Artigo Original

Acute peritonitis: adhesion molecules are regulated by a balance between endogenous and exogenous Annexin A1

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ABSTRACT

This study evaluated the effect of endogenous and exogenous annexin A1 (ANXA1) on cell migration through the modulation of β2-integrin (CD11b) and L-selectin (CD62L) adhesion molecules, using a classic model of peritonitis. Wild type (WT) and ANXA1 null (ANXA1null) mice, pretreated or not with the peptide Ac2-26 (ANXA1 N-terminal; 100 µg) received i.p. administration of zymosan (1.0 mg). After, blood, peritoneal exudates and mesenteries were evaluated by biochemical and cellular analysis at 0, 4 and 24 h. Exogenous administration of Ac2-26 significantly decreased polymorphonuclear cells (PMN) trafficking and TNF-α release 4 h after the peritonitis process. In the inflammation resolution phase (24h), no difference was observed between animals treated with Ac2-26 or not. In addition to the change in cell recruitment, pretreatment with Ac2-26 significantly increased the expression of L-selectin and β2-integrin in blood PMN, but no effect was observed in these cells on ANXA1null. Ultrastructural analysis showed co-localization between ANXA1 and adhesion molecules, particularly CD11b, in the plasma membrane and cytoplasm of neutrophils and endothelial cells. Administration of Ac2-26 significantly reduced CD11b levels in these cells, but ANXA1null neutrophils had a high proportion of CD26L that was not modulated by the exogenous peptide. We envisage that the endogenous levels of ANXA1 are important to control the inflammatory response through CD62L in PMN, whereas exogenous ANXA1 regulates the release of pro-inflammatory cytokines and CD11b in PMN and endothelial cells.

Keywords: β2-Integrin; Immunogold electron microscopy; L-Selectin; Neutrophil; Peptide Ac2-26; Zymosan.

INTRODUCTION

Annexin A1 (ANXA1) was the first cloned member of a superfamily of proteins that is bound to phospholipids in a calcium-dependent manner (1, 2). Structurally, annexins comprise are comprised of two domains: a small N-terminal region, varying in length and composition, and a central domain, the C-terminus, consisting of four or, as in annexin 6 by eight-fold repeat of a conserved sequence of 70-80 amino acids (2). The N-terminal domain is specific for each annexin family member and interacts with different ligands of these proteins, while the C-terminal region is responsible for

the affinity to calcium and binding to phospholipids.

One of the inflammatory process mechanisms that ANXA1 regulates is the reduction of neutrophils in inflammatory sites (3, 4). Human neutrophils have high levels of ANXA1 (about 1-2% of total cytosolic proteins) and may release large amounts of this protein (> 50%) in adherence to the endothelial cell monolayer (5-7). The ANXA1, exposed on the plasma membrane of adherent leukocytes, exerts an inhibitory action, reducing the length of transmigration across endothelial cells (8).

Leukocyte transmigration is regulated by the action of exogenous ANXA1 by shedding of L-selectin (CD62L) (9), adhesion molecule that is responsible for rolling of these cells on the endothelium (10). Experiments using human neutrophils in a flow chamber with a monolayer of endothelial cells from human umbilical cord (HUVEC) demonstrated that the peptide Ac2-26 inhibited the transmigration of PMN through CD62L shedding by binding with the FPRL1 and FPR (11) receptors. In addition, the activity of ANXA1 and the blockade of adhesion molecule β2-integrin (CD11b) by monoclonal antibody were compared in the air pouch model in mice and a complementary effect was demonstrated on the anti-migratory action on PMN into the tissue (12). These results suggest an ANXA1 autocrine and paracrine mechanism in inhibiting firm adhesion during the inflammatory process.

Similarly, the absence of endogenous ANXA1 induced increase in leukocyte transmigration in knockout animals for ANXA1 ($ANXA1^{null}$), an effect associated with increased expression of CD62L in PMN (13). Moreover, PMN $ANXA1^{null}$ animals showed an increase in CD11b expression of in vivo models of inflammation by endotoxin LPS (14) and in vitro after incubation of cells with PAF (activation factor platelets), fMLP (formyl-methionine-leucine-phenylalanine) and PMA (phorbol 12-myristate 13-acetate) mediators (13, 15). The data from these investigations demonstrate the active participation of endogenous ANXA1 in the regulation of expression of adhesion molecules in different experimental inflammation models.

Studies related to the effect of ANXA1 on the expression of adhesion molecules are involved in most peripheral blood PMN flow cytometry analyzes or in in vitro assays. (13, 14). However, the interaction of this protein with adhesion molecules in PMN-endothelial cell interactions during the cell transmigration process has been little explored by morphological and ultrastructural studies. In this respect, the purpose of this study was to investigate the expression and co-localization of ANXA1 and the adhesion molecules CD11b and CD62L during leukocyte-endothelial interactions in the mesentery, using a classic model of acute peritonitis induced by zymosan in mice. Furthermore, we tested the effect of pharmacological pre-treatment with peptide Ac2-26 (the N-terminal ANXA1) in this

experimental model using wild-type (WT) and ANXA1^{null} mice.

MATERIALS AND METHODS

Animals

Male wild-type (WT) littermate and ANXA1 null mice (HANNON et al., 2003) (20–25 g of body weight), maintained on a standard chow pellet diet with tap water ad libitum, were used for all experiments. Animals were housed at a density of five animals per cage in a room with controlled lighting (lights on from 8:00 a.m. to 8:00 p.m.) in which the temperature was maintained at 21–23°C. All experiments were performed according to protocols approved by U.K. Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act 1986) and Committee on Care and Use of Laboratory Animal Resources of the School of Medicine (Protocol nº 3015/06), São Jose do Rio Preto, SP, Brazil.

Zymosan Peritonitis

Experimental peritonitis was induced by i.p. injection of 1 mg of boiled zymosan A (Sigma-Aldrich) in 0.5 ml of sterile saline (16, 17), whereas control animals were injected with an equal volume of saline. Each group consisted of 5 animals (control, 0 h; peritonitis, 4 and 24 hs). At different time points (0, 4 and 24 hs), animals were anesthetized with ketamine and xylazine (100 and 20 mg/Kg, i.p.) for collection of blood aliquots (maximum 1 ml), before euthanasia and washing of the peritoneal cavity with 3 ml of PBS supplemented with 3 mM EDTA. Then, fragments of the mesentery were collected and processed as described below.

In another set of experiments, mice were treated with 100 µg per animal of peptide Ac 2-26 i.p. (Ac-AMVSEFLKQAWFIENEEQEYVQTVK); obtained from Invitrogen, São Paulo, Brazil) in 100 µl of sterile saline 15 min before zymosan injection. This dose was scaled up from a previous study with this model of peritonitis (18). PMN recruitment into the peritoneal cavity, blood and mesentery was assessed at the 4 and 24h time-points.

Cellular analyses and quantification

Aliquots of blood (20 µl) or peritoneal lavage fluids (100 µl) were diluted 1/10 in Turk's solution (0.1% crystal violet in 3% acetic acid); total and differential counting were obtained with a Neubauer chamber using a 40 objective and analyzing on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany). Peritoneal cells and blood were distinguished in polymorphonuclear (PMN) and monocyte/macrophages (mono-M∅). Data were reported as mean ± SEM of the average number of cells x 10^5 / mL from blood samples and number of cells x 10^5 per animal from peritoneal washing.

Blood Flow Citometry.

In order to quantify L-selectin (CD62L) or β2-integrin (CD11b) expression, leukocytes were isolated from abdominal aorta blood collected in EDTA (100 mg/mL). Erythrocyte lysis was performed using an ammonium chloride solution (0.13 M), and leukocytes we 109 recovered after washing the preparation with PBS. The cells (1.0 x 10^6) were incubated for 30 min at 4ºC in the dark with 10 µL of monoclonal antibodies against L-selectin conjugated to PE (anti-mouse CD62L) and β2-integrin conjugated to FITC (anti-mouse CD11b) (BD Pharmigen, San Diego, USA). Immediately after incubation, the cells were analyzed using a FACSCalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Data were obtained from 10,000 cells and only morphologically viable neutrophils were considered for analysis. Leukocytes were separated based on size and granularity. Fluorescence was determined and the results are expressed as the mean fluorescence of two assays performed in duplicate.

Biochemical analyses*: *cytokine levels.

Aliquots of peritoneal lavage fluids were centrifuged at 400x *g*, for 10 min, and tested for TNF-α, IL-1β and IL-6 according to the manufacturer's protocol (R&D Systems).

Histological analyses: mesentery flat mount.

Fragments of WT mesentery were stretched with pins and fixed in formol 10% for 30 min. After, they were washed with distilled water, stained by the toluidine blue solution (30 min), separated from the small intestine and distended on the slides for the histological quantification of inflammatory cells. Analysis of cells in the mesenteric connective tissue was performed at 0, 4 and 24 hs post-zymosan administration with or without prior drug treatment (peptide Ac2-26) with high power objective (x40) counting PMNs in 1 mm^2 areas (analyzing at least 10 sections of tissues per group). Values are reported as mean ± SEM of number of cells per mm^2.

Transmission Electronic Microscopy - Post-embedding Immunogold Labeling

Fragments of the WT and $ANXA1^{null}$ mesenteries at 4h post-zymosan administration were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, 0.1% sodium cacodylate buffer (pH 7.4) for 24 h at 4°C. They were then washed in sodium cacodylate, dehydrated through a graded series of methanol, and embedded in LR Gold (London Resin Co., Reading, Berkshire, UK). Sections (0.5 µm) were stained with 1% Toluidine blue in 1% Borax solution (TAAB Laboratories, Aldermaston, Berkshire, UK). Sections (70 nm) were cut on an ultramicrotome (Reichert Ultracut; Leica, Austria) and placed on nickel grids for immunogold labeling.

To detect the expression of ANXA1 and adhesion molecules (CD11b and CD62L) on PMNs (intravascular and transmigrated cells) and endothelial cells of mesenteries, a post-embedding immunogold labelling double

reaction was performed. Ultrathin sections were incubated: (1) phosphate-buffered solution (PBS) containing 1% egg albumin (PBEA); (2) PBS containing 5% egg albumin (PBEA) for 30 min; (3) the polyclonal sheep anti-ANXA1 (1:200) (Abcam, Cambridge, UK) and the polyclonal goat anti-CD11b (1:200) or monoclonal rat anti-CD62L (1:50) (Santa Cruz Biotechnology, California, USA) for 2h; (4) normal sheep serum and normal goat or rat serum, respectively, were used as control; (5) after washes in PBEA, in order to detect ANXA1, a donkey anti-sheep IgG antibody (1:100 in PBEA) conjugated to 15 nm colloidal gold (British Biocell, Cardiff, UK) was added, and to detect CD11b or CD62L, a rabbit anti-goat or goat anti-rat IgG antibody (1:100 in PBEA) conjugated to 10 nm colloidal gold (British Biocell) was added; and (6) after 1h, sections were washed in PBEA, and then in distilled water.

Ultrathin sections were stained with uranyl acetate and lead citrate before examination on a ZEISS LEO 906 electron microscope (Electron Microscope Centre, IBILCE-UNESP). Randomly photographed sections of intravascular and transmigrated PMNs to the tissue were used for the immunocytochemical analysis. The area of cell compartment was determined with the Axiovision software. The density of immunogold labeling (number of gold particles per μm^2) was calculated and expressed for each cell compartment. Values are reported as mean ± SEM of *n* electron micrographs analyzed.

Statistical Analysis

Statistical differences between means were determined by analysis of variance followed, if significant, by the Bonferroni test. A probability value less than 0.05 was taken as significant.

RESULTS

Effect of pharmacological pretreatment with peptide Ac2-26 on cellular response

As expected, the effect of i.p. zymosan administration in mice, after 4 h, was associated with an intense inflammatory response characterized by an increase in the PMN number in the blood, peritoneal exudate and mesentery, compared to control animals (Table 1). In the later experimental time, 24 h, the levels of PMN decreased in the blood, peritoneal exudate and mesentery, with values similar to the control group (Table 1).

Pretreatment of mice with peptide Ac2-26, after 4 h, significantly decreased the number of PMN in the blood, peritoneal exudate and the mesentery compared to those without drug treatment (Table 1). At 24 h, the effect of exogenous administration of the peptide was similar to untreated animals, by reducing the number of PMN in blood and peritoneal washing (Table 1). However, a significant increase of PMN was observed in the tissue, compared to the untreated group.

Table 1. Quantitative analysis of PMN migration in the mouse [a]

Time (h)/ Treatment	Collected Samples		
	Blood ($x 10^5$ cells/ml)	Peritoneal Lavage ($x 10^5$ cells/animal)	Mesentery (cells/mm^2)
0 (control)	8 ± 3	3 ± 2	1 ± 0,5
4 / Vehicle	16 ± 3[b]	80 ± 16[b]	44 ± 10[b]
24 / Vehicle	5 ± 0.8	7 ± 2	4 ± 2
4 / Peptide Ac2-26	5 ± 1.16[c]	32 ± 5[c]	13 ± 4[c]
24 /Peptide Ac2-26	2 ± 0.3	16 ± 6	127 ± 23[c]

[a] WT mice were treated with zymosan (1 mg i.p.) at time 0 and blood, peritoneal exudates and mesenteries collected at the reported time points. Tissue preparation was done as described in *Materials and Methods.* Values are expressed as mean ± SEM of tissues analyzed from 5 mice/group.
[b] $p < 0.05$ vs control group (0h).
[c] $p < 0.01$ vs corresponding vehicle group (Bonferroni test).

Cytokine Detection

Similarly to cell trafficking, profiles of cytokine levels were markedly altered by zymosan peritonitis at 4h, with expected transient increase in peritoneal exudate TNF-α (91.93 ± 13.50 pg/ml), followed by changes in IL-1β (374.4 ± 44.38 pg/ml) and IL-6 (668 ± 46.94 pg/ml) compared to non detected levels in control group. In mice pretreated with Ac2-26 the TNF-α response was diminished significantly at this time point (27.95 ± 9.26 pg/ml), but the IL-1β (272 ± 49.5 pg/ml) and IL-6 (672 ± 13 pg/ml) response was not changed compared to untreated group. At 24h, cytokine levels were not detected in all experimental groups.

L-selectin and β2-integrin expression on blood PMN

Besides alteration in blood cell counts, PMN of mice pretreated with Ac2-26 displayed a significantly higher degree of activation at 4h, as assessed by L-selectin and β2-integrin expression (Fig. 1A and B). At 24h, the effect of pharmacological treatment was also associated with high significant levels of L-selectin on PMN compared to cells of untreated mice (Fig. 1A).

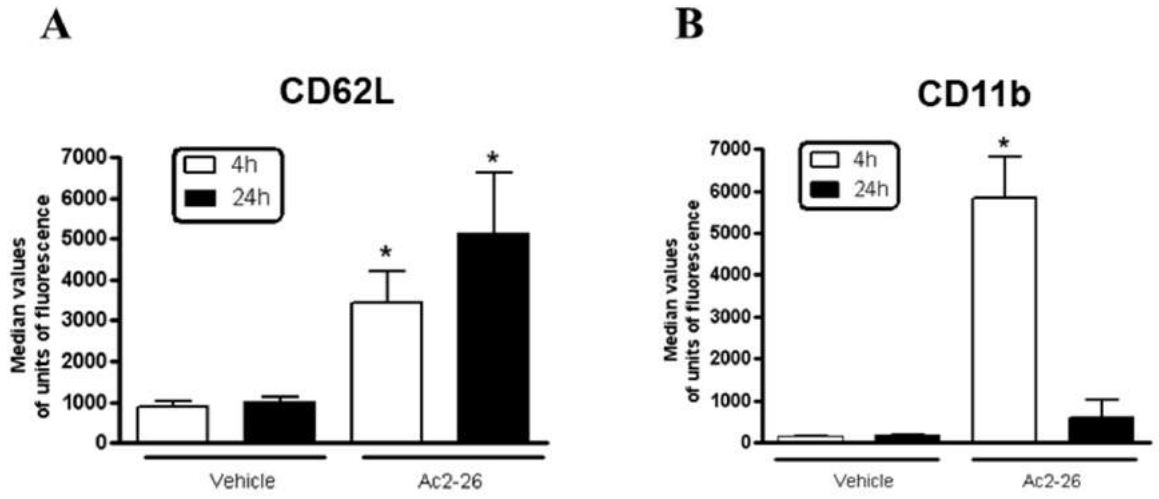

Figure 1. L-selectin and β2-integrin expression in circulating leukocytes. Mice pretreated i.p. with vehicle (PBS) or Ac2-26 (100 μg) received 1 mg of zymosan i.p. at time 0. At different time points after zymosan, blood was collected and CD62L [**A**] and CD11b [**B**] expressions on leukocytes quantified by flow cytometry. Data are mean ± SEM from two experiments performed with three mice each. **P* < 0.05 versus correspondent vehicle group value.

To confirm this result we assessed the expression of adhesion molecules by immunocytochemical analysis on blood neutrophils at 4h of inflammatory response. In addition, we investigate the effect of absence of endogenous ANXA1 by using neutrophils from null mice for ANXA1 (ANXA1^{null}) in the same experimental conditions. As show by Figure 2, wild-tipe (WT) neutrophils from untreated mice (vehicle) presented low levels of L-selectin and β2-integrin in the cytoplasm and plasma membrane (Fig. 2A and D) compared to neutrophils from Ac2-26 treated animals (Fig. 2B and E). Some points of co-localization between ANXA1 and adhesion molecules were detected in these cells (Fig. 2B and E). Quantification of gold particles associated with adhesion molecules confirmed the significant proportion of them in the treated group, while no effect was detected in the ANXA1^{null} cells (Fig. 2C and F).

Ultrastructural analysis of ANXA1 and adhesion molecules

The expression of ANXA1 and adhesion molecules β2-integrin (CD11b) and L-selectin (CD62L) was detected after 4 h in neutrophils and postcapillary venule endothelial cells of the mesentery, through post-embedding immunogold labeling, to show their localization and modulation during the initial inflammatory response in WT and ANXA1^{null} mice.

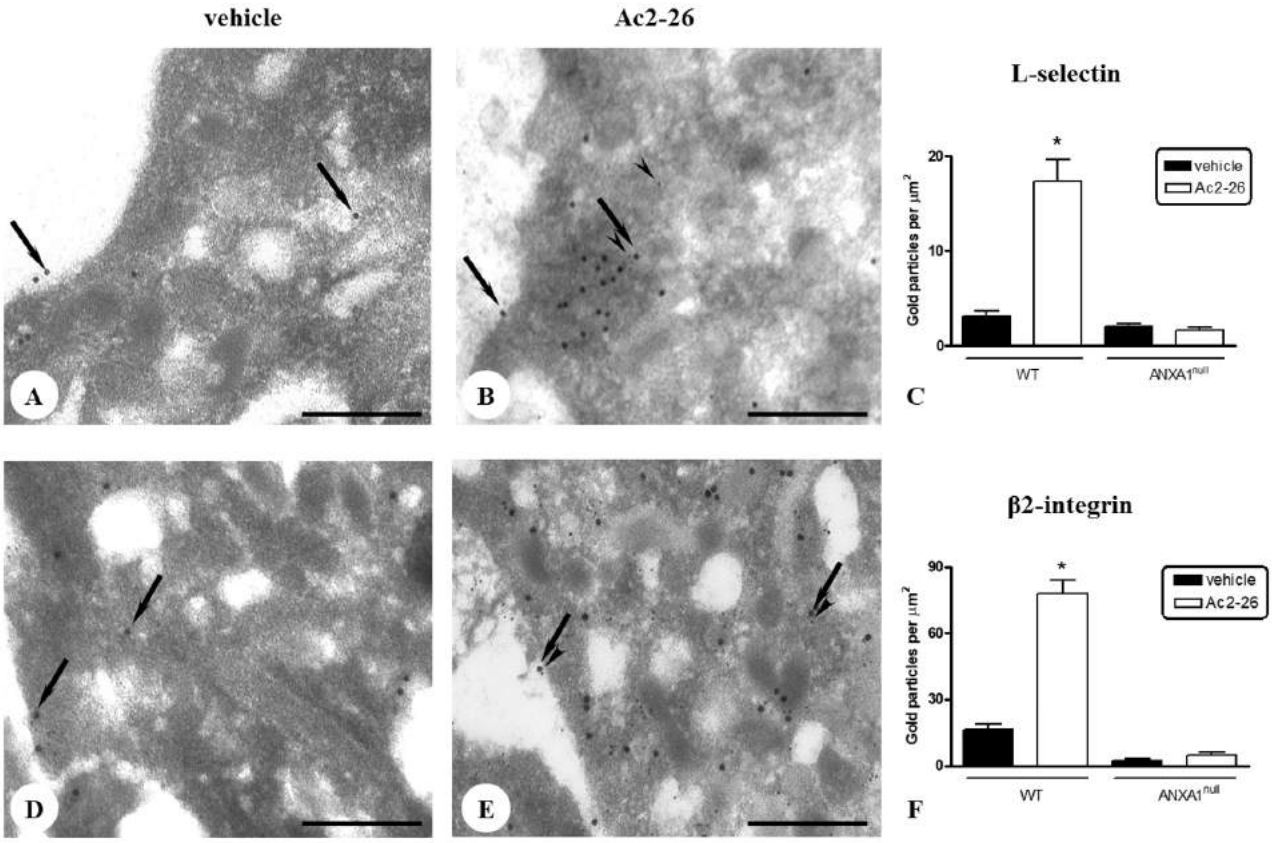

Figure 2. Immunogold detection of L-selectin and β2-integrin in WT and ANXA1^{null} blood neutrophils after 4h of peritonitis. [**A and D**] A low degree of immunoreactivity was obtained with adhesion molecules in the cytoplasm and plasma membrane (arrows) of WT neutrophils from non-treated mice (vehicle). [**B and E**] After pharmacological treatment, WT neutrophils presented a significant proportion of L-selectin and β2-integrin in the subcellular compartments (arrows) with points of co-localization with ANXA1 (arrowheads). Scale bars, 0.5µm. Density of L-selectin [**C**] and β2-integrin [**F**] immunogold particles in neutrophils. Data are mean ± SEM of 10 distinct cells for each group analyzed in the blood of 4 mice. *p < 0.001 *vs.* vehicle WT group.

The analysis of neutrophils (intravascular and transmigrated) and endothelial cells in the WT mesentery showed immunoreactivity of ANXA1 and CD11b mainly in the plasma membrane and cytoplasm, often presenting points of co-localization (Fig. 3A and C). In addition, WT transmigrated neutrophils and endothelial cells next to them showed a significant increase of endogenous ANXA1 with 18.2 ± 1.6 and 31.5 ± 4.5 gold

particles/μm^2 ($p<0.05$), respectively, compared to levels shown by intravascular neutrophils (8.6 ± 1.4 gold particles/μm^2) in contact with endothelial cells (15.9 ± 2.7 gold particles/μm^2).

When CD11b expression of neutrophils was compared between both genotypes, we observed a significant reduction of this adhesion molecule in the ANXA1^{null} cells (Fig. 3B and D) compared with WT (Fig. 3E). The ANXA1^{null} endothelium in interaction with intravascular neutrophils, also showed low expression of CD11b compared to WT endothelium (Fig. 3F).

The effect of pharmacological treatment with peptide Ac2-26 in the initial inflammatory response (4h) was associated with a significant decrease in the CD11b expression on WT neutrophils and endothelial cells (Fig. 3E and F). In ANXA1^{null} animals, pretreatment with peptide provoked opposite effect on CD11b expression causing a significant increase in intravascular neutrophil and endothelial cells (Fig. 3E and F).

CD62L was detected after 4h of experimental peritonitis in the cytoplasm and especially in the plasma membrane of neutrophils from WT animals, showing a few points of co-localization with the ANXA1 protein (Fig. 4A and C). In ANXA1^{null} intravascular neutrophils, we observed stronger immunoreactivity of CD62L in the cytoplasm and plasma membrane (Fig. 4B and D) compared to WT cells, as confirmed by quantitative analysis of gold particles (Fig. 4F). No immunogold labeling was detected in sections used as control of the reaction, incubated with PBEA without primary antibody (Fig. 4E).

Exogenous administration of peptide Ac2-26 showed no change in expression of this adhesion molecule as evidenced in the ultrastructural immunocytochemistry analysis of neutrophils from WT and ANXA1^{null} mesentery, followed by quantification of gold particles associated with CD62L (Fig. 4F).

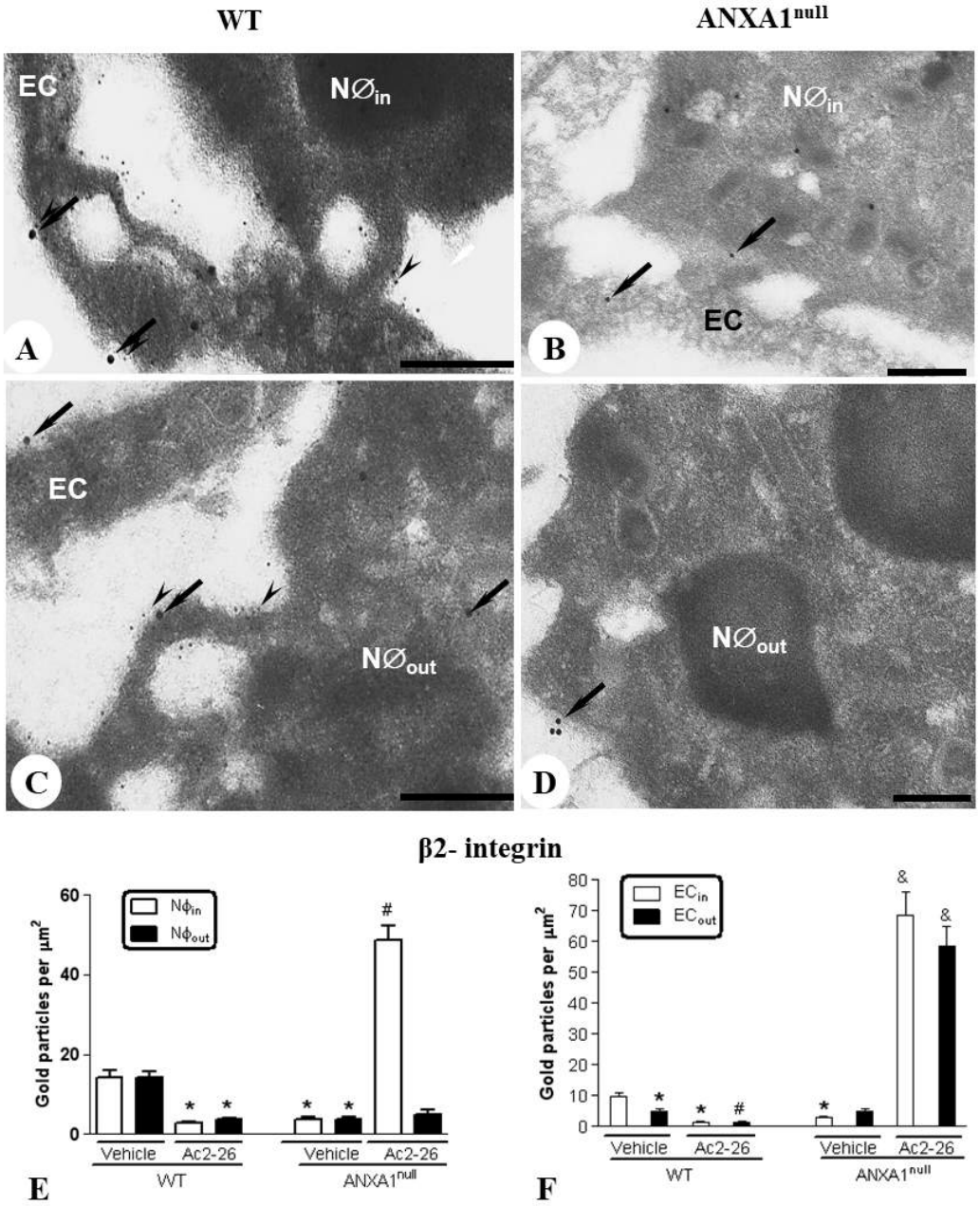

Figure 3 - Immunocytochemical localization of ANXA1 and β2-integrin (CD11b) in neutrophils and endothelial cells from WT and ANXA1^{null} mesenteric tissue. [**A and C**] Interplay between intravascular (Nø$_{in}$) and transmigrated neutophils (Nø$_{out}$) and post capillary venule endothelial cells (EC) showing co-localization of ANXA1 (arrowheads) and CD11b (arrows) in the plasma membrane and cytoplasm at 4h of peritonitis. [**B and D**] Neutrophils and ECs from ANXA1^{null} mesentery presented low expression of CD11b. Scale bars, 0.5µm. Density of CD11b immunogold particles in neutrophils [**E**] and endothelial cells [**F**] at 4h post-zymosan administration in WT and ANXA1^{null} mice pretreatet or not with Ac2-26. Data are mean ± SEM of 10 distinct cells for each group analyzed in the mesenteric tissue samples of 4 mice. (**E**) *$p < 0.05$ *vs.* vehicle WT Nϕ$_{in}$ and Nϕ$_{out}$; #$p < 0.001$ *vs.* all experimental groups. (**F**) EC$_{in}$ = EC interacting with Nø$_{in}$; EC$_{out}$ = EC interacting with Nø$_{out}$. *$p < 0.05$ *vs.* vehicle WT EC$_{in}$; $^{\#}p < 0.05$ *vs.*vehicle WT EC$_{out}$; $^{\&}p < 0.001$ *vs.* all experimental groups.

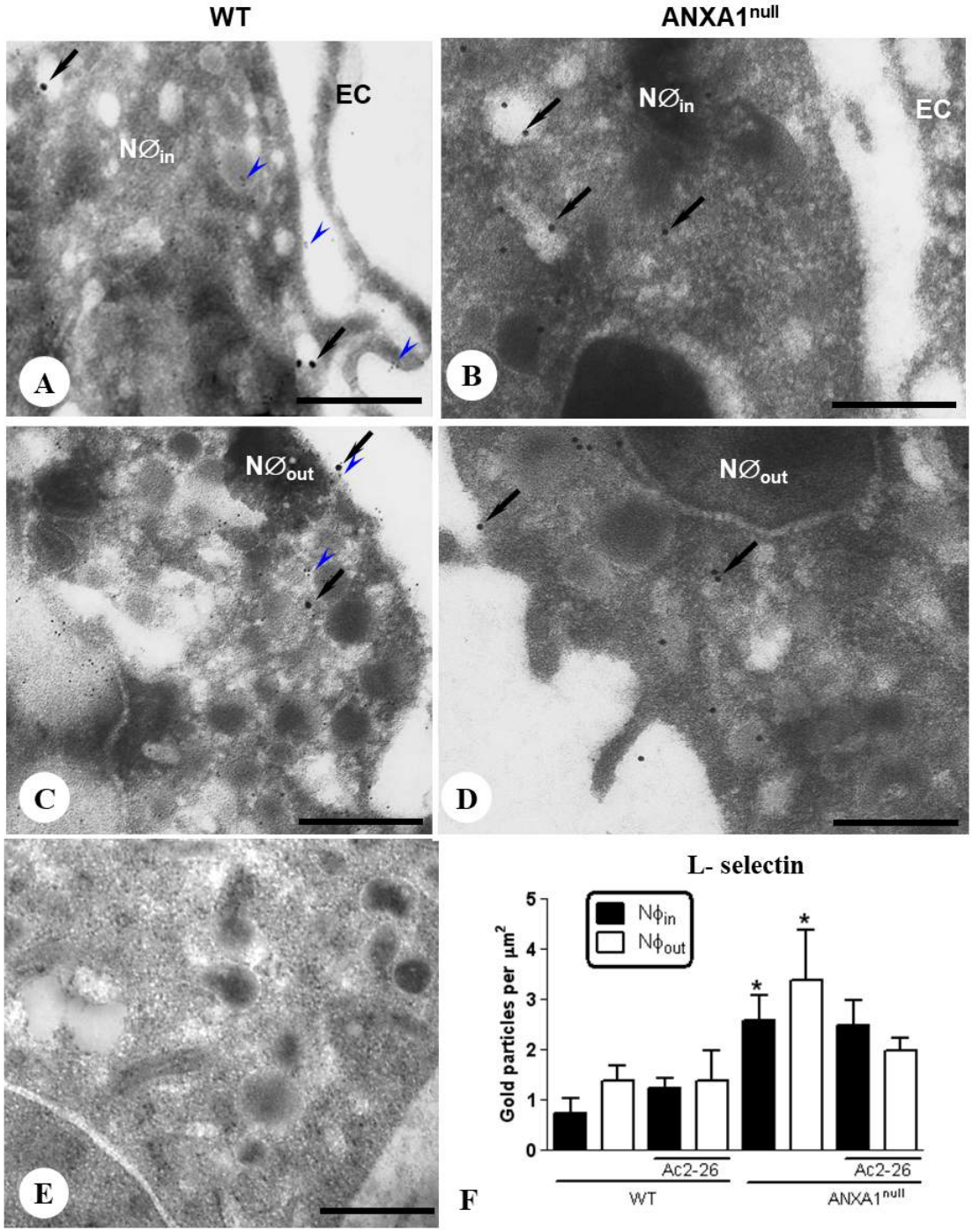

Figure 4 - Expression of ANXA1 and L-selectin (CD62L) on neutrophils from WT and ANXA1^{null} mesentery. (A, C) After 4h of inflammatory response, ANXA1 (arrowheads) and CD62L (arrows) were detected in the cytoplasm and plasma membrane of intravascular ($N\phi_{in}$) and transmigrated ($N\phi_{out}$) neutrophils and poorly co-localized. Endothelial cell (EC). **[B and D]** ANXA1 null neutrophils showed a high immunoreactivity of CD62L (arrows). **[E]** Absence of gold labeling in control section of reaction. Scale bars, 0.5µm. **[F]** Density of CD62L immunogold particles in $N\phi_{in}$ and $N\phi_{out}$ at 4h post-zymosan administration in WT and ANXA1^{null} mice pretreatet or not with Ac2-26. Data are mean ± SEM of 10 distinct cells for each group analyzed in the mesenteric tissue samples of 4 mice. *$p < 0.01$ *vs.* vehicle WT $N\phi_{in}$ and $N\phi_{out}$.

DISCUSSION

This study evaluated the effect of exogenous ANXA1 in the modulation of cell migration and its colocalization with adhesion molecules β2-integrin (CD11b) and L-selectin (CD62L), using a classic model of peritonitis induced by zymosan in mice treated or not with peptide Ac2-26 (19, 20). In addition, we analyzed the expression of these adhesion molecules in the absence of endogenous ANXA1 during transmigration of polymorphonuclear leukocytes (PMN) using the same experimental conditions in null animals for ANXA1 (ANXA1^{null}).

In order to verify the action of exogenous ANXA1 on PMN recruitment during zymosan peritonitis, animals were pretreated with peptide Ac2-26 (N-terminal region of ANXA1) fifteen minutes prior to induction of experimental peritonitis (18). Zymosan administration resulted in the expected effects of this experimental model (19-21), characterized by an increase in the number of PMN after 4h in blood, peritoneal lavage and the mesentery, followed by a significant decrease (> 50%) after 24h. The action of exogenous peptide Ac2-26 promoted an inhibition of ~ 70% and 50% in the recruitment of PMN to the peritoneal cavity and blood, respectively, and a significant decrease in the release of tumor necrosis factor-α (TNF-α) in peritoneal washing after 4 h. Similarly, DAMAZO et al. (2006) using the murine model of zymosan peritonitis, showed that treatment with peptide Ac2-26 also decreased the release of pro-inflammatory cytokines, such as KC and IL-1β. Even in our studies, histological analysis of the mesentery after 4 h, confirmed the anti-migratory role for this protein on the PMN reported in the literature (6, 21, 22).

To understand the mechanisms involved in the inhibition of cell transmigration by ANXA1, detected at 4 h time point of peritonitis, we analyzed by ultrastructural immunocytochemistry the expression of this protein and adhesion molecules, β2-integrin (CD11b) and L-selectin (CD62L) in neutrophils and endothelial cells of animals pretreated or not with peptide Ac2-26. The ANXA1 expression was demonstrated in the cytoplasm and plasma membrane of neutrophils, confirming previous data (6, 23, 24). Furthermore, we observed increased levels of ANXA1 in endothelial cells and transmigrated neutrophils from mesentery compared to intravascular cells. Similar ultrastructural results were described for transmigrated neutrophils in the mesentery of rats after experimental inflammation was induced by carrageenan (6). In addition, an increase in the Anxa1 gene expression was triggered by zymosan after 4 h in ANXA1^{null} animals as assessed by X-gal method in light microscopy (18). The increase in the ANXA1 levels in neutrophils during the transmigration

process, probably contributes to control the number of PMN in tissue (7, 8, 19).

In this investigation, we show the ultrastructural colocalization of ANXA1 and CD11b, mainly in the plasma membrane of neutrophils (intravascular and transmigrated) and endothelial cells. The colocalization of human recombinant ANXA1 and integrin α4β1 was also described in the membrane of U937 monocytic cell line by confocal microscopy studies (25). Moreover, we found that the exogenous administration of peptide Ac2-26 significantly reduced the levels of CD11b in these cellular types but did not alter the expression of CD62L in PMN. Surprisingly, at 4 h high significant levels of CD11b and CD62L were detected by flow cytometry and immunocytochemical analysis on peripheral blood PMN from pretreated animals, but this effect was not associated with an increase in cell transmigration on mesentery at this time point. Probably, the significant down-regulation of CD11b expression on post-capillary venule endothelial cells, also induced by pretreatment, represents an important step to inhibitory effect of PMN firm adhesion to tissue.

In subsequent studies, knowing that the ANXA1^{null} animals have an exaggerated inflammatory response and recovering the responsiveness not exaggerated by the administration of recombinant protein ANXA1 or its peptide Ac2-26 (13-15, 18), we examined the expression of adhesion molecules CD11b and CD62L on neutrophils and endothelial cells in these animals, using the same experimental model. Immunocytochemical analysis showed that the lack of endogenous ANXA1 caused a significant decrease in the expression of CD11b in neutrophils and mesentery postcapillary venule endothelial cells, compared to WT animals, after 4 h of inflammation. However, the exogenous administration of peptide Ac2-26 in these animals significantly increased the CD11b expression on intravascular PMN and endothelial cells. ANXA1 deficiency had been associated with exaggerated PMN activation, as measured by upregulation of CD11b on the plasma membrane after incubation with fMLP, PAF, or PMA (15). In this context, a series of biochemical studies have highlighted a role for ANXA1 and its N-terminal domain in mediating fusion of PMN granules with membrane preparations (5, 23, 26). It is therefore possible that exogenous addition of peptide Ac2-26 could facilitates the fusion areas between specific granules and plasma membrane with consequent increased cell-surface CD11b expression, an important step during subendothelial cell chemotaxis (27).

CD62L expression was also modulated on the mesenteric PMN of ANXA1^{null} animals, showing a significant increase after 4 h of peritonitis induction in response to the absence of endogenous ANXA1. This effect has been considered one of the mechanisms responsible for the exacerbation of the inflammatory

response in ANXA1^{null} animals described in models of acute inflammation induced by carrageenin and zymosan (13, 18). On the other hand, the exogenous administration of peptide Ac2-26 did not cause effects in the CD62L modulation in the neutrophils from both genotypes, showing that endogenous ANXA1 regulates this adhesion molecule.

In this experimental model of acute peritonitis, we showed that endogenous levels of ANXA1 are important to control inflammatory response through CD62L in PMN, while the exogenous ANXA1 regulates pro-inflammatory cytokine (TNF-α) release and CD11b expression on PMN and endothelial cells. These data contribute to better understanding of ANXA1 biology showing that modulation of this protein localization must drive its function in the cellular microenvironment and consequent anti-inflammatory action.

Abbreviations

ANXA1, annexin-A1; ANXA1^{null}, annexin-A1 null mice; CD11b, β2-integrin; CD62L, L-selectin; Nϕ_{in}, intravascular neutrophil; Nϕ_{out}, transmigrated neutrophil.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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