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Brief Conclusive Report

Inflammation-dependent α5β1 (very late antigen-5) expression on leukocytes reveals a functional role for this integrin in acute peritonitis

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ABSTRACT

The potential role of α5β1 (VLA-5) in leukocyte trafficking in zymosan-induced acute peritonitis was determined. In naïve mice, ~98% of Gr1^{high} cells (PMN) in bone marrow and circulation were α5β1-negative; these profiles were modestly affected by peritoneal injection of zymosan. In contrast, ~30% of Gr1^{high} cells recruited by zymosan (24 h) to the peritoneal cavity expressed α5β1. With respect to F4/80^{+} cells, ~60% of bone marrow and peripheral blood populations expressed α5β1, with ~90% positivity in resident cells of noninflamed peritoneum. Analysis of α5β1 expression revealed inflammation-dependent increased expression on Gr1^{high} and F4/80^{+} cells in bone marrow, blood, and peritoneal cavity. Blockade of α5β1, by an anti-α5 mAb, attenuated zymosan-induced 24 h recruitment of Gr1^{high} and F4/80^{+} cells. At least one underlying mechanism of this action was reduction of cell adhesion and transmigration across microvascular vessels, as revealed by intravital microscopy. Confocal analyses indicated that deposition of fibronectin, the principal ligand for α5β1, was up-regulated significantly on and around the inflamed mesenteric microvasculature. These data suggest that the effects of α5-blockade may be a result of inhibition of α5β1-dependent leukocyte adhesion to and migration along the fibronectin matrix. This is the first report that identifies a functional role for α5β1 in leukocyte trafficking during acute inflammation. *J. Leukoc. Biol.* 87: 877–884; 2010.

Introduction

The recruitment of leukocytes from the blood to the site of inflammation is a pivotal event during acute inflammation [1], governed by a series of overlapping molecular and cellular events including release of cytokines, activation of their receptors [2], and involvement of large families of adhesion molecules [3, 4]. Integrins are α/β-heterodimeric transmembrane proteins [5], which upon cell activation, undergo conformational changes, resulting in increased affinity for their ligands [6]. A major role of integrins in leukocytes is in myeloid and lymphoid cell adhesion to endothelial cells; this process relies on at least one of the four integrins: α4β1 (also termed VLA-4 or CD49d/CD29), α4β7, αLβ2 (LFA-1 or CD11a/CD18), and αMβ2 (membrane-activated complex 1 or CD11b/CD18) [6]. In certain conditions, α4β1 and α4β7 and their ligands CD106 and mucosal addressin cell adhesion molecule-1 might also contribute to the initial leukocyte capture [7, 8]. In addition, the formation of a bimolecular complex between CD44 and α4β1 is required for α4β1-mediated, firm adhesion of activated T cells [4]. Moreover, the contribution to firm adhesion by β2 integrin (CD18) family members, e.g., αLβ2 and αMβ2—acting in concert with their counter-receptor CD54 (ICAM-1)—has been described previously [9–11].

Leukocytes extravasated into the inflamed tissue are subjected to an orchestrated series of adhesive, deadhesive, and signaling events that favor their migration toward the site of inflammation: These sequential processes are also reliant on leukocyte integrins [6]. Extracellular matrix integrin ligands, including fibronectin, tenascin, and fibrillar collagen, appear to guide interstitial leukocyte migration and accumulation during inflammation [12]. Thus, the laminin receptor α6β1 (VLA-6) together with the collagen-binding integrins α1β1 (VLA-1) and α2β1(VLA-2) are essential for interstitial cell locomotion and retention in inflamed, nonlymphoid tissues [12]. Leukocytes can bind to fibronectin via α5β1 (VLA-5) [13, 14], which mediates their in vitro migration, evident upon activation [15, 16]. The expression of α5β1 has been reported on different immune cells, including activated macrophages, neutrophils, mast cells, and T lymphocytes [6, 13, 16–21]. In

Abbreviations: DAPI=4′,6-diamidino-2-phenylindole, MFI=mean fluorescence intensity, MPO=myeloperoxidase, PMN=polymorphonuclear leukocytes

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addition, $\alpha5\beta1$ is abundantly expressed on stromal and endothelial cells: Its targeting represents a novel strategy for antiangiogenic therapies, making antagonists to this integrin potentially useful for the treatment of cancer and other angiogenesis-dependent diseases [22–24].

Early studies suggested that targeting the integrin $\alpha5\beta1$ might also have a potential for the treatment of inflammatory diseases, a proposition scantly supported by experimental evidence. For instance, a cyclic Arg-Gly-Asp peptide, with high affinity for $\alpha5\beta1$ and moderate selectivity against other integrins [25], protected against ischemia/reperfusion injury of the liver via inhibition of macrophage invasion into the transplanted organ [26]. In contrast, using an $\alpha5\beta1$ antibody, Burns et al. [18] revealed a redundant role for $\alpha5\beta1$ in endotoxin-induced lung inflammation. In fact, a comprehensive characterization of the contribution of $\alpha5\beta1$ integrin to leukocyte trafficking in inflammation is still missing. To address this point, we have examined $\alpha5\beta1$ expression on leukocytes in relation to their compartmentalization, in resting as well as during an acute inflammatory reaction. Having found $\alpha5$ upregulation after inflammatory challenge, we have applied a validated model of a self-resolving, acute inflammation (hence, without the complication of angiogenesis induction) and studied the effect of an anti-$\alpha5$ antibody on the extent of PMN and mononuclear cell trafficking.

MATERIALS AND METHODS

Zymosan-induced peritonitis

Male Swiss Albino mice (20–25 g; Harlan, UK), maintained on a standard diet with water ad libitum, housed in a room with controlled lighting and temperature, were used. Animals were used 7 days after arrival, according to guidelines laid down by the Ethical Committee for the use of Animals (Barts and The London School of Medicine (UK). Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). Peritonitis studies were performed as described previously [27, 28], using 1 mg boiled zymosan A (Sigma-Aldrich, Poole, UK) in 0.5 ml sterile PBS. At different time-points, blood aliquots were collected by cardiac puncture with heparin (25 U), whereas peritoneal cavities were washed with 3 ml PBS, supplemented with 3 mM EDTA and 25 U/ml heparin. Bone marrows were collected by flushing both femurs with 3 ml DMEM, supplemented with 25 U/ml heparin.

Animals were treated s.c. with a function-blocking rat anti-mouse $\alpha5$ mAb (0.05 mg; clone 5H10-27; BD PharMingen, Oxford, UK), irrelevant isotype control (rat IgG2a; BD PharMingen), 5 min before zymosan challenge.

Other inflammatory stimuli

In selected experiments, mice were inflamed by i.p. injections of carrageenan (500 μg/cavity; λ carrageenan; Sigma-Aldrich), CXCL1 (500 ng; Peprotech Ltd., London, UK), or LPS (10 mg/kg; *Escherichia coli* serotype 0111:B4; Sigma-Aldrich) and killed 4 or 24 h later, as detailed below.

Cellular analyses

Aliquots of blood, bone marrow, or peritoneal lavage fluids were diluted in Turk's solution (3% acetic acid and 0.1% crystal violet), followed by total and differential counting using a Neubauer chamber (Nikon YS2, Tokyo, Japan; 40× objective) [27, 28]. Cell-surface staining for flow cytometry was performed as described before [29]. Briefly, after FcR blockade, cells were labeled for 30 min at 4°C with the PE-conjugated anti-$\alpha5$ antibody (clone 5H10-27, MFR5; BD PharMingen), together with FITC-conjugated anti-Gr1 to detect granulocytes (clone RB6-8C5; BD PharMingen) or FITC-conjugated anti-F4/80 to detect monocytes and macrophages (clone ClA3-1; Serotec, Oxford, UK). Data acquisition was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), using CellQuest™ software (Becton Dickinson). Forward- and side-scatters were set to exclude erythrocytes and dead cells. Granulocytes were monitored with Gr1 staining; this often (e.g., blood and bone marrow samples) revealed an intermediate and a high Gr1-expressing population. Hence, a gate was set to ana-

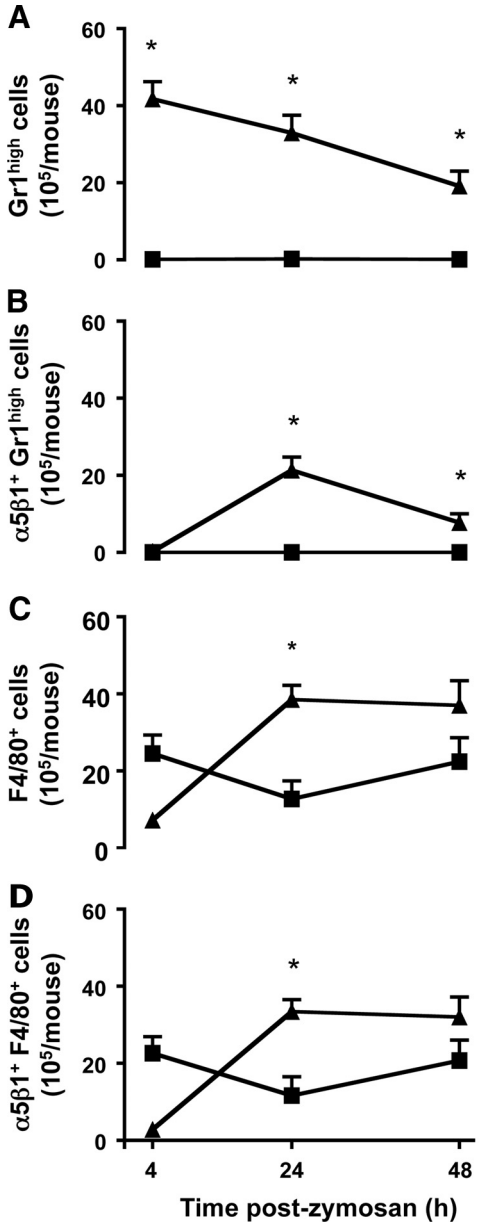

Figure 1. Kinetics of $\alpha5\beta1^+$ cell migration into the mouse peritoneal cavity inflamed with zymosan. PBS (0.5 ml i.p.) or zymosan (1 mg i.p.) was injected to mice and $Gr1^{high}$ (A) or $F4/80^+$ (C) cell accumulation assessed at the reported time-points using FACS as described in Materials and Methods. Dual-staining protocols allowed detection of $\alpha5\beta1$ on $Gr1^{high}$ (B) and $F4/80^+$ (D) leukocyte populations. At least 10^5 events were analyzed per sample. Data are mean ± SEM of six mice/group. *, $P < 0.05$, when compared with the respective PBS group.

lyze only the Gr1^{high} population, corresponding to the PMN population. In all cases, at least 10^5 events were analyzed per sample. Positive and negative populations were identified based on dual-color staining performed with a PE- and FITC-conjugated, irrelevant IgG isotype (eBioscience, Hatfield, UK).

Mesenteric MPO activity

Leukocyte MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine along, according to a published protocol [30]. Lung tissue samples from mice were homogenized in PBS containing 0.5% hexadecyl trimethylammonium bromide; homogenates were centrifuged at 13,000 *g* for 5 min prior adding 20 μl supernatants to 160 μl tetramethylbenzidine and 20 μl H_2O_2 in 96-well plates at 37°C. OD was read at 620 nm using GENios (Tecan, Reading, UK). Assays were performed in duplicate and normalized wet-tissue weight.

Intravital microscopy

Intravital microscopy of the inflamed mesentery was performed as reported previously [30–32]. Briefly, animals were treated with blocking mAb anti-α5 integrin, as stated above. Two hours to 24 h after zymosan (1 mg/cavity) or vehicle (0.5 ml/cavity) injection, mice were anesthetized intramuscularly with 7.5 mg/kg xylazine and 150 mg/kg ketamine hydrochloride and placed in supine position on a heating pad (37°C), and a mesenteric vascular bed was exteriorized and mounted on a Zeiss Axioskop "FS" with a water-immersion objective lens (magnification, ×40; Carl Zeiss MicroImaging, Welwyn Garden City, UK) and an eyepiece (magnification, ×10; Carl Zeiss MicroImaging). Analysis of leukocyte-endothelium interactions was made in three to four randomly selected postcapillary venules (diameter between 20 and 40 μm; visible length of at least 100 μm vessel length) for each mouse. Quantification of leukocyte adhesion and leukocyte emigration was performed off-line by frame-to-frame analysis of the videotaped images as described previously [30–32].

Immunostaining of mouse mesentery

A whole-mount preparation of the isolated mesentery was produced, following a protocol optimized for the rat [33]. Tissues were fixed in ice-cold 4% neutral-buffered formalin for 30 min, blocked with 1% BSA for 15 min, permeabilized in PBS, and supplemented with 1% BSA and 0.1% Triton X-100 for 30 min at 4°C. Following 1 h incubation at 4°C with primary rat anti-mouse CD31 (PECAM-1; clone MEC13.3) and rabbit anti-fibronectin (IgG fraction; Sigma-Aldrich) antibodies, tissues were washed and incubated with appropriate secondary antibodies conjugated to Alexa Fluor 488 or 543 (Invitrogen, Paisley, UK) at 4°C for 1 h. Samples were then mounted using Vectashield Hard mount medium containing DAPI for nuclear staining (Vector Labs, Peterborough, UK). In all studies, appropriate irrelevant control mAb were used in parallel with the specific primary antibodies. Samples were viewed using a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss MicroImaging). Typically, overlapping, 1 μm-thick optical sections in the Z-plane were captured using the same settings for comparisons between control and experimental samples.

Data handing and statistical analysis

Statistical differences between groups in the in vivo experiments were determined by one-way ANOVA, followed if significant, by the Student Newmann-Keuls test using GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA). In all cases, a probability value, $P < 0.05$, was taken as significant.

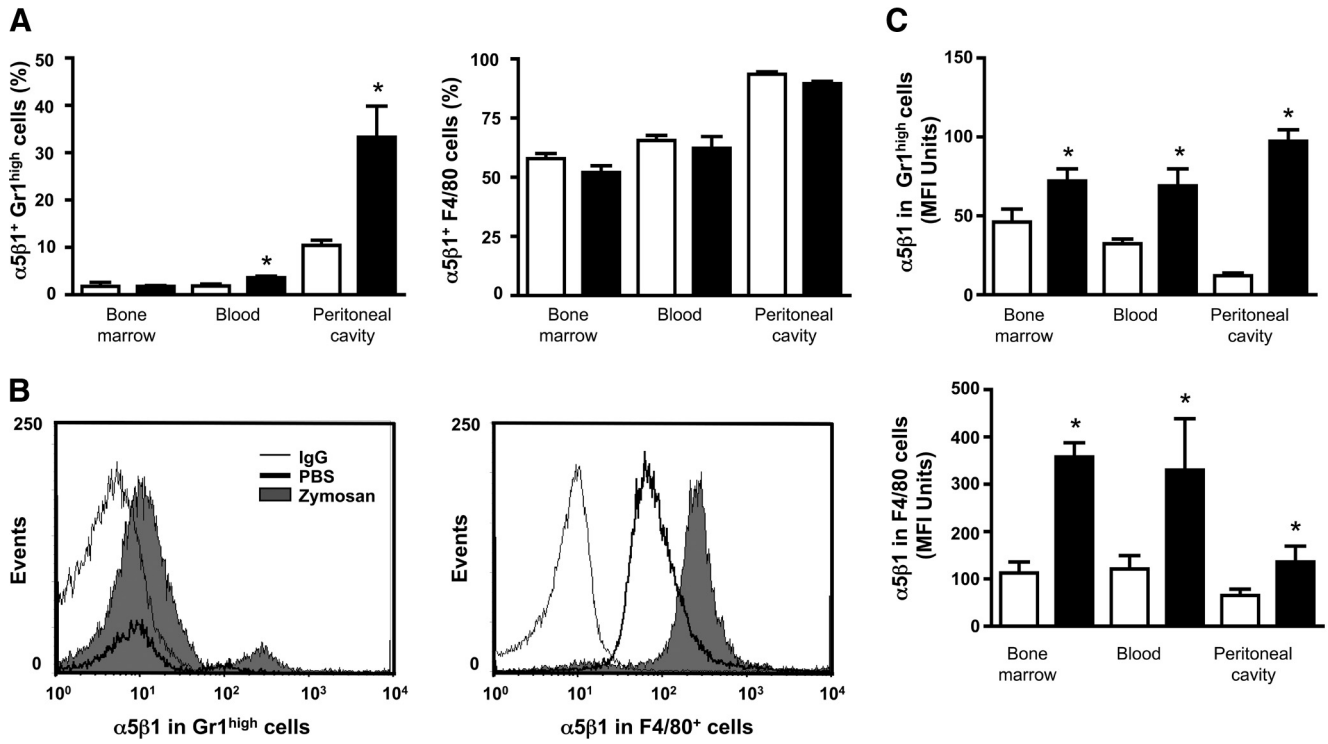

Figure 2. Modulation of α5β1 expression in bone marrow and blood cells during acute inflammation. Zymosan (1 mg i.p.) or PBS (0.5 ml i.p.) was injected into mice. Twenty-four hours later, bone marrow, blood, and peritoneal cavity Gr1^{high} and F4/80$^+$ leukocytes were analyzed for α5β1 expression by FACS, as described in Materials and Methods. At least 10^5 events were analyzed per sample. (A) Percentage of double-positive cells in the distinct compartments; (B) representative profile of α5β1 expression in peritoneal Gr1^{high} or F4/80$^+$ cells (24 h post-zymosan); (C) degree of α5β1 expression on Gr1^{high} or F4/80$^+$ cells, respectively. In all cases, data are mean ± SEM of at least five mice/group. *, $P < 0.05$, when compared with the respective PBS group.

RESULTS AND DISCUSSION

In this study, we set out to determine the potential role for $\alpha5\beta1$ on mouse leukocyte trafficking in inflammation, providing strong evidence that this integrin mediates, in part, the response of leukocytes to zymosan. Some degree of selectivity toward classical, nonselective inflammogens also emerges from these data, likely linked to the exposure of the major $\alpha5\beta1$ counter-receptor, fibronectin, within the inflamed microcirculation.

$\alpha5\beta1$ Expression in inflammatory cells

Initially, we investigated the zymosan peritonitis model [27, 28]. In line with published studies, naïve (data not shown) or PBS-injected (**Fig. 1**) peritoneal cavities provided essentially no $Gr1^{high}$ cells and on average, 2–3 million $F4/80^+$ cells/mouse. After zymosan injection, a $Gr1^{high}$ cell population was recruited promptly within 4 h—the peak of the response in our analyses—with its number declining steadily thereafter (Fig. 1A). Interestingly, whereas at the 4-h time-point, virtually all peritoneal PMN were $\alpha5\beta1$-negative (Fig. 1B), an $\alpha5\beta1$-expressing $Gr1^{high}$ subpopulation was evident at 24 h post-zymosan, with a slight decrease (~60%) by 48 h (Fig. 1B). In contrast to the $Gr1^{high}$ cells, $F4/80^+$ cells represented the majority of the cells resident within the noninflamed peritoneal cavity (PBS group ≥80%): In response to zymosan injection, this population displayed the expected kinetics [27, 34] so that compared with naïve animals, which possessed $2–3 \times 10^6$ $F4/80^+$ peritoneal cells, there was an initial decrease at the 4-h time-point, followed by a steady influx that reached a plateau at the 24-h time-point (Fig. 1C). Notably, the majority of $F4/80^+$ cells stained for $\alpha5\beta1$ (Fig. 1D) within the analyzed time interval.

Altogether, these data prompted us to investigate whether leukocyte $\alpha5\beta1$ expression was modulated in relation to the cell location and/or in response to inflammogen application. Indeed, other integrins, such as $\alpha M\beta2$, are known to be up-regulated during cell trafficking [35]. To address this point, subsequent peritoneal lavage, blood, and bone marrow analyses were carried out at the 24-h time-point to allow comparative assessment of $Gr1^{high}$ and $F4/80^+$ cell populations.

$\alpha5\beta1$ Expression in bone marrow and circulating cells

Figure 2 reports the comparison of $\alpha5\beta1$ expression in noninflamed with inflamed (24 h post-zymosan) mice. In PBS-injected animals, only a small percentage (~2%) of bone marrow and blood Gr-1^{high} cells expressed $\alpha5\beta1$ (Fig. 2A). This was not modified (bone marrow) or minimally elevated (blood, up to ~4%) after peritonitis induction. In contrast, peritoneal $\alpha5\beta1^+$ $Gr1^{high}$ cells, only minimally present in naïve peritoneal cavities, displayed an approximate threefold increase of $\alpha5\beta1$ expression 24 h post-zymosan application, from 10% to ~30% (Fig. 2A). In contrast to the $Gr1^{high}$ population, the majority of bone marrow (70%), blood (60%), and peritoneal (90%) $F4/80^+$ cells of PBS-injected mice was double-positive for $\alpha5\beta1$ (Fig. 2A): The extent of these double-positive populations was not altered following injection of zymosan, as assessed at the 24-h time-point (Fig. 2A).

When these data were analyzed at the single-cell level (see Fig. 2B for representative histograms of peritoneal cells), it was evident that injection of zymosan augmented $\alpha5\beta1$ expression on $Gr1^{high}$ and $F4/80^+$ cells, although only a fraction of the former displayed a marked increment in $\alpha5\beta1$ immunoreactivity (in line with data of Fig. 2A). Cumulative data for intensity of $\alpha5\beta1$ expression are in Figure 2C, which shows the results obtained for bone marrow, blood, and peritoneal compartments. Upon zymosan stimulation, $Gr1^{high}$ cells exhibited a marked increase of $\alpha5\beta1$ expression in all three compartments, and MFI units ranged between 70 and 90 (Fig. 2C, left panel). When ratios for Gr-1^{high} and $F4/80^+$ cells from PBS versus zymosan-treated mice were assessed with respect to $\alpha5\beta1$ expression, peritoneal $Gr1^{high}$ cells showed the most pronounced (approximately eightfold) up-regulation, reaching fluorescence intensities of ~100 MFI units (Fig. 2C, left panel). In contrast, induction of peritonitis triggered an approximate twofold increase in $\alpha5\beta1$expression on $F4/80^+$ peritoneal cells, although maximal MFI values were greater (~300 MFI units) when compared with $Gr1^{high}$ cells.

Collectively, these analyses indicate that treatment with zymosan induces tissue-specific and cell-specific regulation of $\alpha5\beta1$ expression during the course of an acute inflammatory reaction. An important role for de novo synthesis of $\alpha5\beta1$ in $Gr1^{high}$ cells could be an alteration of the cellular fate. Studies conducted with lymphocytes, endothelial cells, and fibroblasts [36–39] indicate that $\alpha5\beta1$ ligation can extend the cellular

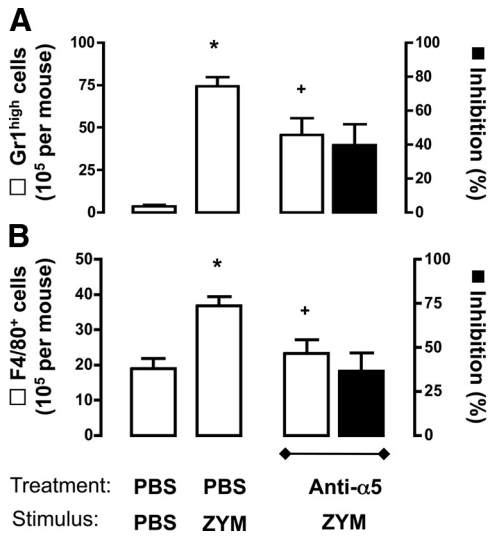

Figure 3. Anti-inflammatory effect of anti-α5 in zymosan peritonitis. Zymosan (ZYM; 1 mg i.p.) or PBS (0.5 ml i.p.) was injected into mice and $Gr1^{high}$ (A) or $F4/80^+$ (B) cell accumulation assessed at the 24-h time-point using FACS as described in Materials and Methods. Some mice were treated with an anti-α5 mAb (50 μg s.c. at time 0) or PBS (5 ml/kg). Open bars show changes in cell number, and closed bars show the percentage inhibition in response to treatment. Data are mean ± SEM of six to eight mice/group. *, $P < 0.05$, versus PBS/PBS (noninflamed) mice; +, $P < 0.05$, versus respective PBS/zymosan-treated animals.

lifespan, but there are no data for recruited PMN. It is possible that up-regulation of α5β1 could be instrumental to the extended survival phenotype acquired by the PMN once migrated into the inflammatory site. Another function of α5β1 may be regulation of the removal of peritoneal macrophages from the site of inflammation to the draining lymph nodes [40], as observed recently in a thioglycollate mouse peritonitis model [40]. The relevance of this mechanism to the findings we report here is uncertain, as this process may not be fully operative within the time-frame of our experiments. Nonetheless, we could observe a congruent (from 50% to 75%) increase in the percentage of $\alpha5\beta1^+/F4/80^+$ cells comparing the blood compartment with the peritoneal cavity; this change, also in consideration that locally produced cytokines and chemokines may enhance α5β1 expression and/or activity further [41], could contribute to subsequent removal of $\alpha5\beta1^+/F4/80^+$ cells during the active resolution phase of the peritonitis. A third possibility could be a direct effect on the process of leukocyte recruitment, and this was tested in the next series of experiments.

Anti-inflammatory effects of α5β1 blockade

Treatment of mice with a function-blocking anti-α5 mAb resulted in a significant decrease in $Gr1^{high}$ and $F4/80^+$ cell numbers by ~37% and 40%, respectively, as determined at the 24-h time-point (**Fig. 3A, B**). in fact, the inhibition on $F4/80^+$ cell-number increase caused by zymosan is nearer to 90% when assessed on net values.

These anti-migratory effects of the α5β1-blocking antibody were supported by the experiments of intravital microscopy, which were conducted over the 2- to 24-h time-course post-zymosan injection. **Figure 4, A–C**, shows representative images of the mesenteric microvasculature at 4 h in animals treated with zymosan and vehicle (Fig. 4B), zymosan, and anti-α5 mAb (50 μg; Fig. 4C), displaying a clear reduction in the extent of cell adhesion and emigration upon α5β1 blockade.

Zymosan injection increased the extent of leukocyte adhesion to the vessel wall and emigration into the mesenteric tissue at 2 and 4 h. By 24 h, cell adhesion in zymosan-injected animals returned to values observed for PBS-injected animals,

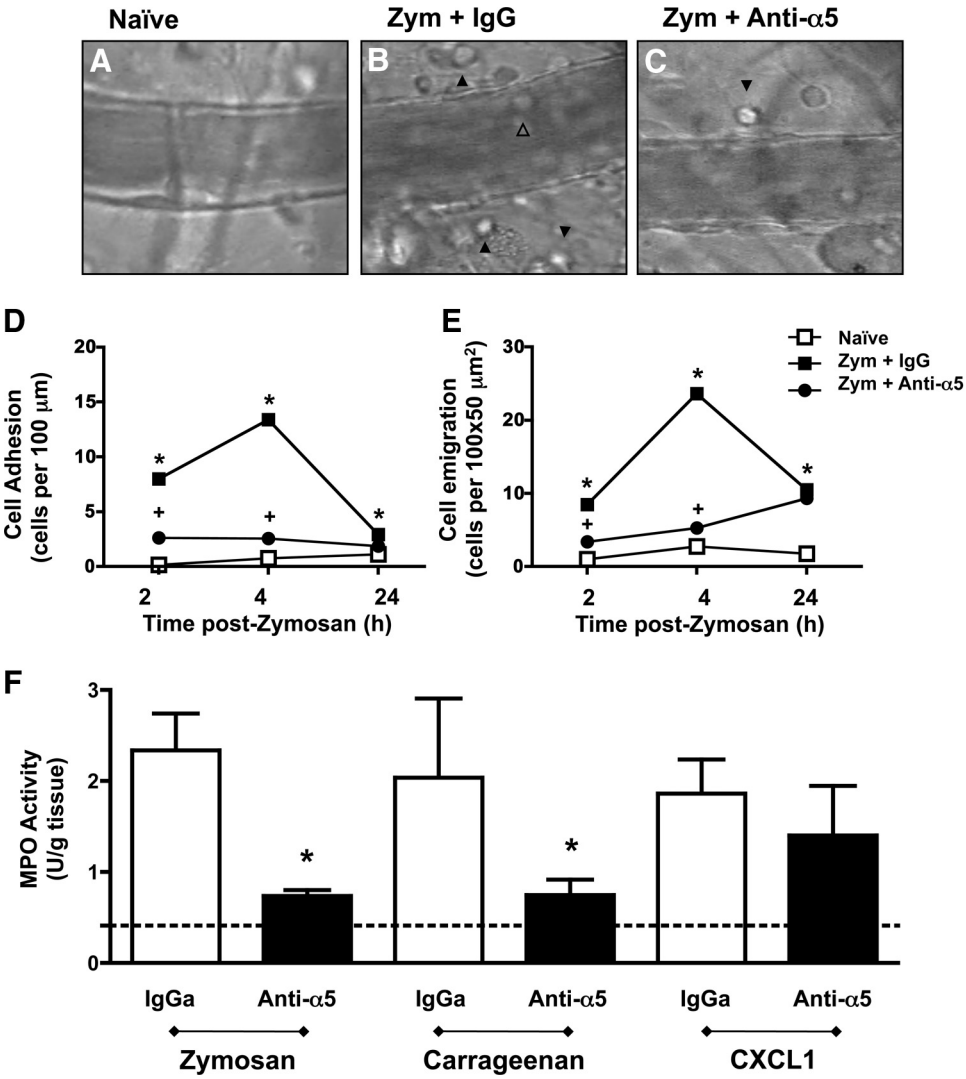

Figure 4. α5β1 sustains cell adhesion and emigration in the inflamed mesenteric microcirculation. Intravital images of a characteristic field of mesenteric microvasculature of animals; 4 h time-point from naïve animals (A) and zymosan-injected vehicle/irrelevant IgG-treated (B; 1 mg) animals treated with anti-α5 (C; 50 μg), showing adhered (open arrowhead) or migrated leukocytes (closed arrowheads). For a kinetic experiment, mice were treated with anti-α5 mAb and inflamed with zymosan. Intravital microcirculation analyses were conducted at 2, 4, or 24 h after zymosan. Quantification of cell adhesion (D) and cell emigration (E) was performed as described in Materials and Methods. Results are mean ± SEM of six mice. *, $P < 0.05$, zymosan-injected animals compared with PBS-injected animals; +, $P < 0.05$, treatments compared with zymosan-injected animals. (F) In selected experiments, peritoneal cavities were inflamed with zymosan (1 mg), carrageenan (500 μg), or CXCL1 (500 ng) 4 h prior to mesenteric tissue collection. Mice were perfused via the heart with 60 ml PBS to wash excess of cells from microvascular beds and mesenteric tissues collected and processed for MPO determinations. Results are expressed as mean ± SEM of six mice/group. *, $P < 0.05$, when compared with IgG-injected animals.

with consequent decrease in leukocyte emigration into the mesenteric tissue (Fig. 4, D and E). In these settings, blockade of $\alpha5\beta1$ activity led to a reduced extent of cell adhesion to the inflamed microvasculature and emigration into the subendothelial matrix in the first hours after zymosan injection. It is difficult to discriminate whether $\alpha5\beta1$ is responsible for leukocyte adhesion and migration, as if it were, blockade of the initial adhesion event would suppress cell migration naturally. Alternatively, $\alpha5\beta1$ may promote only cell adhesion (and therefore, recruitment of inflammatory cells onto the venule endothelium), and some other molecules are promoting migration. These experiments do not allow us to discriminate between these two possibilities, but whichever hypothesis is true, it remains that $\alpha5\beta1$ is a key player in the recruitment of inflammatory leukocytes in this model.

The intravital microscopy data were complemented by MPO analyses, which gave us interesting insights. In fact, treatment of mice with the anti-$\alpha5$ mAb yielded a high degree of inhibition of mesenteric MPO activity incremented by zymosan injection (Fig. 4F). Importantly, this set of experiments revealed that the anti-$\alpha5$ mAb treatment yielded significant inhibition also on carrageenan-induced MPO activity augmentation, whereas there was no effect of the still high degree of tissue inflammation provoked by the chemokine CXCL1 (Fig. 4F). Furthermore, when $Gr1^{high}$ infiltration into the peritoneal cavity was provoked by LPS, the anti-$\alpha5$ mAb was ineffective: $8.8 \pm 2.1 \times 10^5$ $Gr1^{high}$ cells/mouse in LPS + control IgG-treated mice and $10.9 \pm 1.1 \times 10^5$ $Gr1^{high}$ cells in the LPS + anti-$\alpha5$ mAb group (six mice/group; not significant). These latter data are in line with the study conducted in a model of lung inflammation [18]. A pattern is therefore emerging, whereby $\alpha5\beta1$ is endowed with proadhesive and promigratory functions, only when strong, exudative inflammatory agents (zymosan and carrageenan) are applied [42, 43] but not upon application of more subtle inflammogens/chemoattractants (CXCL1 and LPS). Furthermore, inhibition of these early events in the vasculature would translate into a significant attenuation of peritoneal $Gr1^{high}$ and $F4/80^+$ trafficking numbers at later timepoints. The potential contribution of other sites of action in the more complex model of peritonitis (e.g., cell trafficking from the omentum or mesothelial cell activation) cannot be excluded and would deserve further investigation. In the final set of experiments, we monitored expression of the major $\alpha5\beta1$-binding protein, fibronectin, in these settings of experimental inflammation.

Localization of fibronectin in the inflamed mesenteric microvasculature

We examined mesenteric tissue samples stained for PECAM-1 (to detect endothelial cells and identify vessel walls) and fibronectin (**Fig. 5**). Confocal analyses showed fibronectin deposits in extravascular regions of the inflamed microvasculature, and fibronectin is present on the basal and the luminal surface of the endothelial monolayers, including interendothelial spaces (arrow in Fig. 5).

The potential relevance of fibronectin exposure into the vessel augmented by the observation that it was augmented in the mesentery upon treatment with zymosan. As expected, fibronectin immunoreactivity in the subendothelial tissue of noninflamed mice was detected, but upon zymosan application, a marked tissue infiltration of leukocyte associated with large deposits of fibronectin (**Fig. 6**) was observed: Thus, tissue inflammation might increment tissue expression of fibronectin. Deposition of fibronectin in the vasculature and its increase during inflammation have been reported in a model of colitis [44]. Fibroblasts and other stromal cells could respond to activation with fibronectin synthesis and release [45]. It is more likely, at least within the time-frame of our experiments, that plasma exudation contributes markedly to this fibronectin deposition in inflamed tissues [42, 46, 47].

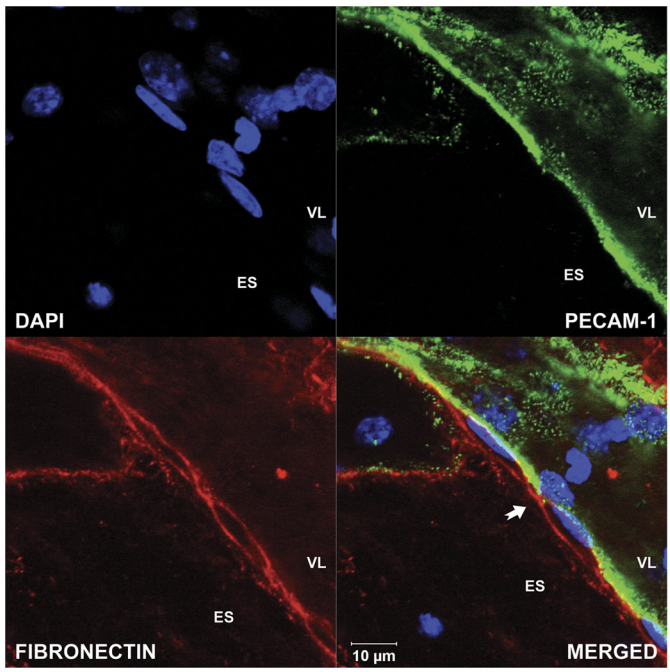

Figure 5. Fibronectin immunoreactivity on the vessel wall. Inflamed mesentery was harvested 4 h after injection of zymosan (1 mg i.p.) and prepared as described in Materials and Methods. (Upper left) Nuclear staining with DAPI; (upper right) endothelial lining (PECAM-1 immunostaining); (lower left) fibronectin is localized, not only in the basal membrane but also in the apical/luminal membrane of the endothelial cell; (lower right) merged image identifying a low PECAM-1 expression spot with the presence of fibronectin, which might have an anchored leukocyte (arrow). ES, Extravascular space; VL, vascular lumen.

Altogether, these integrated intravital and confocal microscopy analyses suggest that leukocyte adhesion, extravasation, and migration, across the deposits of tissue fibronectin scaffolding within the mesenteric microvasculation, are mediated by $\alpha5\beta1$. Based on the new, functional data, underpinned by the intravital observations, it is tempting to propose a wider scenario, where a fine and temporal interplay between $\alpha M\beta2$ and $\alpha5\beta1$ regulates leukocyte recruitment during inflammation in a cell type-specific manner. In addition, a role for $\alpha4\beta1$ in cell trafficking in this peritoneal model cannot be ruled out [48]. These data would beg for novel studies to determine whether a crosstalk between $\beta1$ and $\beta2$ integrins could be taking place at the level of the adherent leukocyte.

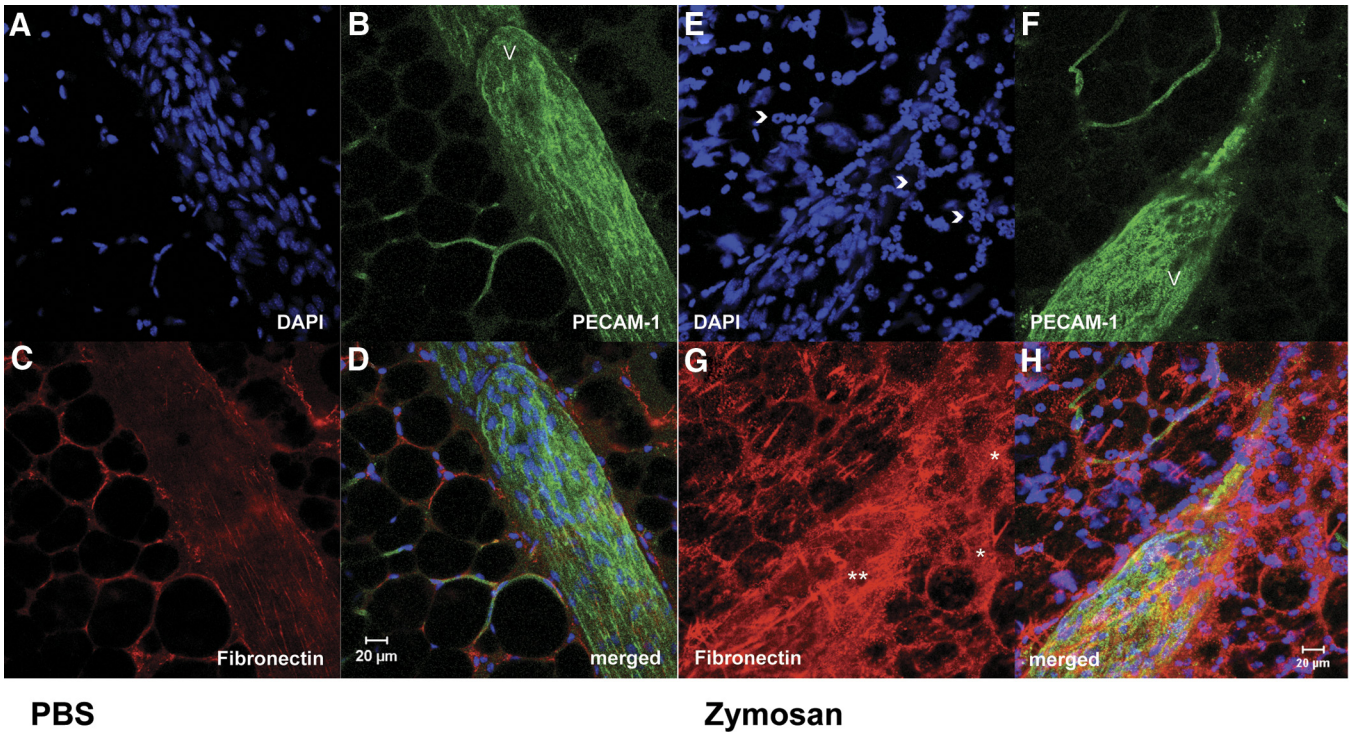

Figure 6. Fibronectin deposits in the inflamed mesenteric microcirculation. Mesentery was harvested 4 h after injection of PBS (0.5 ml i.p.) or zymosan (1 mg i.p.) and prepared as described in Materials and Methods. (A–D) Representative images from one PBS-injected animal with (E–H) reporting images from a zymosan-injected animal. (A and E) Nuclear staining with DAPI; (B and F) staining for PECAM-1; (C and G) fibronectin; (D and H) merged images. Arrowheads point to multi-lobe PMN nuclei, largely evident in zymosan-treated mice. Of note, the large deposition of fibronectin (stained in red) in the tissue surrounding the vessel (V), highlighted in green for PECAM-1. Intravascular and extravascular fibronectin is also indicated by ** and *, respectively. Images are representative of five distinct scans of independent fields.

In conclusion, we have described a detailed pattern of expression for α5β1 on myeloid cells and suggested a functional role in their mobilization from bone marrow to blood and to peritoneal cavity. The trafficking of $\alpha5\beta1^{+}$ cells—$Gr1^{high}$ and $F4/80^{+}$ populations—produced by zymosan injection, prompted us to test the efficacy of α5β1 blockade strategies. We report that blockade of α5β1 integrin revealed an important role for this integrin in cell adhesion and emigration in the inflamed mesentery. In view of the biological activities attributable to α5β1, which include promoting angiogenesis and cell recruitment in inflammation, we propose that anti-α5β1 strategies (e.g., small molecule α5β1 integrin antagonists; refs. [24, 49]) could also be beneficial in more complex inflammatory pathologies, such as rheumatoid arthritis, where both processes might have an etiological function [50, 51].

AUTHORSHIP

André L. F. Sampaio designed experiments, performed experiments, analyzed data, and wrote the first draft. Grit Zahn planned experiments, analyzed data, and contributed to the manuscript writing. Giovanna Leoni performed experiments and analyzed data. Doerte Vossmeyer planned experiments and analyzed data. Claudia Christner analyzed data and wrote the second draft. John F. Marshall designed experiments, performed experiments, and analyzed data. Mauro Perretti planned the project, designed experiments, and wrote the manuscript.

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