutrophil-mediated effects during endotoxemia, we performed another rescue experiment (Fig. 6A), and injected WT and $\alpha_D^{-/-}$ neutrophils (Fig. 6B) to WT or α_D-deficient mice. The purity and activation stage of isolated neutrophils were evaluated before injection. More than 90% of WT and $\alpha_D^{-/-}$ cells were Ly6G/CD11b double-positive, which corresponds to neutrophil phenotype. Isolated cells demonstrate round morphology, and there is no sight of aggregation or NET formation. The level of CD11b expression is a well characterized marker of neutrophil activation.[34-36] The aliquot of isolated neuropils was treated with 100 nM PMA that led to 5-fold increase of CD11b surface expression in 15 min (Supporting Information Fig. S3A) that corresponds to previously described dynamic of neutrophil activation.[34] Based on these data, we concluded that we use purified and nonactivated population of neutrophils for the adoptive transfer.

We did not detect any difference in the survival of WT mice injected with WT or $\alpha_D^{-/-}$ neutrophils (Fig. 6C and D). However, surprisingly, the adoptive transfer of WT neutrophils

dramatically improved the survival of α_D-deficient mice when compared to mice injected with $\alpha_D^{-/-}$ neutrophils (70% [injected WT] vs. 10% [injected $\alpha_D^{-/-}$]) or without any adoptive transfer (Fig. 6F). The dramatic change in the survival rate was also confirmed by significant increase in body temperature of $\alpha_D^{-/-}$ mice injected with WT neutrophils (Fig. 6E).

To verify the quality of adoptively transferred neutrophils, we compared CD11b expression on recruited endogenous and donor neutrophils, which were isolated from the mouse lungs at 48 h after the development of endotoxemia. We found similar levels of CD11b up-regulation on both neutrophil subsets (approximately 10-fold increase to compare with nonactivated neutrophils) (Supporting Information Fig. S3B). Therefore, adoptively transferred neutrophils have similar characteristics with neutrophils from the recipient mice.

Based on the obtained result, we tested if adoptive transfer of WT neutrophils to α_D-deficient mice at 6 h after LPS injection still has a protective effect. We selected time point 6 h, because mice started to develop pathophysiologic conditions, namely, the body temperature dropped and animal activity was markedly reduced. We found that adoptive transfer at this time point did not significantly improve the survival of α_D-deficient mice (Supporting Information Fig. S4). Therefore, the initial presence of $\alpha_D\beta_2$ on neutrophils is critical for the protective mechanism.

3.6 | Integrin $\alpha_D\beta_2$ is up-regulated on neutrophils during endotoxemia and prevents neutrophil necrosis and pyroptosis

To evaluate a potential mechanism of $\alpha_D\beta_2$ function on neutrophils, we tested $\alpha_D\beta_2$ expression during endotoxemia. The expression of $\alpha_D\beta_2$ on neutrophil progenitors is minimal and is only slightly increased on nonactivated neutrophils in circulation.[11] However, the blood neutrophils isolated from mice at 24 h after LPS-induced endotoxemia revealed a 3-fold increase in $\alpha_D\beta_2$ expression (Fig. 7A, Supporting Information Fig. S5A). A similar increase in $\alpha_D\beta_2$ expression was detected on neutrophils incubated in vitro in the presence of 100 ng/ml LPS, thus demonstrating the direct effect of LPS on $\alpha_D\beta_2$ expression (Supporting Information Fig. S5B). Interestingly, the level of $\alpha_D\beta_2$ on monocytes after LPS-induced endotoxemia was not markedly increased (data not shown), which shows a granulocyte-specific effect of $\alpha_D\beta_2$ to endotoxin. Therefore, despite the absence of $\alpha_D\beta_2$ role in neutrophil migration, we found that inflammation, and particularly LPS treatment, significantly increases expression on neutrophils, which contributes to protective effects of $\alpha_D\beta_2$.

Apoptosis, pyroptosis and necrosis are important characteristics during sepsis development that can be regulated via integrin signaling. We evaluated the role of $\alpha_D\beta_2$ in the survival of neutrophils during endotoxemia. WT and $\alpha_D^{-/-}$ neutrophils were incubated 24 h in vitro with or without LPS and necrosis/apoptosis of cells were detected with Annexin V kit by flow cytometry (Fig. 7B). The number of early apoptotic cells was not changed in α_D-deficient samples. However, the percentage of necrotic cells was dramatically increased in α_D-deficient neutrophils, particularly after LPS treatment. Using caspase-1 activity assay, we found that early pyroptosis was also significantly increased in α_D-deficient neutrophils after LPS treatment.

Excessive neutrophil pyroptosis is harmful in the early hyperinflammatory state in sepsis. Necrotic neutrophils are the source of secondary damage and should be removed from the tissue by macrophages. However, our data demonstrate (Fig. 2) that the number of macrophages in lungs of $\alpha_D^{-/-}$ mice is decreased in comparison to WT mice, which complicates the normal efferocytosis of dead cells.

3.7 | Integrin $\alpha_D\beta_2$ on neutrophils stimulate the recruitment of monocytes/macrophages to the lungs

To further understand the protective mechanism of $\alpha_D\beta_2$ on neutrophils, we tested how the adoptive transfer of neutrophils, which improves survival (Fig. 6), modifies leukocyte deposition in inflamed lungs. WT or $\alpha_D^{-/-}$ neutrophils were injected in α_D-deficient mice as described earlier (Fig. 6) and lungs were perfused, isolated, and digested. The leukocyte subsets were detected in lungs using flow cytometry by applying the gating strategy described earlier. To our surprise, we found that injection of WT neutrophils to α_D-deficient mice significantly increased the number of macrophages in lungs (Fig. 8) and reduced the number of neutrophils. At the same time, the injection of $\alpha_D^{-/-}$ neutrophils had no effect.This result provides a molecular mechanism for neutrophil rescue experiments (Fig. 6) and proposes the efferocytosis of necrotic neutrophils as a critical component for the survival mechanism, which is mediated by $\alpha_D\beta_2$.

Notably, the in vitro uptake of necrotic/apoptotic neutrophils by WT and α_D-deficient macrophages was similar (Fig. 8C), which indicates a crucial role of macrophage amount for the protective efferocytosis. To test this hypothesis, we evaluated the efferocytosis in vivo, by analyzing a fate of fluorescently labeled adoptively transferred neutrophils in α_D-deficient mice (Fig. 9). WT and $\alpha_D^{-/-}$ neutrophils were labeled with PKH67 (green) before the adoptive transfer. Mice were injected with neutrophils and a sublethal dose of LPS. After 48 h, lungs were digested, and isolated cells were labeled with anti-CD11b, anti-Ly6G, and anti-F4/80 antibodies. We selected green-positive events and evaluated the expression of neutrophil (Ly6G) and macrophage markers (F4/80) on these cells (Fig. 9B). Interestingly, we found that more than 30% of cells in lung sample injected with WT neutrophils are F4/80 positive, whereas this subpopulation is absent in the lungs of mice injected with α_D-deficient neutrophils. Because the purity of injected PKH67 labeled neutrophils was 90% (Fig. 6B), we hypothesized that green F4/80 positive cells represent macrophages that engulfed PKH67-labeled neutrophils. To test our hypothesis, we analyzed the same samples using imaging flow cytometry (Fig. 9C). We selected PKH67-positive events and evaluated individual cells in $Ly6G^+$ quadrant (Q1) and F4/80-positive quadrant (Q4). We found a dramatic difference in morphology of cells and green signal distribution. Cells in Q1 (Fig. 9C, upper panel) are smaller, with positive Ly6G marker and high expression of CD11b, which corresponds to the phenotype of activated neutrophils. Cells in Q4 (Fig. 9C, lower panel) are larger, more granular, with strong expression of F4/80 and moderate expression of CD11b, which corresponds to macrophage phenotype. Most importantly, the green signal in F4/80-positive cells is located intracellularly and does not cover the entire cell footprint. These distributions confirm the efferocytosis of dead neutrophils by macrophages. We also analyzed double-positive cells (Q2) (Fig. 9C, middle panel) and found cell complexes that consist of macrophages and neutrophils. The images (doubles) represent macrophage, which

engulfed an adoptively transferred PKH67-labeled neutrophil and attached to another neutrophil from the recipient mouse (PKH67-negative). Therefore, we demonstrated the efferocytosis of neutrophils by macrophages in our model of endotoxemia.

4 | DISCUSSION

The members of the integrin family are involved in different functions during pathophysiologic development.[13,19,37,38] The most important and well-known function of integrins is a contribution to cell adhesion and migration. Leukocyte integrins are responsible for the transmigration of immune cells through the endothelial monolayer, migration via ECM and retention within inflamed tissues.[39] However, integrins also participate in a variety of other cell responses during the activation/inhibition of different signaling pathways including apoptosis, proliferation, phagocytosis, and cell activation.[40-42] The specific outcome is regulated by the ligand-binding properties and specificity of expression on different types of cells.

In our previous projects, we have been focused on the contribution of $\alpha_D\beta_2$ to chronic inflammation, particularly during atherogenesis and diet-induced diabetes.[13,14,24] We found that α_D deficiency reduced atherogenesis by decreasing macrophage accumulation and lipid deposition in the vascular wall of atherosclerotic mice.[13] Similarly, macrophage numbers were diminished, and insulin sensitivity was increased in high fat diet-induced diabetic $\alpha_D^{-/-}$ mice.[14] We found that up-regulation of $\alpha_D\beta_2$ on M1-activated macrophages significantly increased macrophage adhesion, which inhibited macrophage migration, promoted retention of M1 macrophages, and prevented the resolution of inflammation. In contrast to chronic inflammation, the development of acute inflammatory response often resolves even before macrophage polarization. Several groups, including ours, have demonstrated that $\alpha_D\beta_2$ has a moderate expression on freshly differentiated macrophages and low expression on circulatory monocytes and neutrophils.[12,13,24,43] Accordingly, one would expect a minimal, if any, role of $\alpha_D\beta_2$ in acute inflammatory response. However, several independent studies demonstrated a pathologic role of $\alpha_D\beta_2$ during acute inflammation such as *P. berghei* malaria infection,[18,19] spinal cord injury,[15,16] and brain injury.[17] It has been shown that α_D deficiency during *P. berghei* malaria infection reduced leukocyte accumulation in the brain, decreased the concentration of inflammatory mediators and diminished cerebral edema.[18] In agreement with these data, the prevention of macrophage accumulation at the site of spinal cord or brain injuries using anti-α_D antibody demonstrates an improved outcome.[44,45] Taken together, the results in models of atherosclerosis, diabetes, malaria infection, and neurotrauma suggest a significant pathophysiologic role for $\alpha_D\beta_2$, which is associated with modulation of macrophage functions.

In contrast to these results, a moderate protective effect of $\alpha_D\beta_2$ was detected during *Salmonella typhimurium* infection.[46] Specifically, the authors showed slightly reduced survival of α_D-deficient mice after *Salmonella* infection, which correlated with an increased concentration of proinflammatory cytokines, enhanced macrophage pyroptosis and a reduced ability to combat Salmonella in $\alpha_D^{-/-}$ mice. Notably, the outcome again was associated with macrophage functions.

In our current study, we tested the role of integrin $\alpha_D\beta_2$ in two acute inflammatory models: LPS-induced endotoxemia (sterile sepsis) and CLP-induced polymicrobial sepsis. We found a similar protective effect of integrin $\alpha_D\beta_2$ in both models, because α_D deficiency significantly increased mortality. Most importantly, we established $\alpha_D\beta_2$-mediated interplay between neutrophils and macrophages, which is critical for the defense mechanism during acute inflammation.

The contribution of other β_2 integrins was previously tested in sepsis models, but the obtained results are limited and cannot be applied to generalize the contribution of specific subsets of leukocytes due to either wide distribution of a particular receptor or narrow ligand binding specificity.[8,47-49] Moreover, the divergence of obtained results demonstrates that α subunit, which is critical for ligand binding, regulates the response. Particularly integrin $\alpha_L\beta_2$, which is expressed on all subsets of leukocytes and interacts only with ICAM receptors, had a protective effect in the CLP model,[48] but a pathologic effect in LPS-induced shock.[50] Integrin $\alpha_X\beta_2$, the classical marker of dendritic cells (which is also expressed on other myeloid subsets), promotes LPS-mediated endotoxemia.[51] Integrin $\alpha_M\beta_2$, which is a major adhesive receptor on myeloid leukocytes, has a controversial outcome based on different reports. Liu et al. demonstrated the protective mechanism of $\alpha_M\beta_2$ in CLP model, because α_M deficiency results in increased mortality, more bacterial load, and apoptosis.[47] However, the results from other groups showed improved survival of WT mice in the presence of anti-α_M blocking antibodies in polymicrobial sepsis and LPS-mediated endotoxin shock,[52].[8] Notably, the protective role of neutrophils was not proposed or detected in any of these studies.

Our data demonstrate that adoptive transfer of WT (integrin $\alpha_D\beta_2$ positive) neutrophils significantly increased the survival of α_D-deficient mice during endotoxemia from 10% (injection of $\alpha_D^{-/-}$ neutrophils) to 70% (injection of WT neutrophils) (Fig. 6). Interestingly, the adoptive transfer of $\alpha_D^{-/-}$ neutrophils to WT mice does not increase mortality rate. Therefore, $\alpha_D\beta_2$-deficient neutrophils are not the source of pathologic signal itself. On the other hand, we found only a moderate contribution of adoptively transferred macrophages to the protective effect of $\alpha_D\beta_2$ during endotoxemia. Specifically, the injection of WT monocytes to $\alpha_D^{-/-}$ mice improved the survival rate from 50% (injection of $\alpha_D^{-/-}$ macrophages) to 70% (injection of WT macrophages) (Fig. 5).

We found that α_D deficiency did not significantly modify the migration of neutrophils (Fig. 4A and B). Hence, the protective effect of $\alpha_D\beta_2$ is not associated with altered migration/accumulation of neutrophils. These results correspond to the well-accepted concept that integrin $\alpha_L\beta_2$, $\alpha_4\beta_1$ and to some extent $\alpha_M\beta_2$, are important for transmigration of neutrophils.[7,53,54] Because neutrophils apply amoeboid movement in 3D ECM,[55] the role of $\alpha_D\beta_2$-mediated neutrophil migration in the tissue is also unlikely.

Neutrophils are the most abundant immune cells, which play the role of a double-edged sword in sepsis. While neutrophil-mediated phagocytosis is critical for microbial clearance, unbalanced cytokine production can initiate a cytokine storm leading to tissue damage and organ dysfunction. A number of studies demonstrate that regulation of neutrophil functions is the most critical part of sepsis treatment. Numerous publications propose a pathologic

contribution of neutrophils to sepsis.[52,56,57] Specifically, the blocking of neutrophil recruitment reduces sepsis development.[56,58] Another neutrophil-mediated effect is related to immunosuppressive functions, which are generated via regulation of T cell activation.[59] At the same time several authors provide evidence of protective outcomes mediated by neutrophils. Specifically, the depletion of neutrophils before CLP reduced survival rate.[60] Our adoptive transfer experiments demonstrated a similar outcome. The injection of neutrophils before the administration of LPS improves the survival rate, whereas the adoptive transfer at 6 h after LPS administration has no protective effect (Supporting Information Fig. S4).

We demonstrate a complex role of $\alpha_D\beta_2$ in neutrophil and macrophage functions during endotoxemia. Neutrophils are shortlived cells that undergo spontaneous apoptosis. In general, neutrophil apoptosis is considered to have a protective effect during sepsis development.[61] However, if the infection is serious enough, some neutrophils undergo necrosis or programmed necrosis, pyroptosis. Uncontrolled release of toxic substances from the dead neutrophils can propagate the inflammatory response leading to tissue damage and sepsis development.[62,63] We did not detect the change in apoptosis of $\alpha_D^{-/-}$ neutrophils. However, we found that α_D deficiency increases necrosis and particularly pyroptosis of neutrophils (Fig. 7B) that can contribute to the increased mortality of α_D-deficient mice during endotoxemia. The increased pyroptosis was previously detected in α_D-deficient macrophages during *Salmonella typhimurium* infection.[46] Apparently, neutrophils and macrophages have a similar $\alpha_D\beta_2$-related molecular mechanism for the prevention of pyroptosis during inflammation. Notably, the up-regulation of $\alpha_D\beta_2$ on neutrophils in circulation during endotoxemia and after LPS treatment in vitro supports our conclusion regarding the importance of $\alpha_D\beta_2$ in neutrophil functions (Fig. 7A).

Most importantly, we revealed that α_D deficiency dramatically decreases the number of monocyte-derived macrophages and increases the number of neutrophils in the lungs. The injection of WT neutrophils to $\alpha_D\beta_2^{-/-}$ mice restores the leukocyte balance, particularly it significantly increases the number of macrophages in the lungs (Fig. 8). Therefore, $\alpha_D\beta_2$-dependent neutrophil response promotes macrophage migration and a macrophage-mediated protective mechanism. The presence of macrophages in tissue is critical for the efferocytosis of apoptotic/necrotic neutrophils, which is essential for the resolution of acute inflammation and improving of an organism's survival during endotoxemia and sepsis. Notably, the recruitment of $\alpha_D^{-/-}$ neutrophils was not diminished compared to WT cells; therefore the augmented number of neutrophils in α_D-knockout lungs is related to unpaired efferocytosis. We demonstrated that $\alpha_D\beta_2$-mediated macrophage accumulation in the lungs promotes efferocytosis, which is markedly decreased in $\alpha_D^{-/-}$ mice, but restored after the injection of WT neutrophils to α_D-deficient mice (Fig. 9). We suggest that $\alpha_D\beta_2$ on neutrophils does not directly affect neutrophil uptake by macrophages, but regulates the macrophage number in tissue. This conclusion is supported by the similar efferocytosis of WT and $\alpha_D^{-/-}$ macrophages in vitro (Fig. 8C). The administration of LPS and CLP surgery are two popular models, which mimic different aspects of sepsis development in mice. Despite a significant difference in pathologic mechanisms, the survival outcome in both models depends on reduced cell necrosis and improved macrophages efferocytosis. Therefore, we propose that

increased mortality rate of α_D-deficient mice in LPS-induced endotoxemia and CLP sepsis (Fig. 1) are regulated via a similar mechanism.

When considered as a whole, our data indicate a significant protective role of $\alpha_D\beta_2$ on the surface of neutrophils during sepsis development, which is manifested in prevention of neutrophil necrosis/pyroptosis and promotion of macrophage migration/accumulation. Further studies are required to understand the link between macrophage accumulation and $\alpha_D\beta_2$ function on neutrophils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CLP	cecal ligation and puncture
ECM	extracellular matrix
WT	wild-type.

REFERENCES

1. Eming SA, Wynn TA, Martin P. Inflammation and metabolism in tissue repair and regeneration. Science. 2017;356(6342):1026–1030. [PubMed: 28596335]
2. Libby P Inflammation in atherosclerosis. Nature. 2002;420(6917):868–874. [PubMed: 12490960]
3. Chmelar J, Chung KJ, Chavakis T. The role of innate immune cells in obese adipose tissue inflammation and development of insulin resistance. Thromb Haemost. 2013;109(3):399–406. [PubMed: 23364297]
4. Machado-Aranda D, Suresh V, Yu B, Dolgachev V, Hemmila MR, Raghavendran K. Alveolar macrophage depletion increases the severity of acute inflammation following nonlethal unilateral lung contusion in mice. J Trauma Acute Care Surg. 2014;76(4):982–990. [PubMed: 24662861]
5. Cochain C, Zernecke A. Macrophages in vascular inflammation and atherosclerosis. Pflugers Arch. 2017;469(3-4):485–499. [PubMed: 28168325]
6. Soehnlein O, Steffens S, Hidalgo A, Weber C. Neutrophils as protagonists and targets in chronic inflammation. Nat Rev Immunol. 2017;17(4):248–261. [PubMed: 28287106]
7. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7:678–689. [PubMed: 17717539]
8. Wolf D, Anto-Michel N, Blankenbach H, et al. A ligand-specific blockade of the integrin Mac-1 selectively targets pathologic inflammation while maintaining protective host-defense. Nat Commun. 2018;9(1):525. [PubMed: 29410422]
9. Wolf D, Bukosza N, Engel D, et al. Inflammation, but not recruitment, of adipose tissue macrophages requires signalling through Mac-1 (CD11b/CD18) in diet-induced obesity (DIO). Thromb Haemost. 2017;117(2):325–338. [PubMed: 27853810]

10. Mitroulis I, Alexaki VI, Kourtzelis I, Ziogas A, Hajishengallis G, Chavakis T. Leukocyte integrins: role in leukocyte recruitment and as therapeutic targets in inflammatory disease. Pharmacol Ther. 2015;147:123–135. [PubMed: 25448040]
11. Van der Vieren M, Le Trong H, St John T, Staunton DE, Gallatin WM. A novel leukointegrin, $\alpha_d\beta_2$, binds preferentially to ICAM-3. Immunity. 1995;3:683–690. [PubMed: 8777714]
12. Miyazaki Y, Vieira-de-Abreu A, Harris ES, et al. Integrin alphaD-beta2 (CD11d/CD18) is expressed by human circulating and tissue myeloid leukocytes and mediates inflammatory signaling. PLoS ONE. 2014;9(11):e112770. [PubMed: 25415295]
13. Aziz MH, Cui K, Das M, et al. The upregulation of integrin alphaDbeta2 (CD11d/CD18) on inflammatory macrophages promotes macrophage retention in vascular lesions and development of atherosclerosis. J Immunol. 2017;198(12):4855–4867. [PubMed: 28500072]
14. Cui K, Ardell CL, Podolnikova NP, Yakubenko VP. Distinct migratory properties of M1, M2, and resident macrophages are regulated by alphaDbeta2 and alphaMbeta2 integrin-mediated adhesion. Front Immunol. 2018;9:2650. [PubMed: 30524429]
15. Bao F, Brown A, Dekaban GA, Omana V, Weaver LC. CD11d integrin blockade reduces the systemic inflammatory response syndrome after spinal cord injury. Exp Neurol. 2011;231(2):272–283. [PubMed: 21784069]
16. Gris D, Marsh DR, Oatway MA, et al. Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. J Neurosci. 2004;24:4043–4051. [PubMed: 15102919]
17. Weaver LC, Bao F, Dekaban GA, et al. CD11d integrin blockade reduces the systemic inflammatory response syndrome after traumatic brain injury in rats. Exp Neurol. 2015;271:409–422. [PubMed: 26169930]
18. de Azevedo-Quintanilha IG, Vieira-de-Abreu A, Ferreira AC, et al. Integrin alphaDbeta2 (CD11d/CD18) mediates experimental malariaassociated acute respiratory distress syndrome (MA-ARDS). Malar J. 2016;15(1):393. [PubMed: 27473068]
19. Miyazaki Y, Bunting M, Stafforini DM, et al. Integrin alphaDbeta2 is dynamically expressed by inflamed macrophages and alters the natural history of lethal systemic infections. J Immunol. 2008;180(1):590–600. [PubMed: 18097061]
20. Peters van Ton AM, Kox M, Abdo WF, Pickkers P. Precision immunotherapy for sepsis. Front Immunol. 2018;9:1926. [PubMed: 30233566]
21. Uhle F, Chousterman BG, Grutzmann R, Brenner T, Weber GF. Pathogenic, immunologic, and clinical aspects of sepsis - update 2016. Expert Rev Anti Infect Ther. 2016;14(10):917–927. [PubMed: 27530423]
22. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. Semin Immunopathol. 2017;39(5):517–528. [PubMed: 28555385]
23. Gris P, Tighe A, Thawer S, et al. Gene expression profiling in anti-CD11d mAb-treated spinal cord-injured rats. J Neuroimmunol. 2009;209:104–113. [PubMed: 19250688]
24. Yakubenko VP, Belevych N, Mishchuk D, Schurin A, Lam SC, Ugarova TP. The role of integrin alpha D beta2 (CD11d/CD18) in monocyte/macrophage migration. Exp Cell Res. 2008;314:2569–2578. [PubMed: 18621369]
25. Yakubenko VP, Yadav SP, Ugarova TP. Integrin alphaDbeta2, an adhesion receptor up-regulated on macrophage foam cells, exhibits multiligand-binding properties. Blood. 2006;107:1643–1650. [PubMed: 16239428]
26. Ozment TR, Ha T, Breuel KF, et al. Scavenger receptor class a plays a central role in mediating mortality and the development of the pro-inflammatory phenotype in polymicrobial sepsis. PLoS Pathog. 2012;8(10):e1002967. [PubMed: 23071440]
27. Cui K, Podolnikova NP, Bailey W, et al. Inhibition of integrin alphaDbeta2-mediated macrophage adhesion to end-product of DHA oxidation prevents macrophage accumulation during inflammation. J Biol Chem. 2019.
28. Wang Q, Imamura R, Motani K, Kushiyama H, Nagata S, Suda T. Pyroptotic cells externalize eat-me and release find-me signals and are efficiently engulfed by macrophages. Int Immunol. 2013;25(6):363–372. [PubMed: 23446850]

29. Tsurubuchi T, Aratani Y, Maeda N, Koyama H. Retardation of early-onset PMA-induced apoptosis in mouse neutrophils deficient in myeloperoxidase. J Leukoc Biol. 2001;70(1):52–58. [PubMed: 11435485]
30. Seemann S, Zohles F, Lupp A. Comprehensive comparison of three different animal models for systemic inflammation. J Biomed Sci. 2017;24(1):60. [PubMed: 28836970]
31. Hu G, Christman JW. Editorial: alveolar macrophages in lung inflammation and resolution. Front Immunol. 2019;10:2275. [PubMed: 31616438]
32. Lee YS, Kim MH, Yi HS,et al. CX3CR1 differentiates F4/80(low) monocytes into pro-inflammatory F4/80(high) macrophages in the liver. Sci Rep. 2018;8(1):15076. [PubMed: 30305672]
33. Van der Vieren M, Crowe DT, Hoekstra D, et al. The leukocyte integrin alpha D beta 2 binds VCAM-1: evidence for a binding interface between I domain and VCAM-1. J Immunol. 1999;163:1984–1990. [PubMed: 10438935]
34. Sengelov H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N. Subcellular localization and dynamics of Mac-1 ($\alpha_m\beta_2$) in human neutrophils. J Clin Invest. 1993;92:1467–1476. [PubMed: 8376598]
35. Borregaard N, Kjeldsen L, Sengelov H, et al. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J Leukoc Biol. 1994;56:80–87. [PubMed: 7517990]
36. Sumagin R, Prizant H, Lomakina E, Waugh RE, Sarelius IH. LFA-1 and Mac-1 define characteristically different intralumenal crawling and emigration patterns for monocytes and neutrophils in situ. J Immunol. 2010;185(11):7057–7066. [PubMed: 21037096]
37. Szpak D, Izem L, Verbovetskiy D, Soloviev DA, Yakubenko VP, Pluskota E. alphaMbeta2 is antiatherogenic in female but not male mice. J Immunol. 2018;200(7):2426–2438. [PubMed: 29459405]
38. Wu H, Gower RM, Wang H, et al. Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. Circulation. 2009;119:2708–2717. [PubMed: 19433759]
39. Bednarczyk M, Stege H, Grabbe S, Bros M. beta2 integrins-multifunctional leukocyte receptors in health and disease. Int J Mol Sci. 2020;21(4).
40. Aoudjit F, Vuori K. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. Oncogene. 2001;20:4995–5004. [PubMed: 11526484]
41. Yakubenko VP, Bhattacharjee A, Pluskota E, Cathcart MK. $\alpha M\beta 2$ integrin activation prevents alternative activation of human and murine macrophages and impedes foam cell formation. Circ Res. 2011;108:544–554. [PubMed: 21252155]
42. Zheng C, Yang Q, Xu C, et al. CD11b regulates obesity-induced insulin resistance via limiting alternative activation and proliferation of adipose tissue macrophages. Proc Natl Acad Sci U S A. 2015;112(52):E7239–E7248. [PubMed: 26669445]
43. Danilenko DM, Rossito PV, Van der Vieren M, et al. A novel canine leukointegrin, alpha d beta 2, is expressed by specific macrophage subpopulations in tissue and a minot CD8+ lymphocyte subpopulation in peripheral blood. J Immunol. 1995;155:35–44. [PubMed: 7541420]
44. Bao F, Dekaban GA, Weaver LC. Anti-CD11d antibody treatment reduces free radical formation and cell death in the injured spinal cord of rats. J Neurochem. 2005;94:1361–1373. [PubMed: 15992367]
45. Mabon PJ, Weaver LC, Dekaban GA. Inhibition of monocyte/macrophage migration to a spinal cord injury site by an antibody to the integrin alphaD: a potential new anti- inflammatory treatment. Exp Neurol. 2000;166(1):52–64. [PubMed: 11031083]
46. Nascimento DO, Vieira-de-Abreu A, Arcanjo AF, Bozza PT, Zimmerman GA, Castro-Faria-Neto HC. Integrin alphaDbeta2 (CD11d/CD18) modulates leukocyte accumulation, pathogen clearance, and pyroptosis in experimental *Salmonella typhimurium* infection. Front Immunol. 2018;9:1128. [PubMed: 29881383]
47. Liu JR, Han X, Soriano SG, Yuki K. The role of macrophage 1 antigen in polymicrobial sepsis. Shock. 2014;42(6):532–539. [PubMed: 25075642]
48. Liu JR, Han X, Soriano SG, Yuki K. Leukocyte function-associated antigen-1 deficiency impairs responses to polymicrobial sepsis. World J Clin Cases. 2015;3(9):793–806. [PubMed: 26380827]

49. Cao C, Gao Y, Li Y, Antalis TM, Castellino FJ, Zhang L. The efficacy of activated protein C in murine endotoxemia is dependent on integrin CD11b. J Clin Invest. 2010;120(6):1971–1980. [PubMed: 20458145]
50. Emoto M, Emoto Y, Brinkmann V, et al. Increased resistance of LFA-1-deficient mice to lipopolysaccharide-induced shock/liver injury in the presence of TNF-alpha and IL-12 is mediated by IL-10: a novel role for LFA-1 in the regulation of the proinflammatory and anti-inflammatory cytokine balance. J Immunol. 2003;171(2):584–593. [PubMed: 12847222]
51. Jawhara S, Pluskota E, Cao W, Plow EF, Soloviev DA. Distinct effects of integrins alphaXbeta2 and alphaMbeta2 on leukocyte subpopulations during inflammation and antimicrobial responses. Infect Immun. 2017;85(1).
52. Asaduzzaman M, Zhang S, Lavasani S, Wang Y, Thorlacius H. LFA-1 and MAC-1 mediate pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis. Shock. 2008;30(3):254–259. [PubMed: 18197144]
53. Mestas J, Ley K. Monocyte-endothelial cell interactions in the development of atherosclerosis. Trends Cardiovasc Med. 2008;18(6):228–232. [PubMed: 19185814]
54. Meerschaert J, Furie MB. The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. J Immunol. 1995;154:4099–4112. [PubMed: 7535821]
55. Lammermann T, Bader BL, Monkley SJ,et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. Nature. 2008;453:51–55. [PubMed: 18451854]
56. Sarangi PP, Hyun YM, Lerman YV, Pietropaoli AP, Kim M. Role of beta1 integrin in tissue homing of neutrophils during sepsis. Shock. 2012;38(3):281–287. [PubMed: 22683734]
57. Shen XF, Cao K, Jiang JP, Guan WX, Du JF. Neutrophil dysregulation during sepsis: an overview and update. J Cell Mol Med. 2017;21(9):1687–1697. [PubMed: 28244690]
58. Zhao YJ, Yi WJ, Wan XJ, et al. Blockade of ICAM-1 improves the outcome of polymicrobial sepsis via modulating neutrophil migration and reversing immunosuppression. Mediators Inflamm. 2014;2014:195290. [PubMed: 24891762]
59. Li Y, Wang W, Yang F, Xu Y, Feng C, Zhao Y. The regulatory roles of neutrophils in adaptive immunity. Cell Commun Signal. 2019;17(1):147. [PubMed: 31727175]
60. Hoesel LM, Neff TA, Neff SB, et al. Harmful and protective roles of neutrophils in sepsis. Shock. 2005;24(1):40–47.
61. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol. 2005;6(12):1191–1197. [PubMed: 16369558]
62. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002;418(6894):191–195. [PubMed: 12110890]
63. Rydell-Tormanen K, Uller L, Erjefalt JS. Direct evidence of secondary necrosis of neutrophils during intense lung inflammation. Eur Respir J. 2006;28(2):268–274. [PubMed: 16510453]

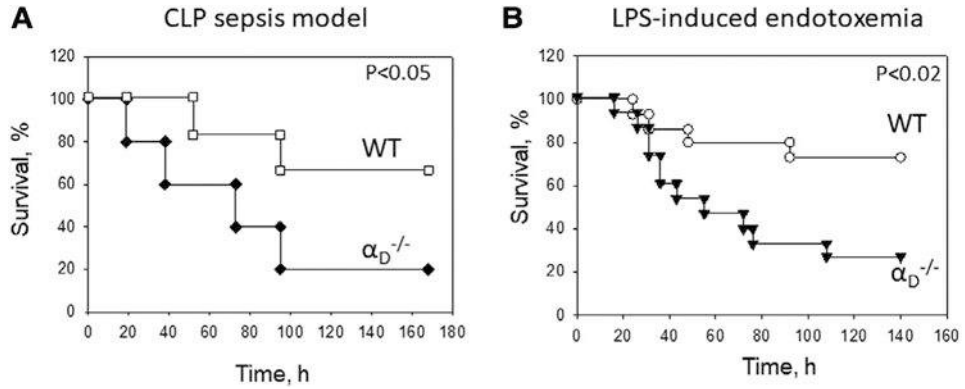

FIGURE 1. Integrin α_D deficiency promotes development of sepsis.

A. Survival curves after surgical induction of a cecal ligation and puncture (CLP) polymicrobial sepsis. Mice that were 16 wk old (wild-type [WT], $n = 6$; $\alpha_D^{-/-}$, $n = 5$) were used for the procedure. The cecum was exteriorized, the contents were massaged distally, and the half of cecum was ligated. The cecum was punctured once with a 25 gauge needle in an avascular region near the distal end and a bleb of cecal contents was extruded from the puncture. **B.** Survival curves after LPS-induced endotoxemia. Ten to twelve weeks old WT($n = 14$) and $\alpha_D^{-/-}$ ($n = 15$) male mice were injected with 10 μg LPS per gram body weight. Statistical significance was assessed by a log-rank Kaplan-Meier method

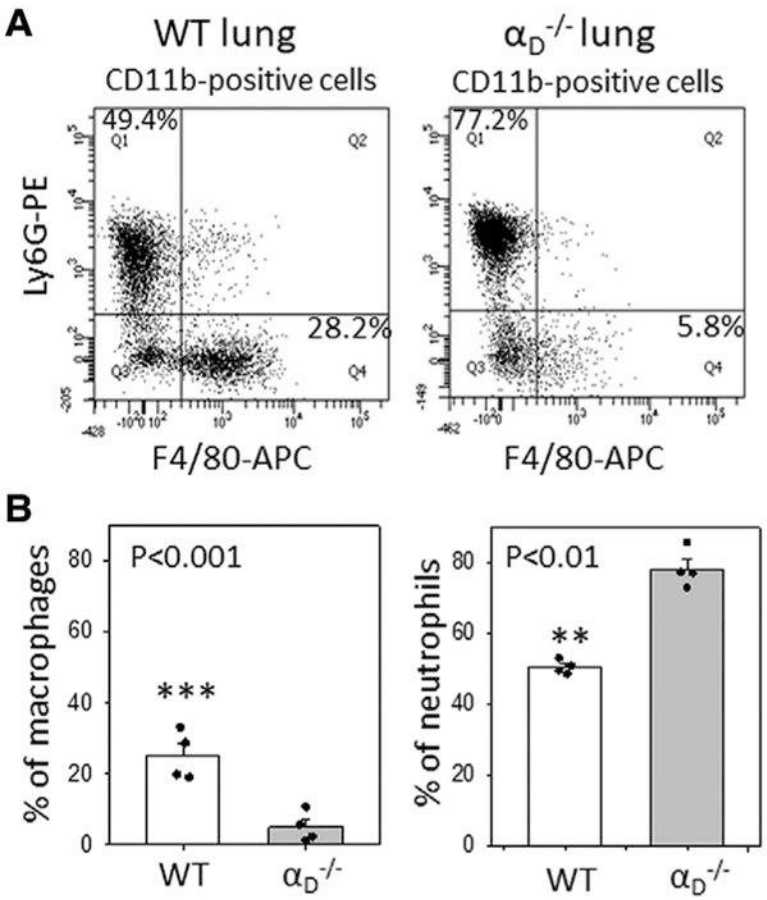

FIGURE 2. Integrin α_D deficiency dramatically decreases the number of macrophages in lungs during endotoxemia.

A. Wild-type (WT) and $\alpha_D^{-/-}$ mice were injected with sublethal dose of LPS. After 48 h lungs were isolated, digested and analyzed using flow cytometry. $CD11b^+$ cells were selected and tested with antibodies against neutrophil (Ly6G) and macrophage (F4/80) markers. **B.** The result of flow cytometry was analyzed and calculated using FACSDiva software. The plots represent the amount of WT and α_D-deficient macrophages (left panel) and neutrophils (right panel) in digested lungs. Statistical analysis was performed using Student's *t*-test. $n = 4$/group. **, $P < 0.01$; ***, $P < 0.001$

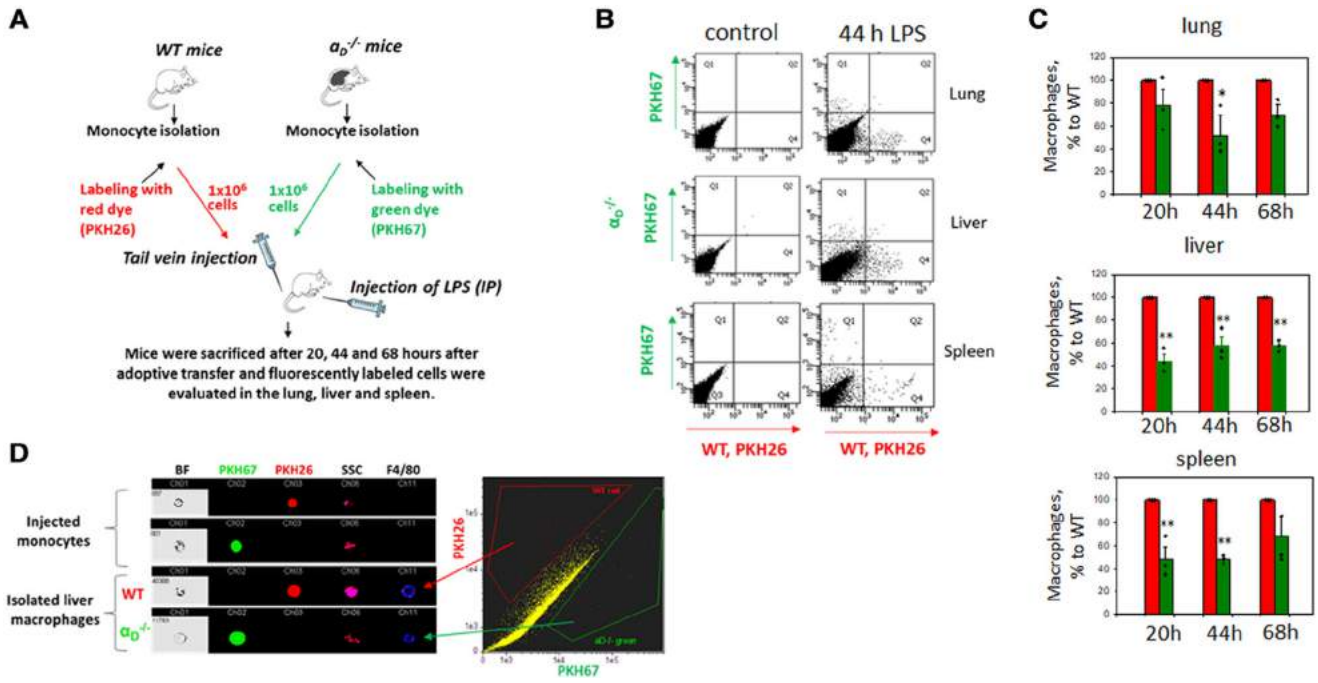

FIGURE 3. Integrin α_D deficiency affects the migration of macrophages to organs during LPS-induced endotoxemia.

A. Schematic representation of experiment. Monocyte progenitors were isolated from bone marrow of wild-type (WT) and α_D-deficient mice. Cells were labeled with red (WT) or green ($\alpha_D^{-/-}$) fluorescent dyes, mixed in equal amount and injected in tail vein of WT mice 20 min before the injection of LPS. After 20, 44, and 68 h lung, liver, and spleen were isolated, digested and analyzed using flow cytometry. **B**. The representative result of flow cytometry analysis after 44 h is shown. Data were analyzed and calculated using FACSDiva software. Migrated WT macrophages (red) were detected in quadrant 4; $\alpha_D^{-/-}$ macrophages (green) were detected in quadrant 1. **C**. The plots represent the amount of WT and α_D-deficient macrophages in different organs based on flow cytometry data. Statistical analysis was performed using Student's *t*-test. *, $P < 0.05$, **$P < 0.01$. **D**. Evaluation of migrated macrophages in liver using Imaging flow cytometry. The population of single, live cells was analyzed on ImageStream Mark II, (Amnis) instrument. The individual cells were analyzed on green and red channels. The expression of the F4/80$^-$ macrophage marker was evaluated on monocytes before the adoptive transfer and on PKH26 and PKH67-labeled cells isolated from the liver at 44 h after monocyte injection. BF- bright field. Channel 2- 488 nm; Channel 3—566 nm; channel 6—side scattering; channel 11- 633 nm

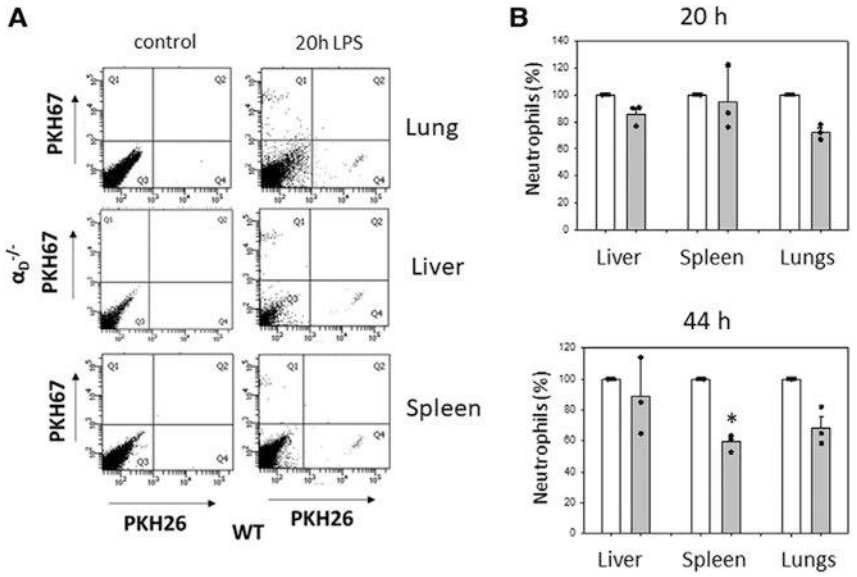

FIGURE 4. α_D deficiency does not significantly change neutrophil migration during endotoxemia.

A. Neutrophil progenitors were isolated from bone marrow of wild-type (WT) and α_D-deficient mice. Cells were labeled with red (WT) or green ($\alpha_D^{-/-}$) fluorescent dyes, mixed in equal amount and injected in tail vein of WT mice 20 min before the injection of LPS. After 20 and 44 h lung, liver, and spleen were isolated, digested, and analyzed using flow cytometry. Migrated WT neutrophils were detected in quadrant 4; migrated $\alpha_D^{-/-}$ neutrophils were detected in quadrant 1. **B**. The results of flow cytometry were calculated using FACSDiva software and plotted as percentage of migrated $\alpha_D^{-/-}$ neutrophils (gray bars) vs. WT neutrophils (open bars). Statistical analysis was performed using Student's *t*-test. $*P < 0.05$, $n = 3$ per group

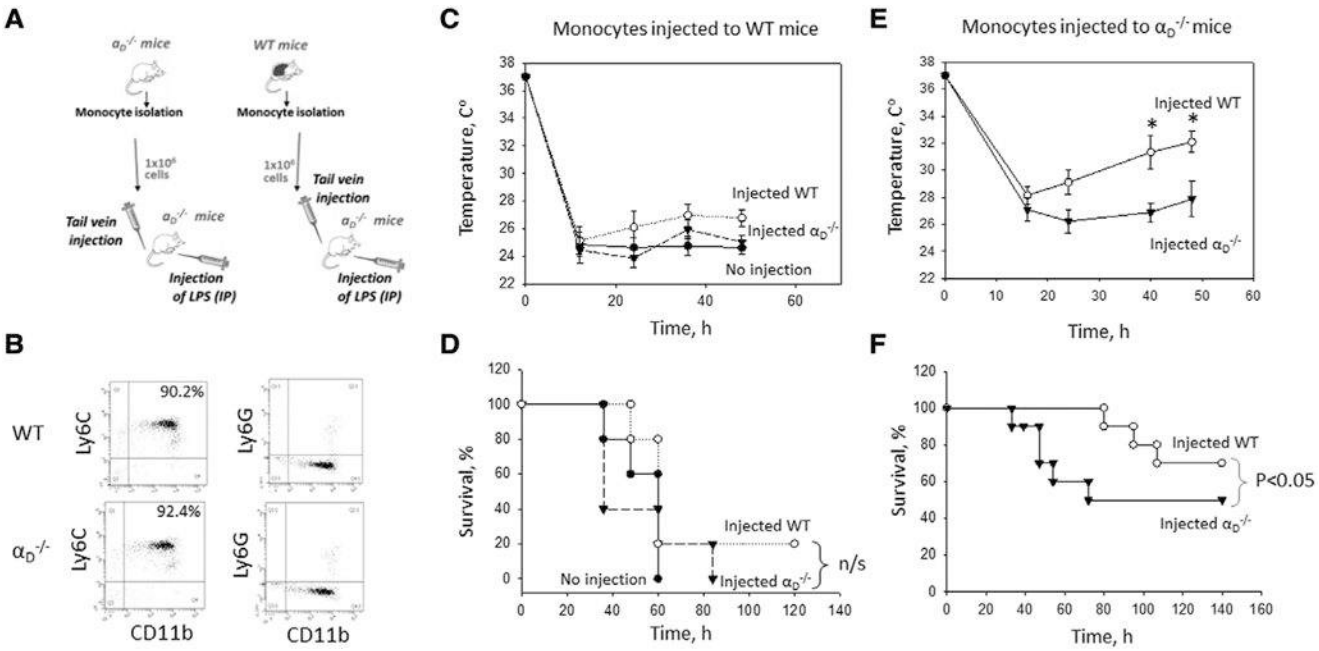

FIGURE 5. Effect of wild-type (WT) and $\alpha_D^{-/-}$ monocyte injection on survival of LPS-challenged mice.

A. Schematic representation of experiment. **B**. Monocytes were isolated by Miltenyi Biotec kit and purity was assessed by flow cytometry with anti-CD11b, anti-Ly6C, and anti-Ly6G antibodies. **C**, **F**. Monocytes isolated from WT (open circle) or $\alpha_D^{-/-}$ (black triangle) mice were injected into the bloodstream of male WT mice (**C**, **D**) (in another experiment $\alpha_D^{-/-}$ mice [**E**, **F**]) 20 min before the injection of LPS. Mice without the injection of adoptively transferred monocytes (black circle) were used as control. Body temperature (**C**, **E**) and survival rate (**D**, **F**) of recipient mice were evaluated after adoptive transfer. $N = 10$ recipient mice per group. Statistical significance for temperature measurements was assessed using Student's *t*-test. *, $P < 0.05$; and for survival rate using log-rank Kaplan-Meier method. *P*-value is not significant for (**D**) and $P = 0.05$ for (**F**)

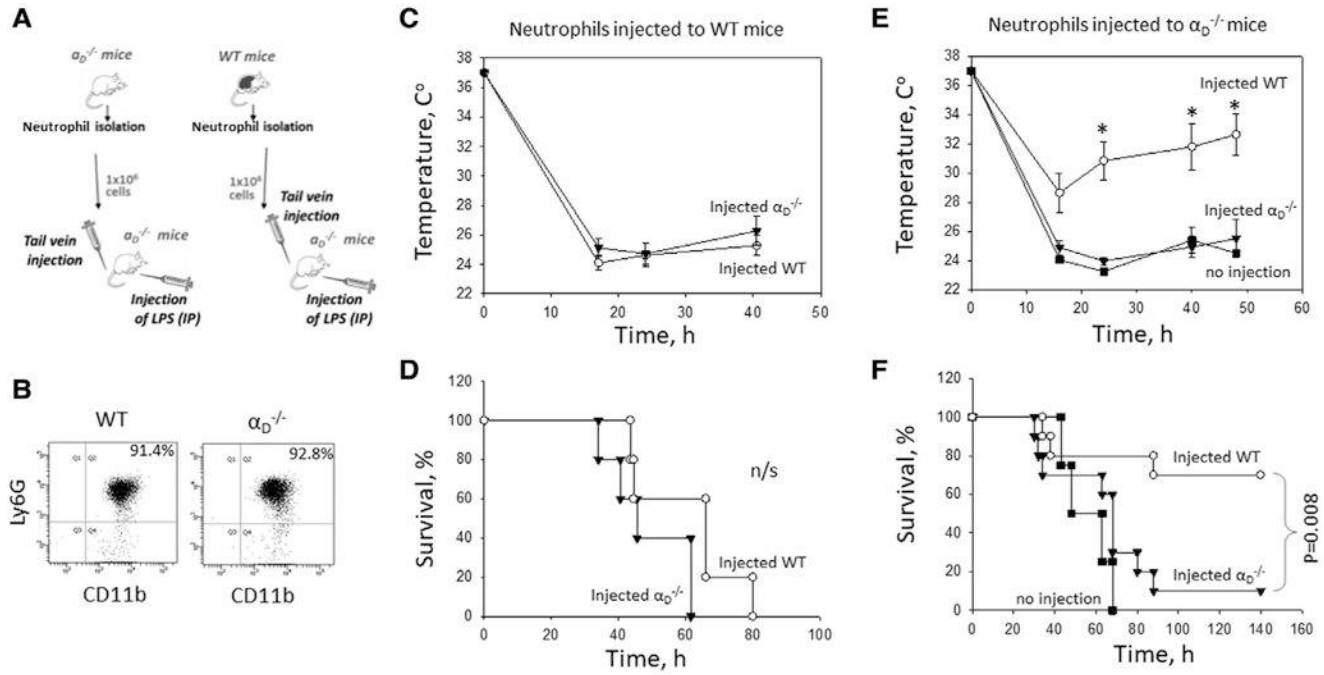

FIGURE 6. Effect of wild-type (WT) and $\alpha_D^{-/-}$ neutrophil injection on survival of LPS-challenged mice.

A. Schematic representation of experiment. **B**. Neutrophils were isolated by Miltenyi Biotec kit and purity was assessed by flow cytometry with anti-CD11b and anti-Ly6G antibodies. **C**, **F**. Neutrophils isolated from WT (open circle) or $\alpha_D^{-/-}$ (black triangle) mice were injected into bloodstream of male WT ($n = 10$ recipient mice per group) (**C**, **D**) or male α_D-deficient ($n = 10$ recipient mice per group; $n = 8$ for "no injection" group) (**E**, **F**) mice 20 min before the injection of LPS. The body temperature (**C**, **E**) and survival rate (**D**, **F**) were evaluated. Statistical significance for temperature measurements was assessed using Student's *t*-test. *, $P < 0.01$ and for survival rate by log-rank Kaplan-Meier method, $P = 0.008$, for α_D-deficientmice(**F**); n/s for WT mice (**D**). $n = 10$/group

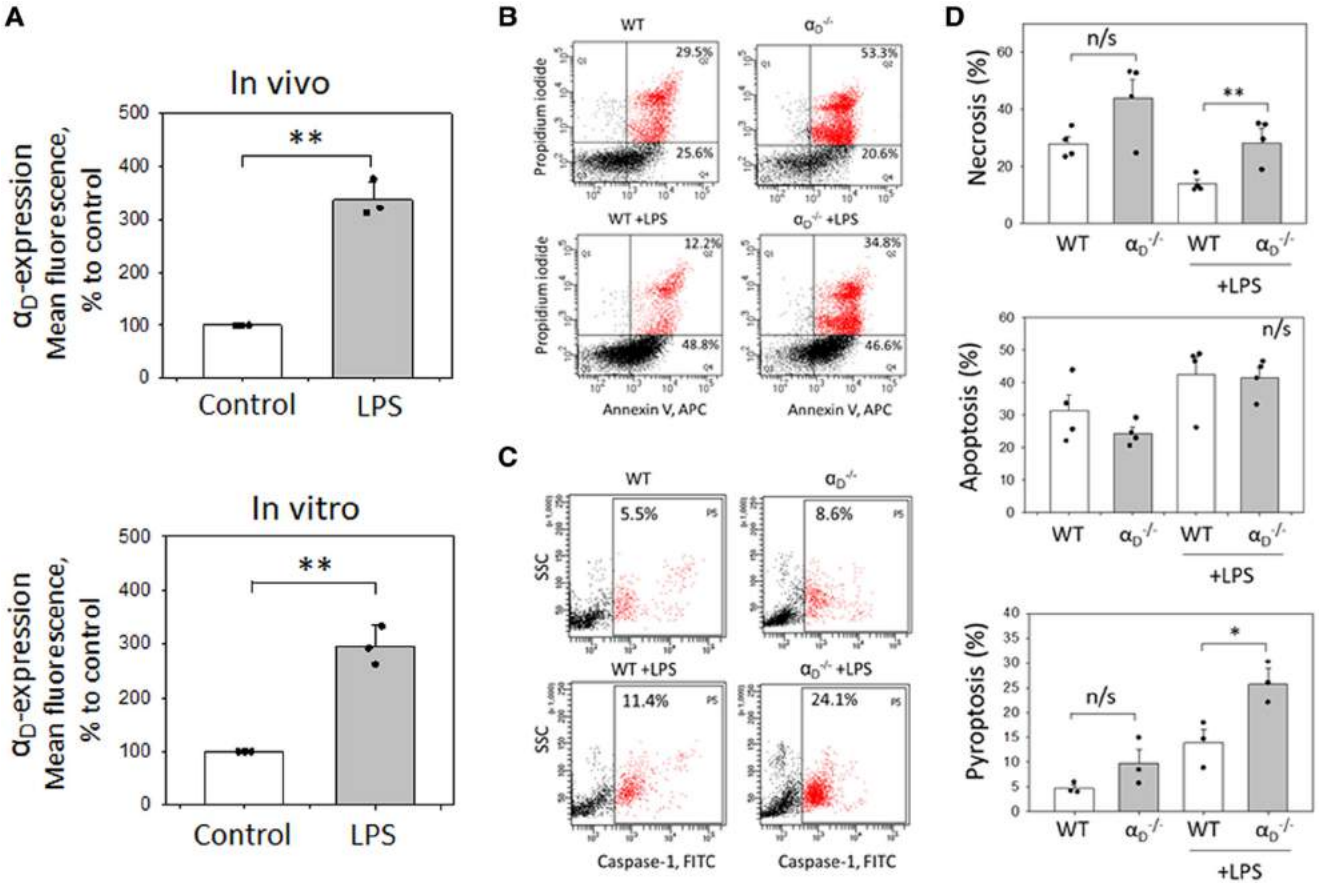

FIGURE 7. A. $\alpha_D\beta_2$ expression is increased on blood neutrophils 24 h after LPS-induced endotoxemia (upper panel) and on neutrophils incubated with LPS in vitro (lower panel).

Upper panel. Leukocytes were collected from the blood of LPS-challenged and control mice. The expression of integrin $\alpha_D\beta_2$ was evaluated on $CD11b^+/Ly6G^+$ cells. $n = 4$. Lower panel. Peritoneal neutrophils were collected 4 h after the injection of thioglycollate and incubated 24 h in the presence of 100 ng/ml LPS before the flow cytometry analysis. $n = 3$. Statistical analyses were assessed using Student's *t*-tests, $^{**}P < 0.01$. **B**, **C**. α_D deficiency increases necrosis (**B**) and pyroptosis (**C**) of neutrophils. Thioglycollate-elicited wild-type (WT; left panels) and $\alpha_D^{-/-}$ (right panels) neutrophils were isolated and incubated with LPS (lower panels) or without LPS (upper panels) for 24 h. Apoptosis was evaluated by FACS using Annexin V kit (**B**). Q2 quadrant represents cells undergoing necrosis. Q4 quadrant shows apoptotic cells. Pyroptosis was assessed using FAM-FLICA-caspase-1 kit (**C**). **D**.The level of necrosis, apoptosis and pyroptosis collected from 3 to 4 independent experiments was calculated. Statistical analyses were performed using Student's paired *t*-tests. $^{**}P < 0.01$, $^{*}P < 0.05$

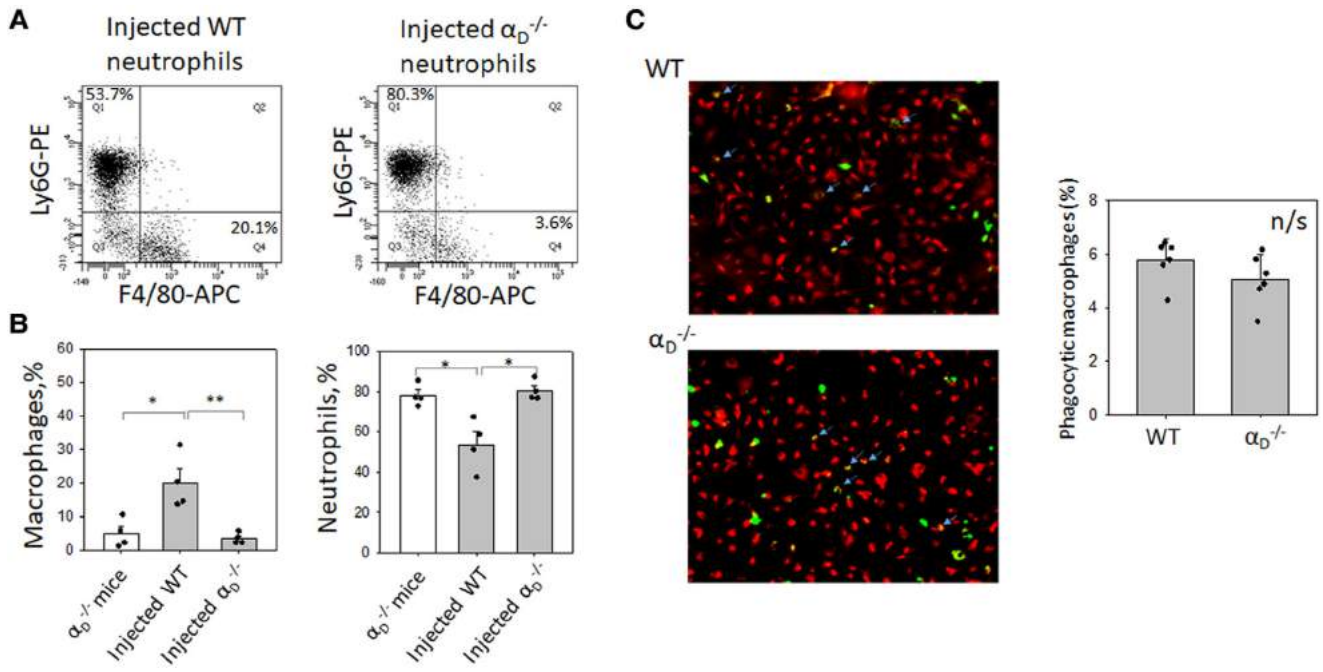

FIGURE 8. Injected wild-type (WT) neutrophils increase macrophage number in lungs of α_D-deficient mice.

A. Neutrophils isolated from WT (left panel) or $\alpha_D^{-/-}$ (right panel) mice were injected into bloodstream of male α_D-deficient mice. After 48 h, lungs were isolated, digested, and analyzed using flow cytometry. $CD11b^+$ cells were selected and tested with antibodies against neutrophils (Ly6G) and macrophages (F4/80) markers. **B**. The result of flow cytometry was analyzed and calculated using FACSDiva software. The plots represent the amount of WT and α_D-deficient macrophages (left panel) and neutrophils (right panel). Statistical analysis was performed using Student's *t*-test. $n = 4$/group. *, $P < 0.05$; **, $P < 0.01$. **C**. In vitro efferocytosis of WT and α_D-deficient macrophages was evaluated. PKH26-labeled (red) macrophages (WT [upper panel] or $\alpha_D^{-/-}$ [lower panel]) were incubated overnight in the presence of apoptotic/necrotic PKH67-labeled (green) neutrophils. Efferocytosis was detected by EVOS Cell Imaging system (blue arrows) and analyzed by Image Analysis Software. $n = 6$ samples per group. Statistical analysis was performed using Student's *t*-tests

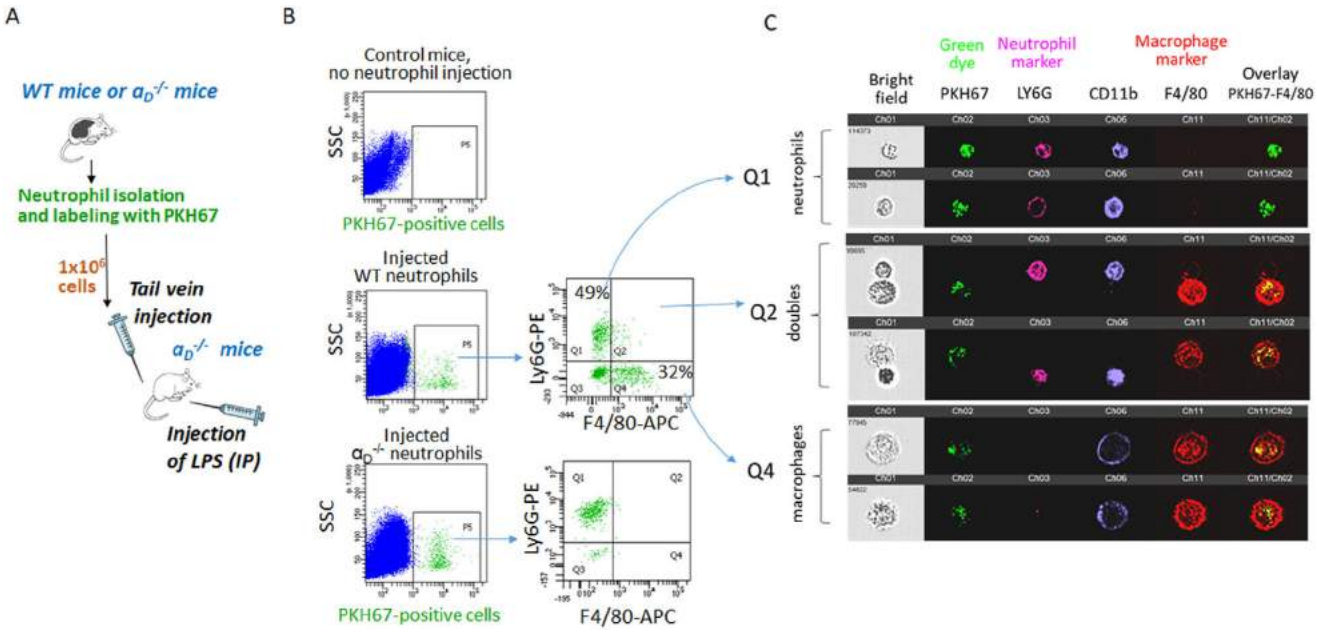

FIGURE 9. Macrophage efferocytosis in lungs of $a_D^{-/-}$ mice after the adoptive transfer.

A. Neutrophils isolated from wild-type (WT) mice were labeled with green fluorescent dye (PKH67) and injected into the bloodstream of male a_D-deficient mice. Lungs were analyzed after 48 h by flow cytometry (**B**) and imaging flow cytometry (**C**). Green-positive cells were selected and tested with antibodies against Ly6G, F4/80, and CD11b. **C**. Efferocytosis was verified by imaging of individual cells in PKH67-positive population using Imaging flow cytometry (Amnis) and analyzed using internalization wizard in IDEA software (Amnis). Bright field, PKH67, Ly6G (PE), CD11b (PECy7), and F4/80 (APC) columns show individual channels. Far right column demonstrates internalization of PKH67-labeled neutrophils by macrophages