

Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis

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ABSTRACT

This study aimed at assessing whether AnxA1, a downstream mediator for the anti-inflammatory effects of GCs, could affect the fate of immune cells in tissue exudates, using LPS-induced pleurisy in BALB/c mice. AnxA1 protein expression in exudates was increased during natural resolution, as seen at 48–72 h post-LPS, an effect augmented by treatment with GC and associated with marked presence of apoptotic neutrophils in the pleural exudates. The functional relevance of AnxA1 was determined using a neutralizing antibody or a non-specific antagonist at FPR/ALXRs: either treatment inhibited both spontaneous and GC-induced resolution of inflammation. Injection of Ac2-26 (100 μ g, given 4 h into the LPS response), an AnxA1-active N-terminal peptide, promoted active resolution and augmented the extent of neutrophil apoptosis. Such an effect was prevented by the pan-caspase inhibitor zVAD-fmk. Mechanistically, resolution of neutrophilic inflammation was linked to cell apoptosis with activation of Bax and caspase-3 and inhibition of survival pathways Mcl-1, ERK1/2, and NF- κ B. These novel *in vivo* data, using a dynamic model of acute inflammation, provide evidence that AnxA1 is a mediator of natural and GC-induced resolution of inflammation with profound effects on neutrophil apoptosis. *J. Leukoc. Biol.* 92: 249–258; 2012.

Introduction

Inflammation is a host-tissue reaction, essential for inactivation of infectious or sterile injurious stimuli, with the main purpose to restore tissue homeostasis. The cardinal signs of inflamma-

tion—dolor, calor, tumor, and rubor—are intrinsically associated to microscope events, such as tissue damage, edema, and leukocyte traffic into the sites of inflammation [1]. In this process, the perpetuation of inflammation represents an important cause for tissue-damage exacerbation and necrosis. Thereafter, unresolved inflammation gives rise to chronic inflammation and autoimmunity, which leads to abnormal repair and alteration in organs with eventual loss of function [2]. It is now evident that the resolution of inflammation is an active and coordinated process, requiring activation of an endogenous program with production of inhibitors and proresolutive molecules, switch in the proteic and lipid mediator class from pro- to anti-inflammatory, and elimination of granulocyte by apoptosis, followed of removal by macrophage phagocytes, restoring tissue homeostasis [2–5].

Annexin A1 is a 37-kDa calcium-dependent phospholipid-binding protein induced by GCs, which bind to and activate FPR [6]. With the use of a combination of pharmacological and transgenic approaches, a role for AnxA1 modulating the inflammatory response has emerged in a variety of rodent inflammatory models [7–12]. Exogenous and endogenous AnxA1 exerts exquisite control on the process of leukocyte recruitment to inflammatory sites, with a modulation of the generation of proinflammatory mediators, including those derived from activation of PLA2, COX-2, and iNOS, as well as of the anti-inflammatory cytokine IL-10. Recent evidence suggests that AnxA1 may control leukocyte apoptosis *in vitro* [13] and favor their removal by macrophage phagocytosis [14, 15].

The apoptosis of neutrophils is an important event in the resolution of acute inflammation [16–19]. Recognition and

Abbreviations: ALXR=lipoxin A₄ receptor, AnxA1=annexin A1, Boc-1=N-t-Boc-Met-Leu-Phe, Dexa=dexamethasone, GC=glucocorticoid, i.p.l.=intrapleural, Mcl-1=myeloid cell leukemia 1, NIS=nonimmune serum, P=phosphorylated, PDE4=phosphodiesterase 4, Wtm=wortmannin

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removal of apoptotic neutrophils by macrophages limit tissue injury caused by neutrophils at the sites of inflammation [20]. Thus, the induction of neutrophil apoptosis could be of potential benefit in the control of acute inflammatory diseases [16–18]. Although the apoptotic program in neutrophils is a cell-intrinsic process, the rate of apoptosis can be altered dramatically by a number of agents [21]. Therefore, understanding the mechanisms involved in neutrophil recruitment, activation, and survival, at the site of inflammation, may provide new clues for the development of effective pharmacological therapies.

In this study, we investigated the role of the AnxA1 pathway (ligand and receptors) in natural and GC-driven resolution of inflammation, establishing the impact of the process of neutrophil apoptosis for resolution of acute pleurisy. We demonstrate that AnxA1 controls the rate of neutrophil apoptosis in vivo during ongoing exudate formation, thus exerting significant modulation on the resolution phase of this model of acute inflammation.

MATERIALS AND METHODS

Animals

All procedures described here had prior approval from the Animal Ethics Committee of Universidade Federal de Minas Gerais (CETEA/UFMG, Protocol Number 148/2006; Brazil). Male BALB/c mice (8–10 weeks), obtained from the Bioscience Unit of Instituto de Ciências Biológicas (Brazil), were housed under standard conditions and had free access to commercial chow and water.

Drugs, reagents, and antibodies

Rolipram (Biomol, Plymouth Meeting, PA, USA), Wtm (Calbiochem, San Diego, CA, USA), and zVAD-fmk (Tocris Bioscience, Minneapolis, MN, USA) were diluted in DMSO and further in PBS. Rabbit anti-P-ERK1/2, anti-Bax, anti-Mcl-1, anti-cleaved caspase-3 and mouse anti-P-I κ B- α were from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-AnxA1 or secondary anti-rabbit and anti-mouse peroxidase-conjugate antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- β -actin, LPS (from *Escherichia coli* serotype O:111:B4), Boc-1, and Dexa were from Sigma-Aldrich (St. Louis MO, USA). Peptide Ac2-26, a synthetic derivative corresponding to aa 2–26 of the N-terminal region of AnxA1, was a gift of Dr. Mauro Perretti (William Harvey Research Institute, Barts and The London School of Medicine, London, USA). Anti-AnxA-1 antiserum was a kind gift from Dr. Steve Poole (Biotherapeutics Group, National Institute for Biological Standards and Control, UK).

Leukocyte migration into the pleural cavity induced by LPS

Mice received an i.p. administration of LPS (250 ng/cavity) or PBS, as described previously [22]. Cells present in the pleural cavity were harvested at different times after administration of LPS by washing the cavity with 2 ml PBS and total cell counts performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytocentrifuge preparations (Shandon Cytospin III), stained with May-Grünwald-Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells/cavity.

Treatment protocols

To evaluate the role of anti-inflammatory agents on the LPS-induced pleurisy, mice were treated with rolipram (6.0 mg/kg, i.p.), the synthetic GC Dexa (2.0 mg/kg, i.p.), or Wtm (1.0 mg/kg, i.p.) in 4-h LPS-challenged

mice, as described previously [22, 23]. To prevent the action of AnxA1, mice were treated with Boc-1 (2.0 mg/kg, i.v.), a nonselective FPR antagonist that blocks the FPR and ALXR activation or with anti-AnxA1 antiserum (0.1 mL hyperimmune serum diluted in 100 μ l PBS/ mice, i.p.), 15 min prior to Dexa [11]. Nonimmune goat serum was used as control. To reproduce the action of AnxA1, 4-h LPS-challenged mice were treated with the peptide Ac2-26 (100 μ g/i.p. or i.p.). zVAD-fmk (1 mg/kg), a broad-spectrum caspase inhibitor [22], was given systemically (i.p.) 15 min before Ac2-26 injection. Drugs were dissolved in DMSO and diluted further in PBS. Control mice received drug vehicle only.

Assessment of leukocyte apoptosis

Apoptosis was assessed morphologically, as reported previously [18, 22–25]. Briefly, cells (5×10^4) collected 8 h after LPS administration were cyto-centrifuged, fixed, and stained with May-Grünwald-Giemsa and counted using oil immersion microscopy ($\times 100$ objective) to determine the proportion of cells with distinctive apoptotic morphology (cells presented chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies, out or inside macrophages). At least 500 cells were counted/slide, and results are expressed as the mean \pm SEM of percentage of cells with apoptotic morphology. The cleavage of caspase-3 and the assessment of the levels of Bcl-2 family proteins (Bax, and Mcl-1) were evaluated by Western blot analysis and used as a biochemical marker of apoptosis. Assessment of apoptosis was also performed by flow cytometry using FITC-labeled annexin-V (ApoDETECT Annexin V-FITC kit; Invitrogen, Carlsbad, CA, USA) and PI as an index of loss of nuclear membrane integrity.

Lysate preparation and Western blot analysis

Inflammatory cells harvested from the pleural cavity were washed with PBS, and whole-cell extracts were prepared as described [22–24]. Protein amounts were quantified with the Bradford assay reagent from Bio-Rad (Hercules, CA, USA). Extracts (40 μ g) were separated by electrophoresis on a denaturing, 10–15% polyacrylamide-SDS gel and electrotransferred to nitrocellulose membranes, as described [26]. Membranes were blocked overnight at 4°C with PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween-20, washed three times with PBS containing 0.1% Tween-20, and then, incubated with specific primary antibodies (cleaved caspase-3, Bax, Mcl-1, P-ERK1/2, P-I κ B- α , AnxA-1, or anti β -actin) using a dilution of 1:1000 in PBS containing 5% (w/v) BSA and 0.1% Tween-20. After washing, membranes were incubated with appropriated HRP-conjugated secondary antibody (1:3000). Immunoreactive bands were visualized by using an ECL detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

All results are presented as the mean \pm SEM. Normalized data were analyzed by one-way ANOVA, and differences between groups were assessed using the Student-Newman-Keuls post-test. A *P* value < 0.05 was considered significant. Calculations were performed using the Prism 4.0 software for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Spontaneous resolution of LPS-induced pleurisy is associated with neutrophil apoptosis

i.p. injection of LPS induced a time-dependent influx of neutrophils into the pleural cavity of mice, which was high at 4 h and peaked between 8 and 24 h. Neutrophil numbers diminished at 48 h, and complete resolution occurred only at 72 h (Fig. 1A). The cellularity of the pleural cavity consisted of 98–100% mononuclear cells in PBS-injected mice, whereas 50–

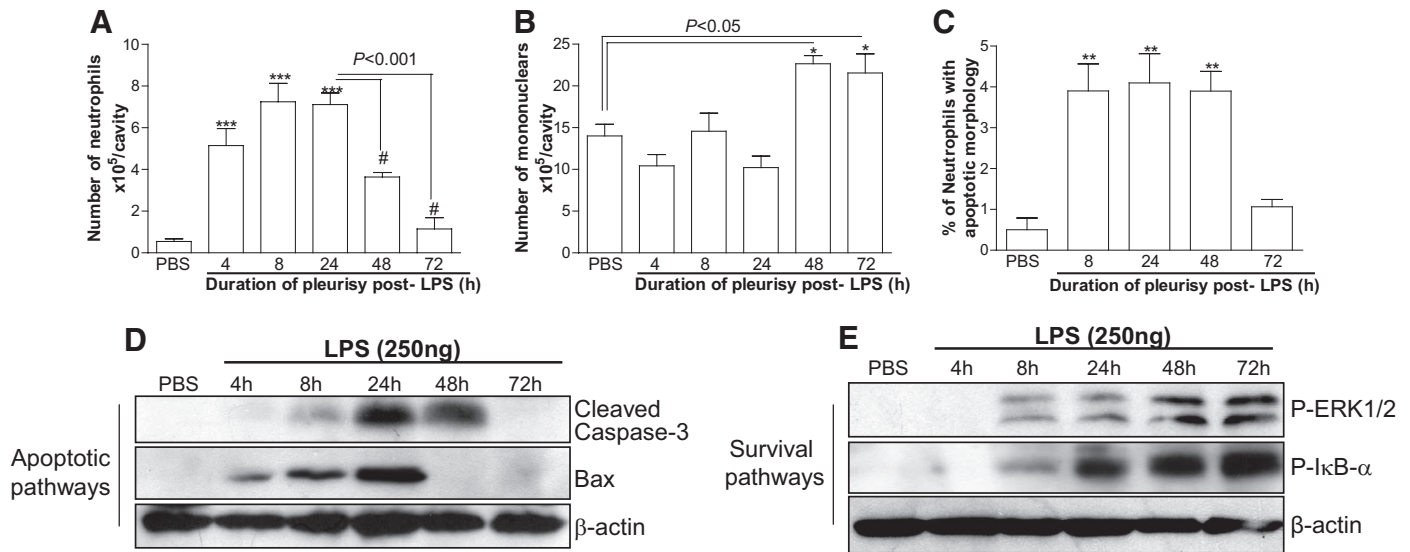


Figure 1. Resolution of LPS-induced pleurisy is associated with increased mononuclear recruitment and activation of a proapoptotic program.

Mice were injected with PBS or LPS (250 ng/cavity, i.pl.), and a number of neutrophils (A), mononuclear cells (B), and cells with distinctive apoptotic morphology (C) were evaluated at various times. Results are expressed as the number of cells/cavity or percent of neutrophils with distinctive apoptotic morphology and are shown as the mean \pm SEM of at least five mice in each group. * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$ when compared with PBS-injected mice; # $P < 0.001$ when compared with 24-h LPS-challenged mice. (D and E) Western blotting analysis of cleaved caspase-3, Bax, P-ERK1/2, and P-I κ B- α . Whole-cell extracts were obtained from inflammatory cells harvested from pleural cavity at various times and processed for Western blot, as described in Materials and Methods. For loading control, membranes were reprobbed with anti- β -actin. Blots are representative of three independent experiments in pools of cells from at least five animals.

60% of neutrophils and 40–50% mononuclear cells could be quantified in LPS-injected mice for the 8- to 24-h period (Fig. 1A). Figure 1B reports absolute cell numbers, showing that there was no increase in mononuclear cells, 8–24 h after LPS challenge; thereafter, there was an important increase of these cells in the pleural cavity, which coincided with the resolution of neutrophil influx (48- to 72-h phase).

The spontaneous resolution of neutrophilic inflammation was associated with an increase in the number of apoptotic cells, as seen from 8 to 48 h, time-points that preceded complete resolution. Apoptosis was demonstrated by morphological criteria (Fig. 1C), caspase-3 cleavage, and Bax accumulation (Fig. 1D). Conversely, the profile of the pro-survival proteins P-ERK1/2 and P-I κ B- α , during LPS-induced pleurisy, is shown in Fig. 1E; these pro-survival proteins are detected in correspondence with neutrophil recruitment, with a marked increase associated with the recruitment of mononuclear cells into the pleural cavity (Fig. 1B).

Spontaneous resolution of LPS-induced pleurisy is paralleled by increased levels of AnxA1

Intact AnxA1 (37-kDa) protein was detected in PBS-challenged mice, but its expression decreased markedly during the active phase of LPS-induced neutrophil recruitment (4- to 24-h postinjection). Intriguingly, intact AnxA1 expression was regained during the resolution phase of this inflammatory response (48–72 h; Fig. 2). Conversely, the 33-kDa breakdown product of AnxA1 was barely detected in PBS-injected mice and strongly detected during the time-points of high neutro-

phil counts in pleural fluid (4–24 h), yet it virtually disappeared during the resolution phase of inflammation (48–72 h; Fig. 2).

Anti-inflammatory drugs up-regulate AnxA1 and activate the neutrophil apoptosis cascade

We have demonstrated previously that treatment of mice with rolipram, a PDE4 inhibitor, after inflammation had been established, decreased the number of neutrophils and increased apoptotic events in the pleural cavity of mice in a PI3K/Akt-dependent manner [22]. Next, we examined the

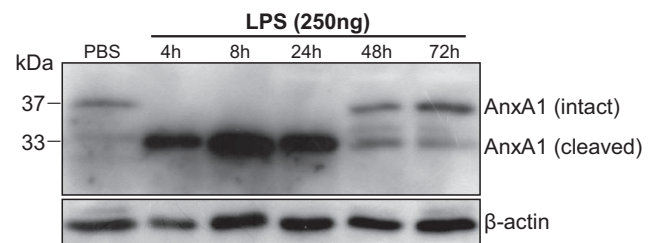


Figure 2. Time-course of AnxA1 expression during LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.), and at several time-points, the inflammatory cells were harvested from pleural cavity and processed for Western blot analysis to detection of AnxA1 levels. For loading control, membranes were reprobbed with anti- β -actin. Blots are representative of three independent experiments in pools of cells from at least five animals.

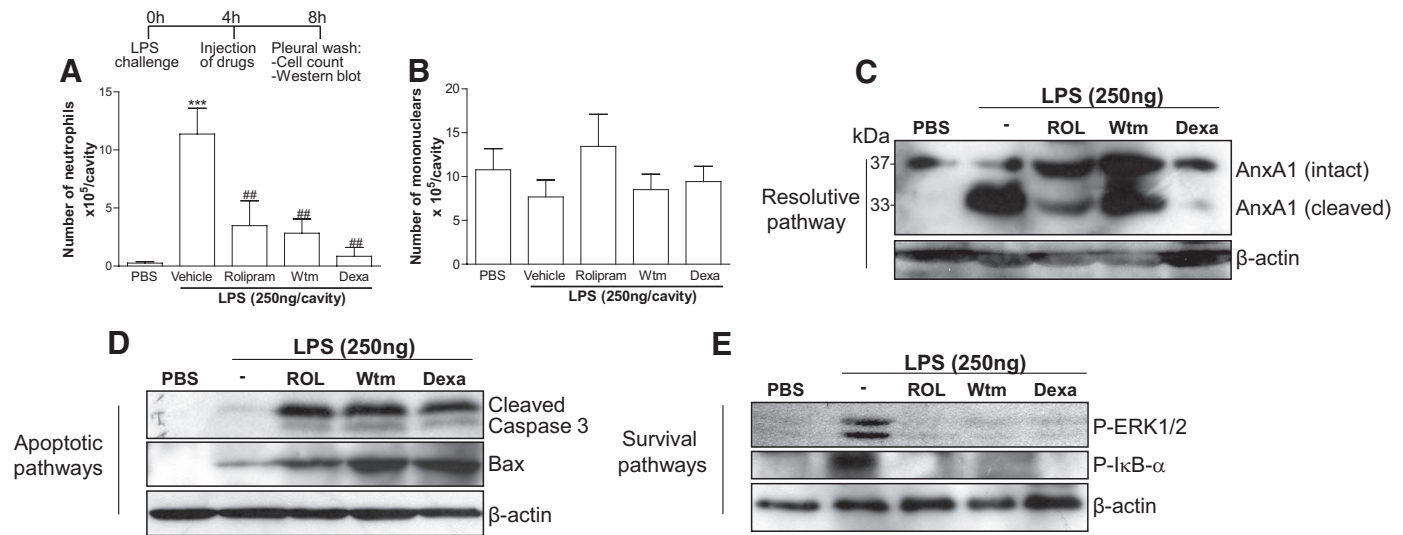


Figure 3. Effect of anti-inflammatory drugs on the levels of AnxA1 and key proteins associated with apoptosis and cell survival. Mice were injected with LPS (250 ng/cavity, i.p.) or PBS and 4 h later, received a systemic injection of rolipram (ROL; 6 mg/kg, i.p.), Dexa (2 mg/kg, i.p.), Wtm (1 mg/kg, i.p.), or drug vehicle. Numbers of neutrophils (A), mononuclear cells (B), and Western blot for detection of proteins associated with resolutive, apoptotic, and survival pathways (C–E) were evaluated, 4 h after drug treatment, i.e., 8 h after LPS challenge. Results are expressed as the number of neutrophils or mononuclear cells/cavity and are shown as the mean \pm SEM of at least five mice in each group. *** $P < 0.001$ when compared with PBS-injected mice; ## $P < 0.01$ when compared with vehicle-treated, LPS-challenged mice. (C–E) Whole extracts from cells harvested from the pleural cavity were analyzed by Western blot for detection of proteins from resolutive (AnxA1), apoptotic (caspase-3 cleavage and Bax), and survival (P-ERK1/2 and P-I κ B- α) pathways. For loading control, membranes were reprobated with anti- β -actin. Blots are representative of three independent experiments in pools of cells from at least five animals.

effect of rolipram, Wtm (a PI3K/Akt inhibitor), and Dexa (a synthetic GC) on the resolution of neutrophilic inflammation in the pleural cavity and whether induction of AnxA1 was associated with this scenario. **Figure 3** shows that treatment of mice with these drugs reduced the number of neutrophils (Fig. 3A), without altering mononuclear cell counts in the pleural cavity (Fig. 3B). Such an effect was accompanied by increased levels of intact AnxA1 in the inflammatory extracts (Fig. 3C). Importantly, drug treatment prevented AnxA1 cleavage, as seen by decreased levels of 33 kDa AnxA1-breakdown product, as compared with LPS alone (Figs. 3C, 4C, and 5C).

Next, we evaluated whether increased levels of intact AnxA1, promoted by treatment with rolipram, Wtm, or Dexa, were associated with an increase in number of apoptotic cells in the pleural cavity. We found a positive correlation between intact AnxA1 and activation of a proapoptotic program centered on caspase-3 cleavage, Bax accumulation, and decreased phosphorylation levels of ERK1/2 and I κ B- α (Fig. 3D and E).

Blockade of the AnxA1 pathway prevents natural and Dexa-driven resolution of neutrophilic inflammation

The functional relevance of AnxA1 in this model of resolving inflammation was then determined. A neutralizing strategy against endogenous AnxA1 was carried out using specific anti-AnxA1 antiserum or a nonselective FPR/ALXR antagonist. Administration of anti-AnxA1 (Fig. 4) or Boc-1 (Fig. 5) prevented

Dexa-induced resolution of neutrophilic inflammation (Figs. 4A and 5A); such effect was associated with decreased expression of intact AnxA1 (Figs. 4C and 5C) and apoptotic events (as seen by decreased Bax and neutrophils with apoptotic morphology; Figs. 4B and C and 5B and C) in pleural exudates. In addition, anti-AnxA1 treatment prevented a Dexa-induced decrease of P-ERK1/2 and P-I κ B- α phosphorylation (Fig. 4C), with very similar data obtained after Boc-1 treatment (Fig. 5C, and data not shown). Of note, treatment of mice with rolipram and Dexa also decreased LPS-induced cleavage of AnxA1 protein (Figs. 4C and 5C).

We also analyzed the effects of anti-AnxA1 serum and Boc-1 on spontaneous resolution of LPS-induced pleurisy. Delivery of these treatments at the 24-h time-point, post-LPS, delayed natural resolution, as determined at 48 h (Figs. 4D and 5D), suggesting that endogenously produced AnxA1 is part of a physiological, proresolutive program. Of note, treatment of mice with a goat NIS had no effect on the resolution of inflammation (Fig. 4D).

An AnxA1 peptidomimetics promotes resolution of LPS-induced pleurisy by inducing neutrophil apoptosis

The data gathered so far indicate that expression of AnxA1 is induced during natural and drug-induced resolution of acute inflammation (Figs. 2 and 3), and strategies that inhibit AnxA1 prevent resolution by decreasing AnxA1-mediated apoptotic events in the pleural cavity (Figs. 4 and 5). Next, we evaluated the effect of exogenously administered

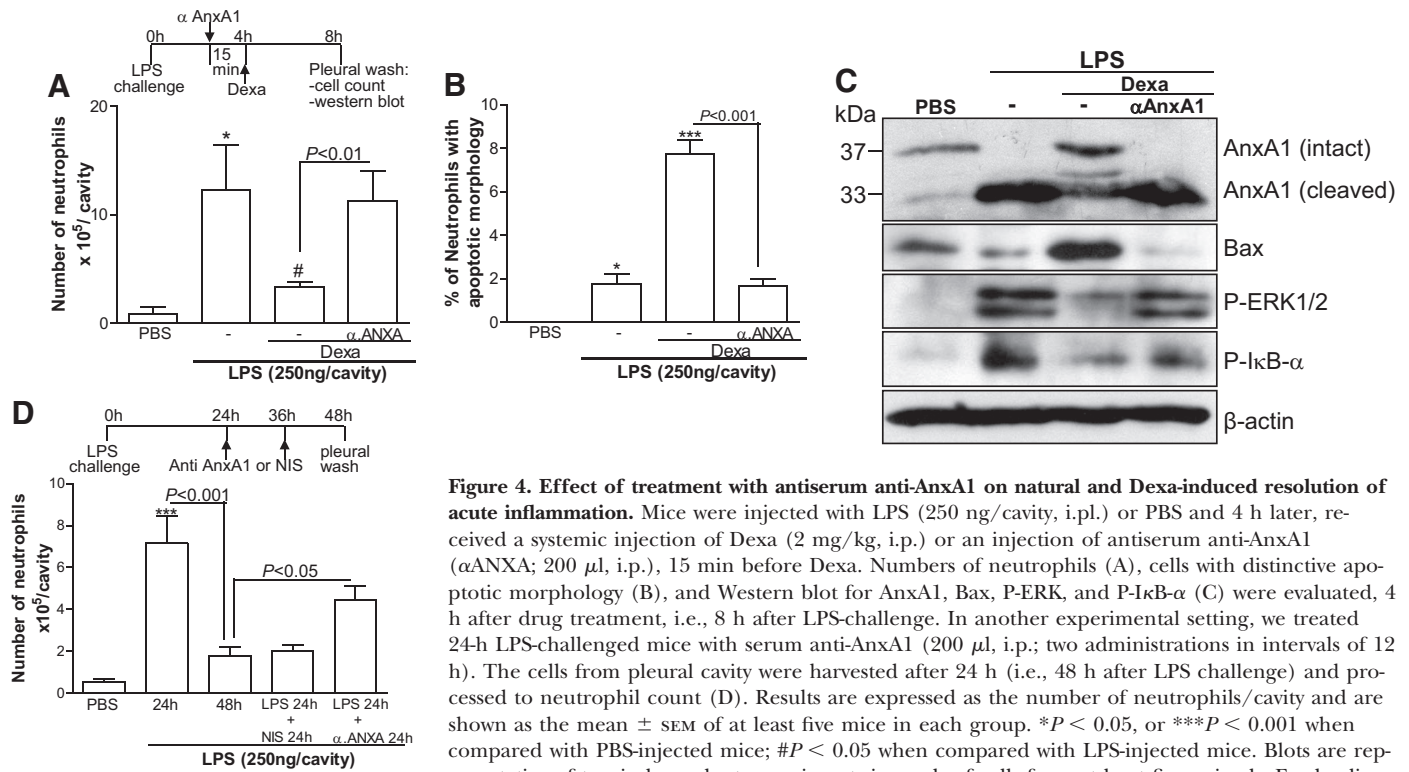


Figure 4. Effect of treatment with antiserum anti-AnxA1 on natural and Dexamethasone-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.p.) or PBS and 4 h later, received a systemic injection of Dexamethasone (2 mg/kg, i.p.) or an injection of antiserum anti-AnxA1 (αANXA ; 200 μl , i.p.), 15 min before Dexamethasone. Numbers of neutrophils (A), cells with distinctive apoptotic morphology (B), and Western blot for AnxA1, Bax, P-ERK, and P-IkB- α (C) were evaluated, 4 h after drug treatment, i.e., 8 h after LPS-challenge. In another experimental setting, we treated 24-h LPS-challenged mice with serum anti-AnxA1 (200 μl , i.p.; two administrations in intervals of 12 h). The cells from pleural cavity were harvested after 24 h (i.e., 48 h after LPS challenge) and processed to neutrophil count (D). Results are expressed as the number of neutrophils/cavity and are shown as the mean \pm SEM of at least five mice in each group. * $P < 0.05$, or *** $P < 0.001$ when compared with PBS-injected mice; # $P < 0.05$ when compared with LPS-injected mice. Blots are representative of two independent experiments in pools of cells from at least five animals. For loading control, membranes were reprobated with anti- β -actin.

AnxA1 on the course of LPS-induced pleurisy. To this end, we treated LPS-inflamed mice with the peptide Ac2-26, which contains the N-terminal, active portion of AnxA1. We found that treatment of 4-h LPS-challenged mice with pep-

tide Ac2-26 (100 μg), by two routes of administration (local and systemic), greatly decreased neutrophil accumulation in the pleural cavity at 24 h (Fig. 6A), without modifying numbers of mononuclear cells (Fig. 6B). Importantly, the ad-

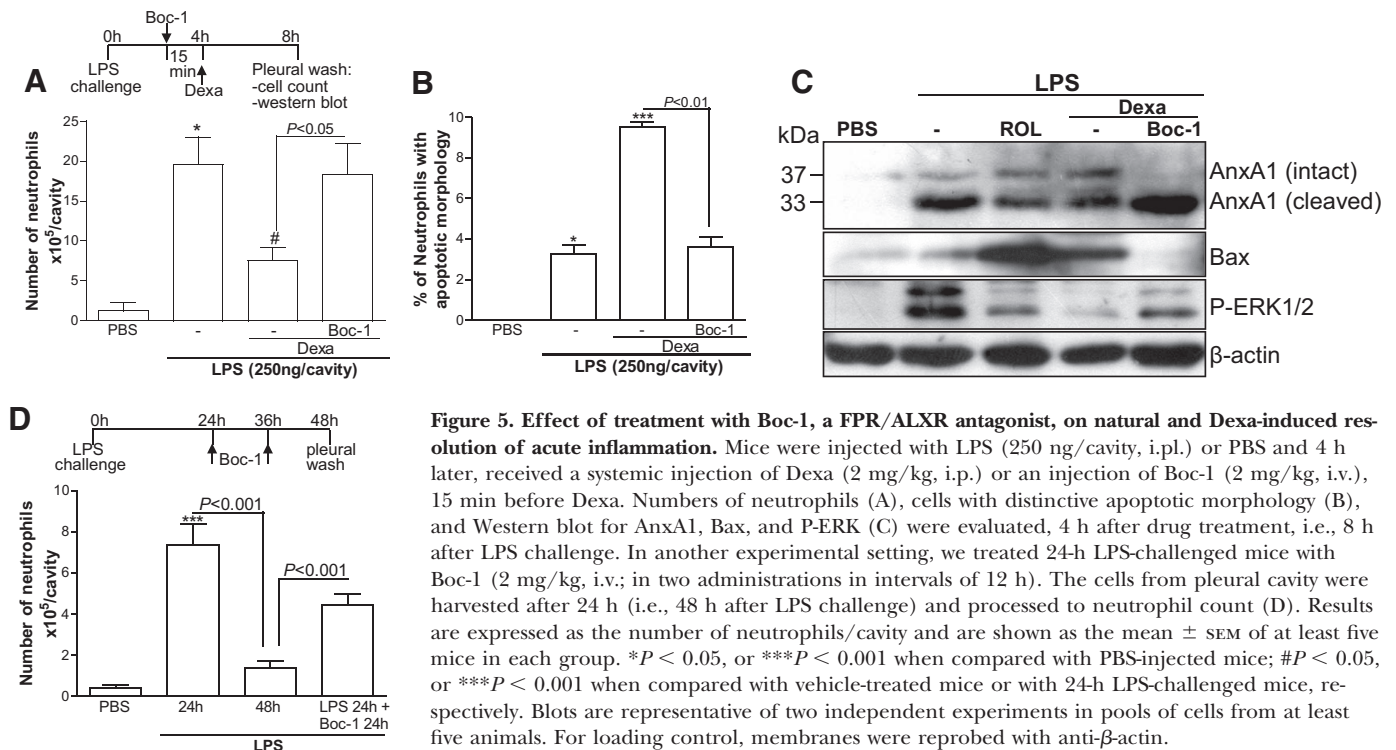


Figure 5. Effect of treatment with Boc-1, a FPR/ALXR antagonist, on natural and Dexamethasone-induced resolution of acute inflammation. Mice were injected with LPS (250 ng/cavity, i.p.) or PBS and 4 h later, received a systemic injection of Dexamethasone (2 mg/kg, i.p.) or an injection of Boc-1 (2 mg/kg, i.v.), 15 min before Dexamethasone. Numbers of neutrophils (A), cells with distinctive apoptotic morphology (B), and Western blot for AnxA1, Bax, and P-ERK (C) were evaluated, 4 h after drug treatment, i.e., 8 h after LPS challenge. In another experimental setting, we treated 24-h LPS-challenged mice with Boc-1 (2 mg/kg, i.v.; in two administrations in intervals of 12 h). The cells from pleural cavity were harvested after 24 h (i.e., 48 h after LPS challenge) and processed to neutrophil count (D). Results are expressed as the number of neutrophils/cavity and are shown as the mean \pm SEM of at least five mice in each group. * $P < 0.05$, or *** $P < 0.001$ when compared with PBS-injected mice; # $P < 0.05$, or *** $P < 0.001$ when compared with vehicle-treated mice or with 24-h LPS-challenged mice, respectively. Blots are representative of two independent experiments in pools of cells from at least five animals. For loading control, membranes were reprobated with anti- β -actin.

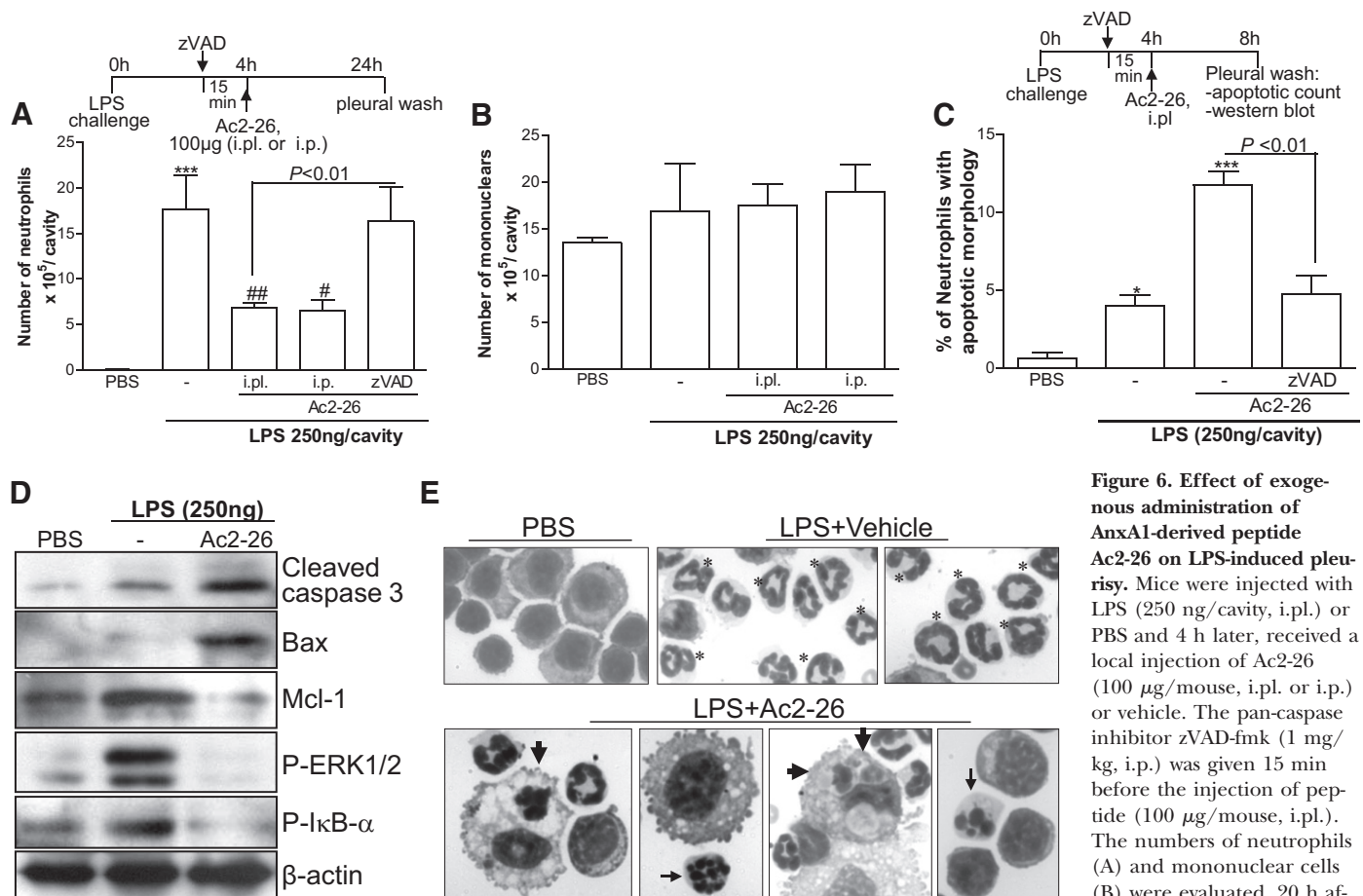


Figure 6. Effect of exogenous administration of AnxA1-derived peptide Ac2-26 on LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.p.) or PBS and 4 h later, received a local injection of Ac2-26 (100 $\mu\text{g}/\text{mouse}$, i.p.) or vehicle. The pan-caspase inhibitor zVAD-fmk (1 mg/kg, i.p.) was given 15 min before the injection of peptide (100 $\mu\text{g}/\text{mouse}$, i.p.). The numbers of neutrophils (A) and mononuclear cells (B) were evaluated, 20 h after drug treatment, i.e., 24 h after LPS challenge. Cells

with distinctive apoptotic morphology (C and E) and Western blot to detection of cleaved caspase-3, Bax, Mcl-1, P-ERK, and P-IkB- α (D) were evaluated, 4 h after drug treatment, i.e., 8 h after LPS challenge. There were at least five mice in each group. * $P < 0.05$, or *** $P < 0.001$ when compared with PBS-injected, and # $P < 0.05$, or ## $P < 0.01$ when compared with vehicle-treated, LPS-injected mice. Blots are representative of three independent experiments in pools of cells from at least five animals. For loading control, membranes were probed with anti- β -actin. (E) Representative figures of nonapoptotic (asterisk) and apoptotic (arrows) neutrophils and apoptotic cells inside macrophages (arrowheads). PBS and vehicle (upper panels) and Ac2-26-treated (lower panels) animals are shown. Original magnifications, $\times 100$.

ministration of the broad-spectrum caspase inhibitor zVAD-fmk prevented the resolution by Ac2-26 of LPS-induced neutrophilic inflammation (Fig. 6A). Of note, treatment with zVAD alone did not alter the kinetics of neutrophil recruitment after injection of LPS [22].

Next, we queried whether apoptosis was the underlying mechanism and established that treatment of mice with Ac2-26 induced neutrophil apoptosis in the pleural cavity, as shown by morphological and biochemical criteria (Fig. 6C and D). Apoptosis was prevented by the broad-spectrum caspase inhibitor zVAD-fmk (Fig. 6C). Annexin-V analysis by flow cytometry showed a similar pattern (data not shown). Examples of apoptotic neutrophils at 4 h after treatment with the peptide Ac2-26 are shown in Fig. 6E.

Neutrophil apoptosis is controlled by a complex network of signaling pathways, which regulate the turnover of key molecules of the Bcl-2 family and activation of the caspases family of proteases [27]. We have shown that rolipram and LY294002 (a PI3K/Akt inhibitor) induced apoptosis of inflammatory

cells, which was accompanied by a decrease in the cellular levels of Mcl-1 [22], a key antiapoptotic Bcl-2 family protein regulating neutrophil survival [28]. Thus, we investigated the ability of Ac2-26 to regulate key apoptotic (Bax and caspase-3) and survival (Mcl-1, ERK1/2, and NF- κ B) pathways, previously shown to be important regulators of neutrophil fate [24, 29, 30]. We observed that Ac2-26-induced apoptosis of neutrophils and resolution of inflammation were accompanied by an increased, proapoptotic Bcl-2 member Bax and caspase-3 cleavage and by decreased cellular levels of Mcl-1, ERK1/2, and P-IkB- α phosphorylation, proteins associated with neutrophil survival.

DISCUSSION

GCs are potent anti-inflammatory and immunosuppressive drugs, largely used for the treatment of various severe inflammatory conditions. The actions of endogenous GC are not un-

derstood completely and may depend on the induction of anti-inflammatory regulatory proteins [6, 31]. In this study, we describe the strict kinetics of PMN apoptosis in a model of LPS-induced acute (and resolving) pleurisy and provide the detailed mechanistic pathways of AnxA1-induced resolution. Our major findings are as follows: (i) AnxA1 is up-regulated during natural and drug-induced resolution of acute inflammation, and such increase is associated with appearance of apoptotic neutrophils in the pleural cavity; (ii) natural and Dexamethasone-induced resolution of neutrophilic inflammation was AnxA1-dependent, as neutralization strategies by use of a specific antiserum or by blocking its receptor, prevented natural and GC-induced resolution, abolishing apoptotic phenomena in the pleural cavity; (iii) injection of Ac2-26, an AnxA1-derived N-terminal peptide, promoted resolution of neutrophilic inflammation in the pleural cavity, an effect associated with the induction of neutrophil apoptosis; and (iv) mechanistically, resolution of neutrophilic inflammation was associated with activation of apoptotic pathways Bax and caspase-3 and inhibition of survival pathways Mcl-1, ERK1/2, and NF- κ B. Therefore, we provide strong evidence that AnxA1 is a mediator of natural and GC-induced resolution of inflammation by promoting apoptosis of neutrophils *in vivo*.

AnxA1 is a known effector of the resolution of inflammation, yet its activities have been studied predominantly in the context of inhibition of leukocyte recruitment, as established in various models of inflammation [8–12]. Our findings are first to show the relevance and the dynamics of AnxA1 in driving natural resolution of inflammation *in vivo*. These data make substantial contribution to the notion that this protein acts physiologically to counteract the inflammatory process [6, 32].

Besides studying pathogenic pathways, it is important to identify therapeutics that can accelerate and/or activate resolution programs. For instance, the concept that attention should be paid to identify resolution-safe drugs, in drug discovery programs, against resolution-toxic therapeutics has been put forward recently (consensus on resolution; see ref. [5]). As an example, compounds, such as GC and PDE4 inhibitors can enhance the resolution of inflammation [22, 23, 33, 34]. Here, we demonstrated that the ability of these compounds to accelerate neutrophilic resolution was associated with increased accumulation of AnxA1. Several actions of GCs are thought to be mediated by AnxA1 [6], and indeed, AnxA1-deficient mice are resistant to some of the anti-inflammatory effects of GCs [35, 36]. In our experiments, a GC-induced increase of AnxA1 expression was associated with resolution of neutrophilic inflammation. More importantly, blockade of AnxA1 with antibodies or the receptor at which AnxA1 binds with Boc-1 decreased the capacity of GCs to accelerate resolution of inflammation. Altogether, these studies do show that AnxA1 is important, not only for natural resolution of inflammation but is also relevant for the proresolutive actions of certain anti-inflammatory drugs *in vivo*.

We and others have shown that apoptosis precedes and plays a major role in the resolution of acute neutrophilic and eosinophilic inflammation *in vivo* [2, 3, 18, 19, 22–25, 37]. Indeed, strategies that prevent apoptosis tend to delay resolution

of neutrophilic inflammation [38]. The first observation that AnxA1 could induce apoptosis was from studies of McKanna [39]. Other studies have also shown a correlation between neutrophil apoptosis and AnxA1 expression *in vitro* [13]. Interestingly, overexpression of AnxA1 in monocytic U937 cells induced spontaneous apoptosis of these cells, and this was associated with activation of caspase-3 [40]. Furthermore, exogenous application of AnxA1 to human neutrophils *in vitro* promoted apoptosis associated with increased calcium concentrations and activation of proapoptotic pathways [41]. However, no studies have yet established what role AnxA1 could play on PMN apoptosis during the dynamic and integrated process of resolving inflammation, as in this *in vivo* model of LPS pleurisy. In our hands, treatment with the AnxA1 peptide Ac2-26 induced resolution of neutrophilic inflammation, which was associated with increased apoptosis of these cells and activation of caspase-3 and the proapoptotic protein Bax. More importantly, blockade of caspases prevented peptide Ac2-26-induced apoptosis and resolution of neutrophilic inflammation. In line with the latter findings, blockade of endogenous AnxA1 prevented Bax accumulation, neutrophil apoptosis, and prolonged neutrophil survival. The receptor at which AnxA1 binds to induce its proresolving effects was not investigated here. However, it is likely that FPR2/ALX is conveying the proapoptotic effects of AnxA1, as full-length AnxA1 binds FPR2/ALX only [42], and this receptor has recently been reported to mediate the *in vivo*-inducing, proapoptotic effects of LXA(4), another FPR2/ALX ligand [43].

It is known that apoptotic neutrophils release AnxA1, which would then act on macrophages to promote efferocytosis, hence, removal of apoptotic cells *in vitro* [15]. Also, macrophages treated with GCs secrete AnxA1, which contributes to the augmented phagocytosis of apoptotic neutrophils [14]. In our experiments, apoptotic bodies were found inside macrophages recovered from the pleural cavity of mice after treatment with peptide Ac2-26 (Fig. 6E), suggesting that an increased uptake of dying cells may also contribute to the global, proresolving effects. A recent study has shown the ability of endogenous AnxA1 to modulate phagocytosis of apoptotic neutrophils in natural, noninflammatory settings, such as in the bone marrow [44]. Collectively, we propose that during ongoing inflammation, endogenous and exogenous AnxA1 can exert exquisite modulation on the fate of recruited neutrophils by promoting their death by apoptosis, as well as their safe removal by phagocytes (efferocytosis). Both processes—apoptosis and efferocytosis—are crucial for resolution and can be modulated *in vivo*, as shown here, by AnxA1.

In our experimental settings, resolution of inflammation and neutrophil apoptosis is associated with an increase of expression of proapoptotic and inhibition of prosurvival pathways. Specifically, we have shown a major role for NF- κ B in mediating survival and permanence of neutrophils [24] and to play an important role in the proapoptotic mechanisms of Dexamethasone [23]. In this regard, the capacity of AnxA1 to induce apoptosis in cancer cells was associated with inhibition of NF- κ B [45]. Also, we have been shown that the loss of Mcl-1 contributes to the induction of neutrophil apoptosis caused by rolipram and PI3K inhibitors [22]. Indeed, in the present

work, both drugs were able to increase an AnxA1-active band, and injection of an AnxA1 peptidomimetic into the pleural cavity of mice was accompanied by decreasing levels of Mcl-1. The relevance of MAPKs for survival in our system has not been evaluated in great detail, although inhibition of ERK1/2 is known to promote resolution of carrageenan-induced pleurisy by inducing neutrophil apoptosis [29]. Of note, increased levels of ERK1/2 phosphorylation and NF- κ B activity in response to LPS are found in AnxA1^{-/-} macrophages [46]. In our experiments, strategies that increased AnxA1 expression or administration of AnxA1 peptide decreased activation of NF- κ B and ERK1/2 in vivo, and this was associated with an increase of neutrophil apoptosis. Importantly, blockade of AnxA1 prevented the inhibition of NF- κ B and ERK1/2 phosphorylation, as induced by treatment with GCs. In contrast with our in vivo results, AnxA1 peptide Ac2-26 induces transient ERK1/2 phosphorylation of macrophages in vitro [47]. It is difficult at first to reconcile these findings, but clearly, there are differences in experimental settings (in vitro vs. in vivo), major cell type studies (macrophages vs. neutrophils), and kinetics (few minutes vs. few hours). Moreover, neutrophil numbers decrease after treatment, suggesting that changes in ERK1/2 phosphorylation could be a result of the resolution of neutrophils or a result of downstream events triggered early by AnxA1 treatment. Altogether, our data clearly show that the ability of AnxA1 to regulate the expression and/or activity of NF- κ B and ERK1/2 underlies the proapoptotic and proresolutive effects of this protein in vivo. This is akin to the effects of GCs in vivo [48].

Intact AnxA1 (37 kDa), the biologically active form of the protein, is found predominantly on the plasma membrane of intravascular adherent neutrophils in vivo [49]. Once neutrophils extravasate into sites of inflammation, the majority of intact AnxA1 is cleaved to its inactive form of 33 kDa. Neutrophil proteases, including elastases and proteinase 3, which are found in sites of inflammation, are thought to be the major contributors to cleave AnxA1 [49–51]. We found here that when neutrophils accumulated in the pleural cavity, the intact AnxA1 band disappeared, and the AnxA1-cleaved product of 33 kDa was then present. However, strict monitoring of the kinetics of this model allowed us to establish that at times of resolution of inflammation, the AnxA1-active band was increased and protein cleavage was minimal. Of interest, compounds, which we have previously shown to increase resolution of neutrophilic [22] and eosinophilic [23] inflammation, augmented the AnxA1-intact band and prevented its degradation, making us suggest that modulation of endogenous AnxA1 might be a more general mechanism to resolve inflammatory responses than solely associated with GC treatment. We can speculate about the possible mechanism by which anti-inflammatory drugs increase intact protein and decrease AnxA1 cleavage. These compounds could be acting to stimulate the release of additional AnxA1 (increasing protein expression or mobilizing the intracellular pool to export and secretion by influencing AnxA1 phosphorylation) [52], decreasing the appearance or activity of the protease or

both. Whether steroids and PDE4 inhibitors resolve inflammation and enhance intact AnxA1 levels in a similar way is also not known and clearly deserves further investigation. In this regard, PDE4 inhibitors, such as rolipram, may act by releasing endogenous corticosterone [53]. We have shown that other cAMP mimetics or elevating agents also resolve neutrophilic inflammation [22], suggesting that cAMP may contribute to the effects of rolipram.

Cleavage is a way to generate fragments (peptides) with diverse biological activities. Williams and colleagues [54] have reported that the 33-kDa N-terminally truncated AnxA1 form, found in the soluble protein fractions of activated neutrophils, displays proinflammatory effects by promoting ERK1/2 activation and neutrophil transendothelial migration. In the latter study, neither the full-length protein of 37 kDa nor the N-terminal 26-aa peptide was able to activate ERK1/2 [54]. Moreover, the concept that the cleaved product of AnxA1 might be proinflammatory has been suggested by findings of the 33-kDa form in BAL fluid samples from cystic fibrosis patients [55]. Of note, a cleavage-resistant AnxA1 displayed a stronger anti-inflammatory effect over time compared with the parental protein [50]. In contrast with these potential proinflammatory roles of AnxA1 cleavage products, a recent study using necrotic PMN demonstrated an inhibitory effect of released AnxA1, proposing a fail-safe role in dampening overexuberant cellular reactivity upon cell necrosis in the environment [56]. At the present, it is unclear what the biological function of these AnxA1-generated peptides is. It appears that AnxA1 cleavage by protease-armed neutrophils is a mechanism that over-rides the anti-inflammatory action of the endogenous protein, including diminished neutrophil transmigration and induced neutrophil detachment on vascular beds. In the context of resolution of inflammation, as in our studies, an intact product was associated with a higher degree of neutrophil apoptosis and the ensuing resolution.

To conclude, our data demonstrate that endogenous AnxA1 plays a fundamental role in driving natural and GC-induced resolution of established, acute neutrophilic inflammation in the pleural cavity of mice. Mechanistically, AnxA1 resolves inflammation by inducing apoptosis of inflammatory cells, an effect mediated by activation of caspase-3 and correlated with loss of Mcl-1, Bax accumulation, and inhibition of ERK1/2 and NF- κ pathways. This is the first observation that AnxA1 promotes apoptosis of neutrophils in vivo. Hence, these results reinforce the idea that AnxA1 or its peptidomimetic may represent a powerful anti-inflammatory strategy for the treatment of diseases, in which neutrophil accumulation plays a relevant role.

AUTHORSHIP

L.P.S., V.P., and M.M.T. designed research, analyzed data, and wrote the paper. L.P.S., J.P.V., C.R.C.N., and L.P.T. performed experiments and analyzed data. F.M.S., F.L., and R.C.R. carried out experiments.

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