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Evidence for an anti-inflammatory loop centered on polymorphonuclear leukocyte formyl peptide receptor 2/lipoxin A4 receptor and operative in the inflamed microvasculature

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Summary

The importance of proresolving mediators in the overall context of resolution of acute inflammation is accepted and prominent, though little is known on whether these anti-inflammatory and pro-resolving molecules act in concert. Here we focused on lipoxin A_4 (LXA_4) and annexin A1 (AnxA1) since these two very different mediators converge on a single receptor, formyl peptide receptor type 2 (acronym FPR2/ALX). Addition of LXA_4 to human PMN provoked a concentration- and time-dependent mobilization of AnxA1 onto the plasma membrane, as determined by western blotting and flow cytometry analyses. This property was shared by another FPR2/ALX agonist, antiflammin-2 (AF2), and partly by fMLP or peptide Ac2-26 (an AnxA1 derivative which can activate all three members of the human FPR family). An FPR2/ALX antagonist blocked AnxA1 mobilization activated by LXA_4 and AF2. Analysis of PMN degranulation patterns and phospho-AnxA1 status suggested a model where the two FPR2/ALX agonists mobilize the cytosolic (and not the granular) pool of AnxA1 through an intermediate phosphorylation step. Intravital microscopy investigations of the inflamed mesenteric microvasculature of wild type and $AnxA1^{-/-}$ mice revealed that LXA_4 provoked leukocyte detachment from the post-capillary venule endothelium in the former (>50% within 10 min; $P<0.05$), but not the latter genotype (~15%; NS). Furthermore, recruitment of $Gr1^{+}$ cells into dorsal air pouches, inflamed with IL-1β, was significantly attenuated by LXA_4 in wild type, but not $AnxA1^{-/-}$ mice. Collectively, these data prompt us to propose the existence of an endogenous network in anti-inflammation centred on PMN AnxA1 and activated by selective FPR2/ALX agonists.

Keywords

Anti-inflammatory G-protein coupled receptor; Lipoxin A_4; Antiflammin; Leukocyte Adhesion; Neutrophil Activation; Intravital Microscopy

Introduction

It is now appreciated that the process of acute inflammation relies on the active involvement of a series of pro-resolving mediators which assure temporal and spatial containment of the

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host reaction[1]. Therefore, a pro-inflammatory phase is followed by an anti-inflammatory and pro-resolving phase in line with “the beginning programmes the end” concept recently put forward[2]. However, whereas it is well accepted that pro-inflammatory mediators (eg. cytokines, chemokines, autacoids) often act in concert in a network-like fashion[3], the significance of such network(s) to inflammatory resolution has been poorly appreciated and specific mediators/pathways have often been investigated in isolation.

Lipoxin A4 (LXA_4)[2] and Annexin A1 (AnxA1) are two effectors of endogenous anti-inflammation[4] being able to halt leukocyte migration[5, 6] and promote macrophage phagocytosis of infective agents as well as apoptotic leukocytes[7,8,9]. In 2002, we reported that this short-lived lipid, produced by trans-cellular synthesis and cooperation between lipoxygenases[10], and the glucocorticoid-regulated protein both converged on a specific receptor target[11], the formyl peptide receptor type 2 (the acronym FPR2/ALX is currently used to identify the human receptor[12]).

LXA_4 and AnxA1 are very different not only chemically, but also with respect to their biosynthesis (produced on demand or stored in cell sources) and metabolic fate; we questioned whether there was a functional link between them, that justified a single receptor being the main transducer for their, essentially similar, pro-resolving properties. In addition, comparing the time course of generation of these two mediators during acute inflammation in mice and men reveals distinct windows for synthesis and secretion, with exudate LXA_4 concentrations peaking at a much earlier time-point than AnxA1. In murine air-pouches, inflamed with the TLR2 agonist zymosan, LXA_4 peaks at 4 h whereas exudate AnxA1 expression is highest at 24 h, coinciding with the peak of polymorphonuclear cells (PMN) influx[11]. The same holds true in the zymosan peritonitis model[13,14]. Furthermore, 15-epi-lipoxin induced by low-dose aspirin treatment inhibits leukocyte recruitment in a human skin blister model of acute inflammation[15]; in the same model, a delay in the onset of the inflammatory response, seen in ~40% of individuals, is associated with abnormal production of 15-epi-lipoxin[16].

AnxA1 is very abundant in human PMN, representing between 2 and 4% of total intracellular proteins[17]. In resting PMN, a proportion (~60%) of intracellular AnxA1 is localized in gelatinase and azurophilic granules[18,19], with the remaining being cytosolic in location. PMN adhesion to endothelial cell monolayers provokes a rapid mobilization of AnxA1 onto the cell surface[20], where this agonist could encounter its receptor to activate juxtacrine and/or autocrine signals[6], thus curtailing the extent of neutrophil trafficking[21]. Nonetheless, the cytosolic non-granular pool of the protein can also be mobilized, for instance in response to drug application[22-24], and the latter process entails a phosphorylation step at Serine 27 (and possibly other residues) which seems to be a pre-requisite for secretion of the protein onto the cell surface[25].

Antiflammin 2 (AF2) is a nonapeptide corresponding to region 246-254 of AnxA1[26]. In experimental models of acute inflammation, AF2 produces inhibitory properties which, at variance from initial observations[27], are not associated with inhibition of phospholipase A_2 but rather to an interference with PMN activation, chemotaxis and adhesion to endothelial cells[28, 29]. In the AnxA1 protein structure, residues 246-254 are exposed on the outer side of the protein[30] hence available for interaction, in conjunction with the N-terminal region[31], with molecular targets. Congruent with this model, we found that AF2 binds to FPR2/ALX,

[2]**Abbreviations:** AF2, antiflammin 2; AnxA1, Annexin A1; CsH, cyclosporin H; Cyt, cytosolic pool; FPR2/ALX, formyl peptide receptor 2/Lipoxin A_4 receptor; GPCR, G-protein coupled receptor; LXA_4, lipoxin A_4; Mem, membrane pool; PMN, polymorphonuclear leukocytes; WRW_4; peptide WRWWWW.

eliciting genuine post-receptor signaling responses and inhibiting PMN interaction with endothelial monolayer under flow[32].

The present study was undertaken to determine whether LXA_4 and AF2 might affect AnxA1 localization in human PMNs. The data obtained indicate the existence of an anti-inflammatory network centred on FPR2/ALX that operates to regulate PMN activation and trafficking in the microcirculation.

Materials and methods

Human PMN isolation

Peripheral blood was collected from healthy volunteers by intravenous withdrawal in 3.2% sodium citrate solution (1:10). PMNs were isolated from blood by density centrifugation on Histopaque 1119/1077 (Sigma-Aldrich, Poole, UK) gradient according to the manufacturer's instructions and suspended in PBS containing 0.5% BSA. Contaminating erythrocytes were removed by lysis with cold milliQ water. All healthy volunteers gave oral and written consent and cell separation was covered by ethical approval 05/Q0603/34 (East London and The City Research Ethics Committee 1).

PMN incubation with formyl-peptide receptor agonists

To monitor AnxA1 mobilization, peptide Ac2-26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK; Tocris, Bristol, UK), AF2 (HDMNKVLDL, Tocris, Bristol, UK) LXA_4 (EMD chemicals, Darmstadt, Germany) or fMLP (Sigma-Aldrich, Dorset, UK) were added to freshly prepared PMNs (4×10^6 cells/well) for 10-60 min; incubations were then stopped by rapid centrifugation at 4°C and freezing. In some experiments, PMNs were stimulated with selected agonists in presence of the FPR1 antagonist cyclosporin H (CsH, −10min; Biomol GmbH, Hamburg, Germany), which was used at 10 μM as recently reported [33, 34]. In other cases, the FPR2/ALX antagonist WRWWWW[35] (WRW_4, 10μM, −10 min; EMD chemicals, Darmstadt, Germany) was used. Altogether, these experiments aimed at investigating the involvement of FPR1 and FPR2/ALX in PMN AnxA1 mobilization after application of the four agonists used in the present study. Finally, in order to measure rapid formation of phospho-Serine[27]-AnxA1, PMNs were incubated with selected agonists for 10 min. In all cases, cell-free supernatants were collected with cells being processed for membrane and cytosolic fractions. AnxA1 localization and expression was monitored by Western blotting and FACS analysis (see below).

Isolation of PMN membrane (Mem) and cytosolic (Cyt) fractions

After treatment, cells (4×10^6) were re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, with a protease and phosphatase inhibitor cocktail; Sigma-Aldrich, Dorset, UK) and processed as previously shown[36]. Briefly, 100μl of lysis buffer have been used and the cells were sheared through a 25-gauge needle ten times. Lysates were subsequently centrifuged for 2 min at 300x*g*, and supernatants centrifuged again for 45 min at 800 x *g*. The resultant supernatant (cytosolic fraction, Cyt) was collected and the remaining pellet re-suspended in 20 mM Tris-HCl, 1% Triton X-100, pH 7.5 (100 μl) for 15 min (membrane fraction, Mem). Membrane-bound AnxA1 was recovered by washing pelleted cells twice with 50 μl of 1 mM EDTA.

Western blot analysis

Samples boiled in 6x Laemmli buffer were subjected to standard SDS-polyacrylamide gel electrophoresis (12%) and electrophoretically blotted onto polyvinylidene difluoride membranes (PVDF, Millipore, Watford, UK). Membranes were incubated with mouse

monoclonal antibodies (mAb) anti-human AnxA1 (clone 1B; dilution 1:1,000[37]) and anti-human actin (1:10,000; Sigma-Aldrich) in Tris-buffer saline solution containing 0.1% Tween 20 (TBST) and 5% (w/v) non-fat dry milk overnight at 4°C. Membranes were washed for 30 min with TBST with the solution being changed at 10-min intervals; membranes were then incubated with secondary antibody (HRP-conjugated goat anti-mouse 1:5000, Dako, Cambridge, UK), for 2 h at room temperature. Phospho-Ser^{27}-AnxA1 was detected with rabbit Ser^{27} phosphospecific antibody (kind gift from Dr. Egle Solito, 1:1000 in TBST, 1% BSA solution[23, 25]). Proteins were then detected using the ECL detection kit and visualized on Hyperfilm (Amersham Biosciences, Amersham, UK).

FACS Analysis

Granule markers and FPR receptors—PMN were treated with the different FPR agonists, as described above, and incubated with mAb against the different granule/vesicle markers CD35, CD66b, CD63 (all FITC-conjugated monoclonal antibodies, 1:10 final dilution; Serotec, Abingdon, UK). CD35 recognizes secretory vesicles, CD66b is a marker for specific granules and CD63 is a specific marker for azurophilic granules. These intracellular organelles are mobilized in a sequential manner by activated PMNs[38,39].

FPR1 and FPR2/ALX expression—FPR1 and FPR2/ALX cell surface expression was measured by incubation with anti-FPR1 or anti-FPR2 mAb (1:10 dilution in both cases; from R&D System, Abingdon, UK and Genovac, Brussels, Belgium, respectively); a final staining with a rabbit anti-mouse IgG (1:200 dilution, clone STAR9B; Serotec) was then conducted.

Flow cytometry analysis was performed analyzing ≥10,000 events using a FACScalibur flow cytometer (Becton Dickinson, San José, CA) using CellQuest software (Becton Dickinson). Results are reported as median fluorescence intensity (MFI) units.

In vivo analyses in AnxA1 deficient mice

AnxA1 deficient (AnxA1$^{-/-}$)[40] and wild type C57BL/6J (AnxA1$^{+/+}$) mice were obtained from B&K Limited (Hull, UK). Male age-matched 5-8 weeks old mice were used for all experiments, approved and performed under the guidelines of the Ethical Committee for the Use of Animals, Barts and The London School of Medicine and Home Office regulations (Scientific Procedures Act, 1986).

PMN recruitment of post-capillary venules: the detachment effect—Intravital microscopy was performed as previously reported[41,42]. Briefly, mice were anesthetized and the left jugular vein was cannulated with polyethylene tubing (PE 10, internal diameter 0.28mm). A cautery incision was made along the abdominal region, the superior mesenteric artery (SMA) exposed and clamped with a microaneurysm clip to induce ischemia in the mesentery for 30 min, followed by a 45-min reperfusion phase. Toward the end of the reperfusion period (ie, after approximately 35 min), the mesenteric vascular bed was exteriorized, placed on a viewing Plexiglas stage, and mounted on a Zeiss Axioskop “FS” with a water-immersion objective lens (magnification 40; Carl Zeiss, Welwyn Garden City, United Kingdom) and an eyepiece (magnification x10; Carl Zeiss). Tissue preparations were transilluminated with a 12 V, 100W halogen light source. A Hitachi charge-coupled device color camera (model KPC571; Tokyo, Japan) acquired images that were displayed onto a Sony Trinitron color video monitor (model PVM 1440QM; Tokyo, Japan) and recorded on a Sony super-VHS videocassette recorder (model SVO-9500 MDP) for subsequent offline analysis. A video time-date generator (FOR.A video timer, model VTG-33, Tokyo, Japan) projected the time, date, and stopwatch function onto the monitor. Mesenteries were superfused with thermostated (37°C) bicarbonate-buffered solution (g/L: NaCl, 7.71; KCl,

0.25; $MgSO_4$, 0.14; $NaHCO_3$, 1.51; and $CaCl_2$, 0.22, pH 7.4, gassed with 5% CO_2/95%N_2) at a rate of 2 mL/min. When a suitable post-capillary venule was selected (diameter 20 - 40 μm; with equivalent numbers of 5-8 adherent leukocytes per 100 μm vessel length), PBS (100 μl), LXA_4 or AF2 were administered (1 μg dose) *via* the jugular vein, and the fate of the adherent leukocytes was monitored for up to 10 min. Offline analysis monitored i) the number of adherent cells that rejoined the bloodstream and ii) the number of newly adherent leukocytes, as described previously[41,42].

PMN trafficking into inflamed air-pouches—Air-pouches were formed on the dorsal area of C57Bla6 mice by injection of 2.5 ml air on day 0 and 3. On day 6, 10ng of mouse IL-1b (Peprotech, London, UK) were injected in 0.5 ml of 0.5% carboxymethyl-cellulose as described[43]. Four hours later, air-pouches were lavaged and exudate fluids stained with Turk's solution for total cell counting in light microscopy. The extent of PMN influx was determined by FACS analysis by using a FITC-conjugated mAb for Ly-6G/Gr1 (clone RB6-8C5; 10 μg/ml final concentration; eBioscience, San Diego, CA) or isotype controls (eBR2a; eBioscience). FACS analysis was conducted as above, determining the percentage of Gr1 positive ($Gr1^+$) cells and calculating the total number of PMN per air-pouch taking into account total cell number.

Data analysis

Western blot data are reported as mean ± SEM, expressing them as percentage of vehicle-treated cytosolic AnxA1 expression from at least three experiments performed in triplicate and plotted as X(time)/Y(expression) graph. The *n* number of mice has been reported for the *in vivo* experiments. Differences were evaluated using the Mann-Whitney U test for non-parametric data (*in vitro* analyses) or by ANOVA followed by Dunnett post-test (*in vivo* analyses). In all cases, a probability value <0.05 was taken as threshold for rejecting the null hypothesis.

Results

Agonist-dependent mobilization of intracellular AnxA1 in human PMN

Resting PMN contained large amounts of AnxA1, which was largely intracellularly located, with a small proportion in the membrane pool. This can be seen in Figure 1*A* and the following figures, with densitometric values of cumulative experiments showing a ratio of ~80±12% intracellular AnxA1 *vs.* 20±12% membrane AnxA1 (10 experiments, performed in triplicate). This is congruent with the predominant intracellular localization of the protein in resting PMN[20]. However, cell activation with soluble stimuli would mobilize the protein onto the cell surface in an EDTA-labile pool, where partial cleavage would occur, hence the appearance of the doublet[36].

Incubation of PMN alone over a 60-min time-course provoked only mild changes in AnxA1 expression in the absence of receptor agonists. Cell treatment with peptide Ac2-26 (10 μM), AF2 (10 μM), LXA_4 (100 nM) or fMLP (1 μM) provoked significant changes in AnxA1 intracellular location though they occurred to a different extent and pattern (Fig. 1*B*). Over the time course, LXA_4 rapidly (10 min) reduced AnxA1 cytosolic content and this was mirrored by an increase in the membrane pool at 30 min (Fig. 1*A*-*B*). AF2 provoked qualitatively similar – yet slower - effects, with significance only being reached at the 30 min time-point. Peptide Ac2-26 seemed to provoke changes similar to LXA_4 but its effects were lost by 60 min, with the pool of intracellular AnxA1 apparently replenished. The FPR1 agonist fMLP mobilized AnxA1 with kinetics similar to those produced by peptide Ac2-26: at 30 min there was significant reduction of intracellular AnxA1, which was partially

replenished by 60 min, possibly consequence to a reduction of the membrane pool by this time-point (Fig.1*B*).

Changes in AnxA1 concentrations in cell supernatants were detected only after 30 min treatment (data not shown) implying some delay in the completion of the AnxA1 externalization process after its exposure on the plasma membrane[20, 36]; normally AnxA1 is not detectable in the culture medium of resting PMN (data not shown). From this set of experiments, we selected the 30 min time-point and the two FPR2/ALX agonists AF2 and LXA_4, for further studies.

Characterization of AF2 and LXA_4 induced AnxA1 mobilization

Incubation of human PMN with AF2 (1-10μM) provoked a reduction of cytosolic AnxA1 and a simultaneous increase in either membrane fraction and cell supernatant (Fig 2*A*) AnxA1. This effect was evident at 1μM AF2, and robustly observed at 3-10 μM concentrations. Treatment of cells with LXA_4 (10-100 nM) induced changes in AnxA1 location similar to AF2; notably, these effects were concentration-dependent and optimal at ~10 nM (Fig. 2*B*).

Pre-treatment of human PMN with the antagonist WRW_4 (10μM, 10 min pre-incubation) affected both LXA_4 and AF2-induced AnxA1 mobilization. In particular, the marked mobilization of AnxA1 on the cell surface induced by either FPR2/ALX agonists was essentially abolished by WRW_4; representative blots are shown (Fig. 3*A*) with cumulative data for cytosolic, membrane and supernatant pools of AnxA1, respectively (Fig. 3*B-D*). Peptide Ac2-26 was also tested in these experiments however its modulation of PMN AnxA1 localization was not affected by WRW_4 (Fig. 3*B-D*).

Experiments done in presence of the selective FPR1 antagonist CsH, showed that AnxA1 mobilization induced by fMLP was genuinely due to engagement of this receptor (Fig. 4). A different scenario emerged for peptide Ac2-26 which retained its ability to mobilize AnxA1 (Fig. 4). Of interest, even when a combination of CsH and WRW_4 was tested, peptide Ac2-26 used at 10 μM, yielded marked mobilization of AnxA1 (Fig. 1S).

To determine whether AnxA1 mobilization was secondary to, or accompanied by, granule mobilization, antibodies specific for PMN granules, such as CD35 (secretory vesicles), CD66b (specific granules) and CD63 (azurophil granules) were used. Flow cytometry analysis showed that, following 30 min incubation, unlike PMA (positive control) neither LXA_4 nor AF2 induced changes in the PMN cell surface expression of any of these markers (Fig. 5*A*). Interestingly, both agonists caused internalization of FPR2/ALX over the 30 min incubation time, with no effect on FPR1 (Fig. 5*B*). In these experiments, fMLP (1 μM) provoked significant up-regulation of CD66b (+60%; Fig. 5*A*).

Since membrane AnxA1 did not appear to derive from intracellular granules, we monitored AnxA1 phosphorylation status in the cytosolic pool[25,24], using an antibody against phospho-Ser^{27}-AnxA1[25]. Shorter time-points were tested here, with Fig. 6 reporting data obtained at 10 min. In resting cells there was little membrane phosphorylated AnxA1; addition of LXA_4 and AF2 provoked rapid increase in this species (Fig. 6*A*). Incubation with fMLP augmented AnxA1 on the membrane surface (Fig. 6*B*), but this did not appear to be phosphorylated.

Cell-surface AnxA1 in stimulated PMN could also be quantified by flow cytometry. At the optimal 30 min incubation time, both LXA_4 and AF2 augmented AnxA1 expression at the single cell level (>2-fold increase in MFI Units; Fig. 7*A-B*) and there was a marked increase

in AnxA1 positive PMN, with values close to 70% versus control (resting PMN) levels of ~15%. A similar pattern was seen following incubation with fMLP.

Anti-inflammatory properties of LXA_4 and AF2 are tightly coupled to AnxA1

Next, we tested if the rapid non-genomic modulation of PMN AnxA1 might be of relevance to PMN trafficking in inflammation. To this end, we used a validated protocol whereby LXA_4[42] and AnxA1 itself exquisitely modulate the fate of adherent leukocytes, a biological effect referred to as 'the detachment phenomenon'[41]. The mesenteric microcirculation of $AnxA1^{+/+}$ mice was inflamed with an ischaemia/reperfusion procedure, allowing the selection of post-capillary venules with a ~6-8 adherent leukocytes, in contrast to the minimal leukocyte interaction with the post-capillary venule endothelium observed in sham operated animals (0-2 cells per vessel) (see Figure 2S).

Injection of a single bolus of LXA_4 (1μg i.v., corresponding to 2.8nmol) to $AnxA1^{+/+}$ mice provoked a marked (>50%) detachment of PMN from the endothelium within the 10 min observation period (Fig. 8*A*). This effect was drastically reduced in $AnxA1^{-/-}$ mice, with no significant alteration in the fate of the adherent leukocytes (~15%; Fig. 8*C*). A similar result was obtained with AF2 (1μg i.v., corresponding to 1 nmol) which afforded significant detachment of adherent leukocytes in $AnxA1^{+/+}$, but not $AnxA1^{-/-}$, mice (Fig. 8*B-C*).

It should be noted that in line with the reported alterations within the microcirculation of in $AnxA1^{-/-}$ [43] absence of AnxA1 was associated with a slightly higher degree of adherent cells in both sets of experiments (e.g. Fig. 8*A-B*).

Finally, we investigated whether the crucial role of endogenous AnxA1 in LXA_4-induced effects was also evident in a more severe type of inflammation. IL-1β injection into mice dorsal air-pouches provoked an intense recruitment of $Gr1^+$ cells at 4h time-point, which was inhibited by treatment with LXA_4 in $AnxA1^{+/+}$, but not $AnxA1^{-/-}$, mice (Fig. 8*D*).

Discussion

In this study we provide strong evidence that engagement of FPR2/ALX by selective agonists would induce AnxA1 phosphorylation and mobilization in human PMN. We propose this cellular response operates to control PMN recruitment to post-capillary venules and migration to inflamed sites, as demonstrated with proof-of-concept experiments in $AnxA1^{-/-}$ mice, in which LXA_4 and AF2 lost their anti-migratory properties.

An effective inflammatory reaction relies on the concerted action of several pathways which attract white blood cells from the vasculature into specific tissue sites. A classical example is that of the synovial joint; here TNF-α initiates a cascade of mediator generation and release (e.g. IL-1 or IL-6)[44,45] culminating in the recruitment of blood borne leukocytes into the synovial tissue and joint space. Blocking TNF-α activity is therefore a very successful therapeutic strategy [44,46]. In recent years an innovative approach to anti-inflammatory therapeutic development has emerged, which aims to capitalize on the action of endogenous anti-inflammatory and pro-resolving mediators[47, 48]. The evidence that the pro-inflammatory phase is required for an appropriate induction of the endogenous anti-inflammatory phase is now well accepted[2]. As an example, pro-inflammatory prostanoids induce LXA_4 synthesis in, what has been proposed to be, a 'class switching' from the pro-inflammatory to the pro-resolving phase of an acute experimental inflammation[49]. However, the option that a network might be operative within anti-inflammatory mediators themselves has been poorly explored: we demonstrate here a functional link between LXA_4 and endogenous AnxA1. Incubation of human PMN with LXA_4 induced a rapid translocation of AnxA1 to the cell surface with a time profile different from that provoked by peptide

Ac2-26 or fMLP. The effect of LXA_4 was mimicked by the anti-inflammatory synthetic nonapeptide AF2.

It is intriguing that both LXA_4 and AF2 inhibit neutrophil activation *in vitro* as well as recruitment of these cells *in vivo*. AF2 reduces HL-60 cells adhesion to endothelial cells, suggesting that it dampens inflammation by blocking leukocyte trafficking and the subsequent eicosanoid production[29]. Furthermore, AF2 is effective in suppressing endotoxin-induced uveitis in rats[50]. The data presented here indicate that such an effect might occur at least partly *via* endogenous AnxA1. Our new data and ensuing hypothesis may provide a mechanistic explanation for the observation that endogenous LXA_4 and AnxA1 are both operative in germ-free mice for optimal generation of IL-10 in the gut[51].

It should also be noted that very few examples of crosstalk in resolution are beginning to emerge, with resolvin E_1 promoting exudate expression of LXA_4, although the specific cell target(s) of this mediator were not identified[52]. In addition, there is indication that endogenous epi-LXA_4, synthesized upon aspirin unique blockade of cyclo-oxygenase 2, can mobilize nitric oxide from the endothelium (constitutive nitric oxide synthase activation) and macrophages (inducible nitric oxide synthase activation) to elicit anti-inflammatory effects in a model of pleurisy as well as in the microcirculation[53]. We cannot exclude that an involvement of endogenous nitric oxide might be occurring also in our settings, though the experiments of leukocyte detachment reported here took place within 10-min. In any case, the recent involvement of rapidly produced endothelial nitric oxide and prostacyclin in the anti-migratory effects of resolvin D_2[54] would suggest to keep this option viable also upon the acute application of LXA_4 and AF2. Finally, the positive association between endogenous AnxA1 and expression of mouse Fpr2 during resolving colitis should be reported[55].

Most of the studies summarized above begin to propose the existence of positive non-genomic loops in resolution, however, there is also emerging evidence for genomic associations. One classical example is the one linking glucocorticoids to over-expression of AnxA1, with increments in gene promoter activity being measured with a reporter gene[14]. In addition, LXA_4 could induce two specific signaling molecules, NGFI-A binding protein 1 (NAB1)[56] and suppressor of cytokine signaling 2[57] to re-programme the phenotype of PMN and dendritic cells, respectively. Among the biological effects elicited by LXA_4, its analogs and other pro-resolving lipid mediators, it is worth highlighting the augmented expression of CCR5 on apoptotic leukocytes, a response that facilitate removal of migrated cells and tissue resolution[58]. De novo gene synthesis has, clearly, been ascribed to glucocorticoids; in this context it must be noted that they will not only augment AnxA1 synthesis and release, but would also increase expression of anti-inflammatory FPR2/ALX[59,60].

The observation that exogenously applied LXA_4 mobilizes AnxA1 and that this contributes, at least in part, to its inhibitory properties on PMN trafficking, adds another layer of complexity to the molecular and functional inter-relationship between these two endogenous effectors of anti-inflammation and resolution. Moreover, it is intriguing how a pattern of distinct inducers of AnxA1 externalization is emerging. Processes such as cell adhesion and activation, which induce re-arrangement of intracellular organelles within the PMN, would mobilize preferentially the pool of AnxA1 contained in granules and vesicles[20,61] (see Fig. 9 for a schematic summary). On the other hand, the typical mobilization induced by glucocorticoids utilizing a receptor-dependent non-genomic pathway [62,63,25] seems to be replicated by other classes of drugs including cromones [23, 24] and, as described here, LXA_4 and AF2. Mobilization of this cytosolic pool of AnxA1 is downstream PKC activation and phosphorylation at Ser^{27} on the AnxA1 N-terminus[25] (Fig. 9). It is unclear whether PKC isoform(s) - advocated for dexamethasone and nedocromil action [23, 25] – also operates

downstream FPR2/ALX activation by LXA_4 and AF2. In any case, the involvement of this specific GPCR is unambiguous, as shown by the effects produced with the receptor antagonist and the observation of FPR2/ALX internalization after agonist application. It should be noted that a recent study indicated selective internalization of the receptor upon application of LXA_4 as a prerequisite for stimulation of phagocytosis by this anti-inflammatory lipid[64].

As stated above, LXA_4 is a potent regulator of PMN trafficking in experimental inflammation, with its ability to inhibit recruitment of this cell type to a variety of tissue sites and in response to several distinct inflammogens[6]. The same is partially true for AF2, though this nonapeptide has been used more often as an inhibitor of PMN activation *in vitro* [27,29] rather than of cell recruitment *in vivo*[65]. For instance, AF2 inhibits PMN adhesion to endothelial cells[28] and leukocyte chemotaxis[66]. Both compounds were tested in the IVM protocol which we reported being sensitive to LXA_4 treatment[42]. The results we present here indicate a crucial role for endogenous AnxA1 in the detachment phenomenon promoted by either FPR2/ALX agonist.

Peptide Ac2-26 was not tested in the leukocyte detachment phenomenon here, in view of the *in vitro* profile we obtained with human PMNs. However, it has been previously shown that administration of this peptide to mice can activate this process in an Fpr1-independent fashion[42] and peptide Ac2-26 also retains its anti-migratory effects in AnxA1-null mice[21]. In the present *in vitro* settings, this AnxA1 mimetic induces AnxA1 mobilization in a different manner with respect to LXA_4 and AF2 (and fMLP) since it does not appear to evoke involvement of either FPR1 and FPR2, as shown by experiments performed with FPR1 or FPR2/ALX selective antagonists. It should be noted that PMN activating effects associated to AnxA1 N-terminal derived peptides (Ac9-25) do not require activation of FPR1 or FPR2/ALX[67].

Finally, it should be noted that the inhibitory properties exhibited by LXA_4 in the air-pouch model were lost in AnxA1$^{-/-}$ mice. Coupled with the data recent observation of LXA_4 lack of effect in Fpr2$^{-/-}$ mice, using the same experimental model and inflammogen[68], we propose a model whereby intravascular activation of PMN Fpr2, in response to the agonists tested here, would bring about AnxA1 mobilization. This event would in turn act in a juxtacrine/autocrine fashion[6], reinforcing the overall attenuation of the process of leukocyte recruitment. Clearly, other intracellular pathways [69,70] and mediators might also act downstream LXA_4 application [5,66], nonetheless the data presented and discussed here point to the non-genomic mobilization of AnxA1 as one of the major downstream effectors of the LXA_4, and AF2, actions that negatively modulate the cell trafficking process. Networks of pro-resolving mediators and receptors are an emerging feature of the endogenous homeostatic response; once more loops are discovered and dissected, these ‘anti-inflammatory networks’ are likely to become an accepted paradigm in the area of inflammatory resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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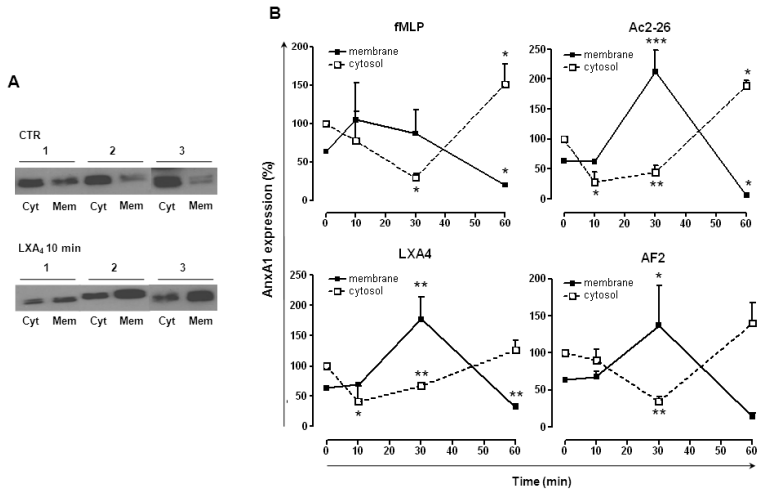

Figure 1. AnxA1 disposition in human PMN upon agonist stimulation

A, Representative blots showing AnxA1 localization in unstimulated and LXA_4 treated human PMN; AnxA1, mainly present in cytosol (Cyt), is mobilized towards the membrane surface (Mem) upon stimulation with agonists (LXA_4). Data are from 3 distinct PMN preparations. *B*, Human PMN were treated with vehicle (CTR), AF2 (10 μM), peptide Ac2-26 (10 μM), LXA_4 (100nM) or fMLP (1 μM) for 10-60 min and then expression of AnxA1 in cytosolic and membrane fraction was determined by Western blotting. Blots are showed as line graphs reporting expression *vs.* time. Data, mean ± SEM, are calculated as percentage of vehicle-treated cytosolic AnxA1 expression taken as 100 (*****p<0.05; ******p<0.01; *******p<0.001 *vs.* CTR; n=3).

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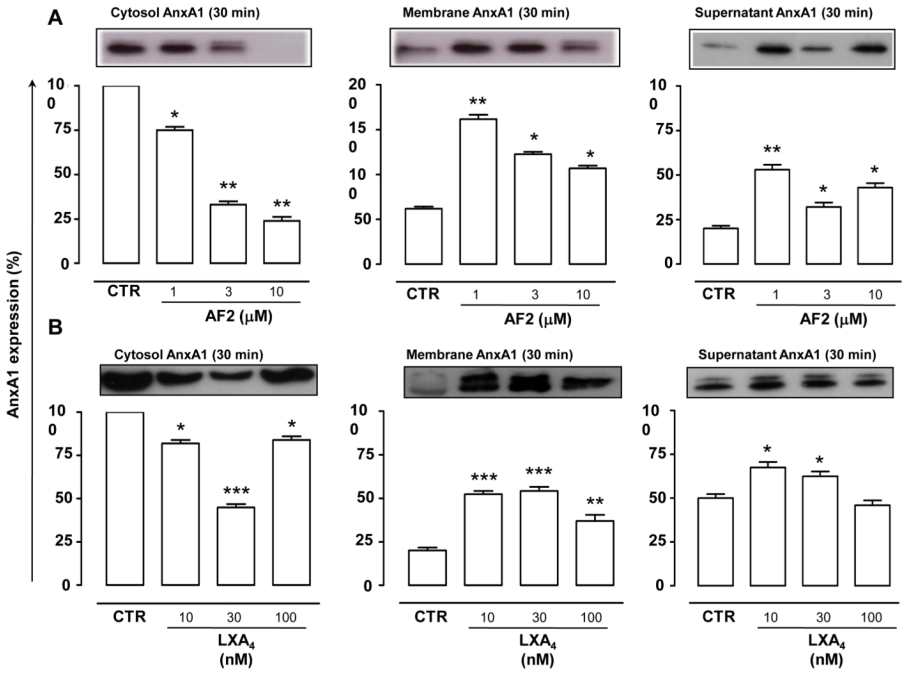

Figure 2. AnxA1 mobilization in human PMN: concentration-response studies

Treatment of human PMN either with AF2 (1-10 μM, *A*) or LXA_4 (10-100 nM, *B*) for 30 min provoked a decrease in cytosolic AnxA1 levels and a parallel increase in AnxA1 membrane surface expression. Blots are representative of three different experiments. Data, mean ± SEM, are calculated as percentage of vehicle-treated cytosolic AnxA1 expression taken as 100 (*****p<0.05; ******p<0.01; *******p<0.001 *vs.* vehicle, CTR).

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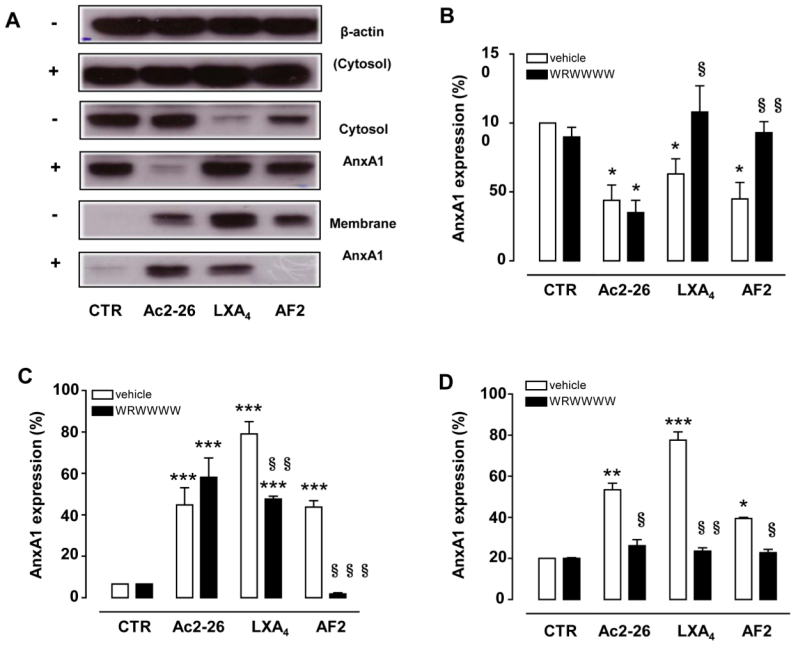

Figure 3. WRW_4 prevents LXA_4 and AF2-elicited mobilization of AnxA1 in human PMN

Human PMN were pre-treated with WRW_4 (10 μM, 10 min), prior addition of vehicle (CTR), Ac2-26 (10 μM), LXA_4 (100 nM) or AF2 (10 μM) for further 30 min. Subcellular fractions were then processed and analyzed by Western blotting. *A*, Representative blots from three different experiments in the presence (+) or absence (−) of WRW_4. *B-D*, Densitometric analysis for AnxA1 expression in cytosol (*B*), membrane (*C*) and supernatant (*D*), respectively. Data, mean ± SEM, are calculated as percentage of vehicle-treated cytosolic AnxA1 expression taken as 100 (*****p<0.05; ******p<0.01; *******p<0.001 vs. CTR, i.e. no WRW_4; §*p*<0.05; §§*p*<0.01; §§§*p*<0.001 *vs.* vehicle, i.e. in presence of WRW_4 only).

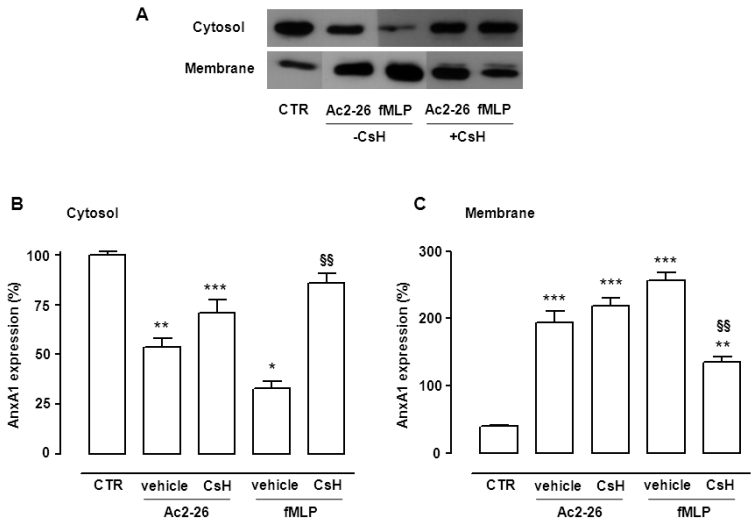

Figure 4. CsH affects fMLP-elicited mobilization of AnxA1 in human PMN

Human PMN were pre-treated with CsH (10 μM, 10 min), prior addition of vehicle (CTR), Ac2-26 (10 μM) or fMLP (1μM) for further 30 min. Subcellular fractions were then processed and analyzed by Western blotting. *A*, Representative blots from three different experiments in the presence (+) or absence (−) of CsH. *B-C*, Densitometric analysis for AnxA1 expression in cytosol (*B*) and membrane (*C*) respectively. Data, mean ± SEM, are calculated as percentage of vehicle-treated cytosolic AnxA1 expression taken as 100 (*****p<0.05; ******p<0.01; *******p<0.001 *vs.* CTR, i.e. no CsH; §*p*<0.05; §§*p*<0.01; §§§*p*<0.001 *vs.* vehicle, i.e. in presence of CsH).

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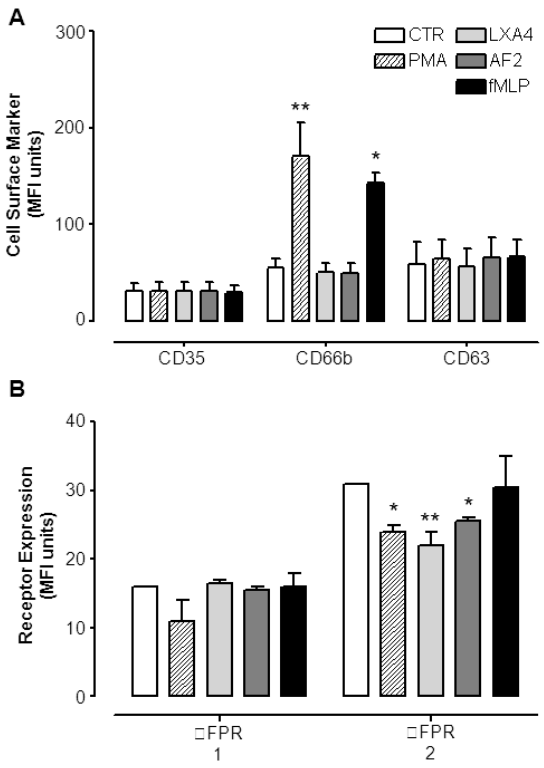

Figure 5. FACS analysis on human PMN after treatment with LXA_4 and AF2

Human PMN were treated with vehicle (CTR), AF2 (10 μM), LXA_4 (100nM) or fMLP (1 μM) for 30 min prior staining with a specific mAbs. PMA (100 nM) was as positive control for PMN degranulation. *A*, Mean fluorescence intensity (MFI) determination for cell surface staining for PMN granule markers CD35, CD66b and CD63. *B*, Mean fluorescence intensity for FPR1 and FPR2/ALX receptors surface expression upon treatment with FPR2/ALX agonists LXA_4 and AF2. Data, reported as MFI unites, are mean ± SEM from three different experiments (*****p<0.05; ******p<0.01 vs. CTR).

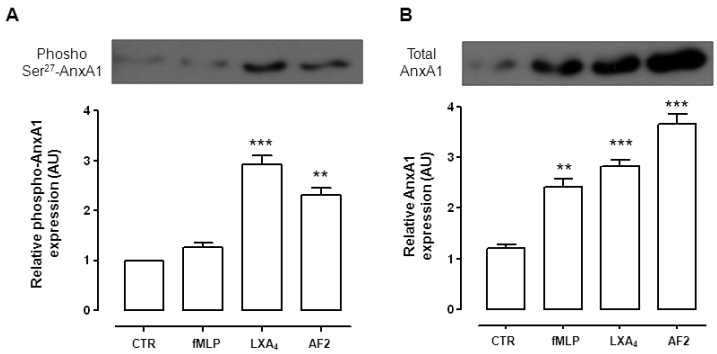

Figure 6. Phospho-Serine ^{27}-AnxA1 detection in PMN membrane fractions

Human PMN were treated with vehicle (CTR) AF2 (10 μM), LXA_4 (100nM) or fMLP (1 μM) for 10 min prior to recovery of membrane-bound AnxA1 by EDTA. *A*, Phospho Serine ^{27}-AnxA1 with representative western blotting and densitometric cumulative values. *B*, total AnxA1 in the membrane pool with representative western blotting and densitometric cumulative values, Data reported as relative optical density arbitrary unit (AU), are mean ± SEM from three experiments (******p<0.01; *******p<0.001 vs. CTR).

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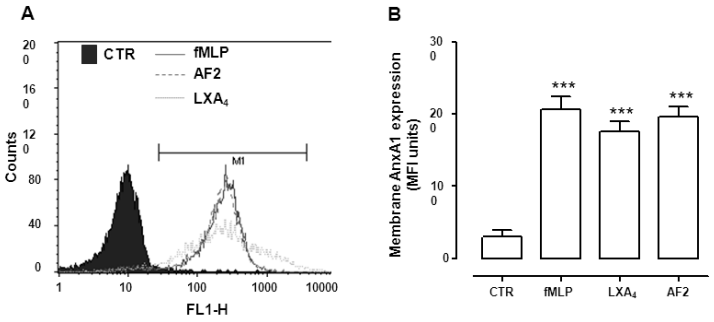

Figure 7. Detection of cell surface AnxA1 by flow cytometry

Human PMN were treated with vehicle (CTR), AF2 (10 μM), LXA_4 (100nM) or fMLP (1 μM) for 30 min prior to staining with a specific anti-AnxA1 mAb. *A*, Representative histograms depicting the increase in fluorescence intensity as AnxA1 becomes externalized. *B*, Cumulative data for AnxA1 expression. Data, reported as MFI unites, are mean ± SEM from three different experiments (***p<0.001 vs. CTR).

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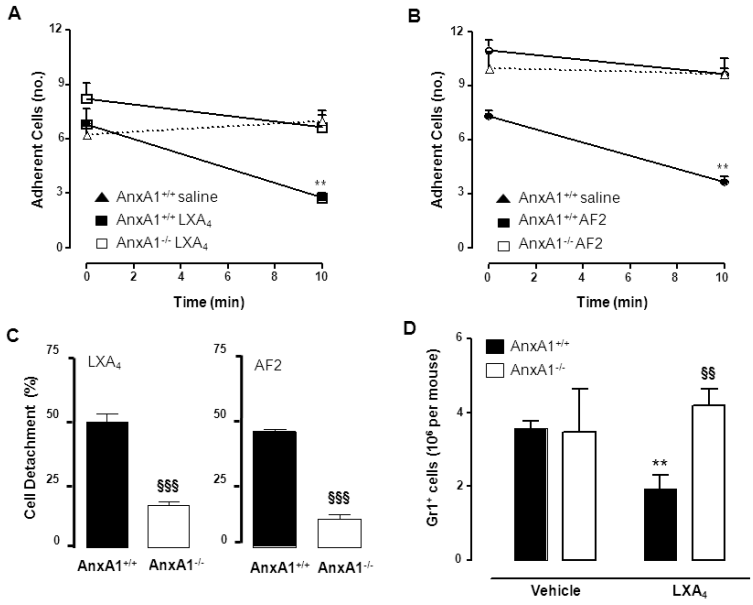

Figure 8. LXA_4 and AF2 provoke leukocyte detachment by mobilizing endogenous AnxA1

Intravital microscopy analyses of $AnxA1^{+/+}$ and $AnxA1^{-/-}$ mouse mesenteric microcirculation inflamed by an ischaemia-reperfusion procedure. *A-B*, Administration of LXA_4 and AF2 (1 μg i.v. at time 0, that is once the preparation is on stage and post-capillary venule selected) induced detachment of adherent leukocytes in $AnxA1^{+/+}$ but not in $AnxA1^{-/-}$ mice (**p<0.01 *vs.* time 0). *C*, Cumulative modulation of the detachment process, as recorded at the end of the 10 min observation period. Data are Mean ± SEM of 6 mice per group ($^{§§§}p$<0.001 vs. $AnxA1^{+/+}$). *D*, Dorsal air pouches of $AnxA1^{+/+}$ and $AnxA^{-/-}$ mice were injected with IL-1β (10 ng in 0.5 ml) at time 0; vehicle (100 μl) or LXA_4 (1 μg/100μl) were injected i.v. *via* the tail vein immediately after. In all cases, air-pouches were washed 4 h later and cells were stained to determine amount of recruited PMN ($Gr1^+$ cells). Data are mean ± SEM of 6 mice per group (**p<0.01 vs. vehicle; $^{§§}p$<0.01 vs. $AnxA1^{+/+}$).

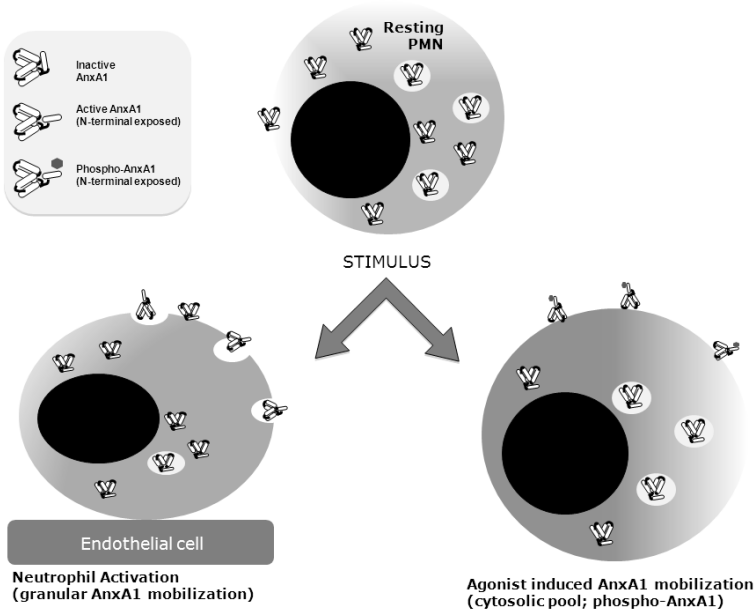

Figure 9. Schematic model for AnxA1 mobilization in human PMN

Proposed scheme of AnxA1 mobilization whereby cell-to-cell interaction (e.g. adhesion to endothelial cells) would externalize AnxA1 by mobilizing predominantly the granular pool[18, 19,20] , whereas the non-granular (cytosolic) pool seems to be mobilized in response to soluble compound addition, e.g. glucocorticoids, cromones and inhibitors of the cystic fibrosis transducer factor[22-24]. The present study adds FPR2/ALX to the list of compounds known to 'activate' the AnxA1 pathway in the neutrophil. The latter mode of mobilization seems to have, as a consistent pre-requisite, phosphorylation of Ser[27] on the AnxA1 N-terminus. It is yet unclear if PMN adhesion to endothelial cells[20] leads to externalization of phosphorylated AnxA1 species.