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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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The importance of proresolving mediators in the overall context of the resolution of acute inflammation is well recognized, although little is known about whether these anti-inflammatory and proresolving molecules act in concert. In this article, we focused on lipoxin A4 (LXA4) and annexin A1 (AnxA1) because these two very different mediators converge on a single receptor, formyl peptide receptor type 2 (FPR2/ALX). Addition of LXA4 to human polymorphonuclear leukocytes (PMNs) 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, and partly by fMLF or peptide Ac2-26 (an AnxA1 derivative that can activate all three members of the human FPR family). An FPR2/ALX antagonist blocked AnxA1 mobilization activated by LXA4 and antiflammin-2. Analysis of PMN degranulation patterns and phospho-AnxA1 status suggested a model in which 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 LXA4 provoked leukocyte detachment from the postcapillary 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 LXA4 in wild-type, but not AnxA1−/−, mice. Collectively, these data prompt us to propose the existence of an endogenous network in anti-inflammation centered on PMN AnxA1 and activated by selective FPR2/ALX agonists. The Journal of Immunology, 2011, 186: 000–000.

It is now appreciated that the process of acute inflammation relies on the active involvement of a series of proresolving mediators that ensure temporal and spatial containment of the host reaction (1). Therefore, a proinflammatory phase is followed by an anti-inflammatory and proresolving phase in line with the “beginning programs the end” concept recently put forward (2). However, whereas it is well accepted that proinflammatory mediators (e.g., cytokines, chemokines, autacoids) often act in concert in a network-like fashion (3), the significance of such networks to inflammatory resolution has been poorly appreciated, and specific mediators and pathways have often been investigated in isolation.

Lipoxin A4 (LXA4) (2) and annexin A1 (AnxA1) are two effectors of endogenous anti-inflammation (4) that are able to halt leukocyte migration (5, 6) and promote macrophage phagocytosis of infective agents as well as apoptotic leukocytes (7–9). In 2002, we reported that this short-lived lipid, produced by transcellular 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 [FPR2/ALX; currently used to identify the human receptor (12)].

LXA4 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 a functional link existed between them that justified a single receptor as the main transducer for their essentially similar proresolving 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 LXA4 concentrations peaking at a much earlier time point than AnxA1 concentrations. In murine air-pouches, inflamed with the TLR2 agonist zymosan, LXA4 peaks at 4 h, whereas exudate AnxA1 expression is highest at 24 h, coinciding with the peak of polymorphonuclear leukocyte (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).

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Abbreviations used in this article: AF2, antiflammin 2; AnxA1, annexin A1; CsH, glucocorticoid-regulated protein both converged on a specific receptor target; CTR, control; FPR2/ALX, formyl peptide receptor 2/lipoxin A4 receptor; LXA4, lipoxin A4; MFI, median fluorescence intensity; PMN, polymorphonuclear leukocyte; WRW4, peptide WRW4.

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AnxA1 is very abundant in human PMNs, representing between 2 and 4% of total intracellular proteins (17). In resting PMNs, 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 juxta and/or autocrine signals (6), thus curtailing the extent of neutrophil trafficking (21). Nonetheless, the cytosolic nongranular pool of the protein can also be mobilized, as, for instance, in response to drug application (22–24), and the latter process entails a phosphorylation step at Ser27 (25) (and possibly other residues), which seems to be a prerequisite for secretion of the protein onto the cell surface (25).

Antiflavin 2 (AF2) is a nonapeptide corresponding to region 246–254 of AnxA1 (26). In experimental models of acute inflammation, AF2 produces inhibitory properties that, at variance from initial observations (27), are not associated with inhibition of phospholipase A2 but rather with 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) and are 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, eliciting genuine postreceptor signaling responses and inhibiting PMN interaction with endothelial monolayer underflow (32).

The present study was undertaken to determine whether LXA4 and AF2 might affect AnxA1 localization in human PMNs. The data obtained indicate the existence of an anti-inflammatory network centered 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 i.v. withdrawal in 3.2% sodium citrate solution (1:10). PMNs were isolated from blood by density centrifugation on Histopaque 1119/1077 (Sigma-Aldrich, Poole, U.K.) 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 (1). Peripheral blood was collected from healthy volunteers by i.v. withdrawal in 3.2% sodium citrate solution (1:10). PMNs were isolated from blood by density centrifugation on Histopaque 1119/1077 (Sigma-Aldrich, Poole, U.K.) 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 (1).

PMN incubation with FPR agonists

To monitor AnxA1 mobilization, peptide Ac-A2-26 (Ac-AMVSEFLQAW-FIENEQEQYQTVK; Tocris, Bristol, U.K.), AF2 (HDMMNKVLDL; Tocris, Bristol, U.K.), LXA4 (EMD Chemicals, Darmstadt, Germany), or IML2 (Sigma-Aldrich, Dorset, U.K.) was added to freshly prepared PMNs (4 × 10^5 cells/well) for 10–60 min; incubations were then stopped by rapid centrifugation at 4°C and freeze. In some experiments, PMNs were stimulated with selected agonists in the presence of the FPR1 antagonist cyclosporin H (C3; 10 μM; Biomol, Hamburg, Germany), which was used at 10 μM, as recently reported (33, 34). In other cases, the FPR2/ALX antagonist WRW44WW (15) (WRW44; 10 μM; 10 min; EMD Chemicals) 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 current study. Finally, to measure rapid formation of phospho-Ser27-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 were monitored by Western blotting and FACS analysis (see below).

Isolation of PMN membrane and cytosolic fractions

After treatment, cells (4 × 10^6) were resuspended in lysis buffer (20 mM Tris-Cl, pH 7.5, with a protease and phosphatase inhibitor mixture; Sigma-Aldrich) and processed as previously shown (36). Briefly, 100 μl lysis buffer was used, and the cells were sheared through a 25-gauge needle 10 times. Lysates were subsequently centrifuged for 2 min at 3000 g, and supernatants centrifuged again for 45 min at 8000 g. The resulting supernatant (cytosolic fraction) was collected and the remaining pellet resuspended in 20 mM Tris-HCl, 1% Triton X-100, pH 7.5 (100 μl), for 15 min (membrane fraction). Membrane-bound AnxA1 was recovered by washing pelleted cells twice with 50 μl 1 mM EDTA.

Western blot analysis

Samples boiled in 6 × Laemmli buffer were subjected to standard SDS-PAGE (12%) and electrophoretically blotted onto polyvinylidene difluoride membranes (PVDF; Millipore, Watford, U.K.). Membranes were incubated with the mouse mAbs anti-human AnxA1 (clone 1B; dilution, 1:1000; Sigma-Aldrich) in TBST and 5% (w/v) nonfat 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 Ab (HRP-conjugated goat anti-mouse 1:5000; Dako, Cambridge, U.K.), for 2 h at room temperature. Phospho-Ser27-AnxA1 was detected with rabbit anti-Ser27 phosphate-specific Ab (a kind gift from Dr. Egle Solito [Imperial College London, London, U.K.], 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, U.K.).

FACS analysis

Granule markers and FPR receptors. PMNs were treated with the different FPR agonists, as described above, and incubated with mAbs against the different granule/vesicle markers CD35, CD66b, and CD63 (all FITC-conjugated mAbs, 1:10 final dilution; Serotec, Abingdon, U.K.). 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 Systems, Abingdon, U.K., and Genovec, Brussels, Belgium, respectively); a final staining with a rabbit anti-mouse IgG (1:200 dilution; clone STAR9B; Serotec) was then conducted.

With flow cytometry, ≥10,000 events were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with 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/6 (AnxA1+/+) mice were obtained from B&K Limited (Hull, U.K.). Male age-matched 5- to 8-wk-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 postcapillary 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, 0.28 mm). A cautery incision was made along the abdominal region, and the superior mesenteric artery was 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 (i.e., after ~35 min), the mesenteric vascular bed was exteriorized, placed on a viewing Flexiglas stage, and maintained on a Zeiss Axiostop FS with a water-immersion objective lens (Carl Zeiss, Welwyn Garden City, U.K.) and an eyepiece (Carl Zeiss). Tissue preparations were trans-illuminated with a 12-V, 100-W halogen light source. A Hitachi charge-coupled device color camera (model KPC571; Hitachi, Tokyo, Japan) acquired images that were displayed on a Sony Trinitron color video monitor (model PVM 1440QM; Sony, Tokyo, Japan) and recorded on a Sony super-VHS video cassette recorder (model SVO-9500 MDP) for subsequent offline analysis. A video time-date generator (FOR.A video time-date generator, model VTG-33; JVC, Tokyo, Japan) projected the time, date, and stopwatch function onto the monitor. Mesenteries were superfused with thermostated (37°C) bicarbonate-buffered solution (grams per liter: NaCl, 7.71; KCl, 0.25; MgSO₄, 0.14; NaHCO₃, 1.51; and CaCl₂, 0.22; pH 7.4, gassed with 5% CO₂/95% N₂) at a rate of 2 ml/min. When a suitable venules (diameter, 20–40 μm) was made along the abdominal region, and the superior mesenteric artery was exteriorized and placed on a viewing Flexiglas stage, and maintained on a water-immersion objective lens (Carl Zeiss). Tissue preparations were trans-illuminated with a 12-V, 100-W halogen light source. A Hitachi charge-coupled device color camera (model KPC571; Hitachi, Tokyo, Japan) acquired images that were displayed on a Sony Trinitron color video monitor (model PVM 1440QM; Sony, Tokyo, Japan) and recorded on a Sony super-VHS video cassette recorder (model SVO-9500 MDP) for subsequent offline analysis. A video time-date generator (FOR.A video time-date generator, model VTG-33; JVC, Tokyo, Japan) projected the time, date, and stopwatch function onto the monitor. Mesenteries were superfused with thermostated (37°C) bicarbonate-buffered solution (grams per liter: NaCl, 7.71; KCl, 0.25; MgSO₄, 0.14; NaHCO₃, 1.51; and CaCl₂, 0.22; pH 7.4, gassed with 5% CO₂/95% N₂) at a rate of 2 ml/min. When a suitable postcapillary venule was selected (diameter, 20–40 μm; with equivalent numbers of 5–8 adherent leukocytes per 100-μm vessel length), PBS (100 μl), LXA₄, or AF2 was 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 days 0 and 3. On day 6, 10 ng mouse IL-1β (Peprotech, London, U.K.) was injected in 0.5 ml 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 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+) PMNs.

**FIGURE 1.** AnxA1 disposition in human PMNs upon agonist stimulation. A, Representative blots showing AnxA1 localization in unstimulated and LXA4-treated human PMNs; AnxA1, mainly present in cytosol (Cyt), is mobilized toward the membrane surface (Mem) upon stimulation with agonists (LXA4). Data are from three distinct PMN preparations. B, Human PMNs were treated with vehicle (control [CTR]), AF2 (10 μM), peptide Ac2-26 (10 μM), LXA4 (100 nM), or fMLF (1 μM) for 10–60 min; then expression of AnxA1 in cytosolic and membrane fractions was determined by Western blotting. Blots are shown as line graphs reporting expression versus 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 versus CTR; n = 3. fMLP, formyl-methionyl-leucyl-phenylalanine.

**FIGURE 2.** AnxA1 mobilization in human PMN: concentration-response studies. Treatment of human PMNs either with AF2 (1–10 μM) (A) or LXA4 (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 versus vehicle, CTR.
cells and calculating the total number of PMNs per air-pouch, taking into account total cell number.

Data analysis

Western blot data are reported as mean ± SEM, expressed as percentage of vehicle-treated cytosolic AnxA1 expression from at least three experiments performed in triplicate and plotted as an X(time)/Y(expression) graph. The $n$ number of mice has been reported for 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 posttest (in vivo analyses). In all cases, a $p$ value $<0.05$ was taken as the threshold for rejecting the null hypothesis.

Results

Agonist-dependent mobilization of intracellular AnxA1 in human PMN

Resting PMNs contained large amounts of AnxA1, which was, for the most part, intracellularly located, with a small proportion in the membrane pool. This arrangement can be seen in Fig. 1A and the following figures, with densitometric values of cumulative experiments showing a ratio of $\sim 80 \pm 12\%$ intracellular AnxA1 versus $20 \pm 12\%$ membrane AnxA1 (10 experiments, performed in triplicate). This finding is congruent with the predominant intracellular localization of the protein in resting PMNs (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 PMNs 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 $\mu$M), AF2 (10 $\mu$M), LXA4 (100 nM), or fMLF (1 $\mu$M) caused significant changes in AnxA1 intracellular location, although they occurred to a different extent and in a different pattern (Fig. 1B). Over the time course, LXA4 rapidly (10 min) reduced AnxA1 cytosolic content, and this was mirrored by an increase in the membrane pool at 30 min (Fig. 1A, 1B). AF2 provoked qualitatively similar—yet slower—effects, with significance being reached only at the 30-min time point. Peptide Ac2-26 seemed to provoke changes similar to those of LXA4, but its effects were lost by 60 min, with the pool of

FIGURE 3. WRW4 prevents LXA4- and AF2-elicited mobilization of AnxA1 in human PMNs. Human PMNs were pretreated with WRW4 (10 $\mu$M, 10 min), prior to addition of vehicle (CTR), Ac2-26 (10 $\mu$M), LXA4 (100 nM), or AF2 (10 $\mu$M) for an additional 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 WRW4. B–D, Densitometric analysis for AnxA1 expression in cytosol (B), membrane (C), and supernatant (D). Data, mean ± SEM, are calculated as percentage of vehicle-treated cytosolic AnxA1 expression taken as 100. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus CTR (i.e., no WRW4), $^p p < 0.05$, $^b p < 0.01$, $^{b b} p < 0.001$ versus vehicle (i.e., in presence of WRW4 only).

FIGURE 4. CsH affects fMLF-elicited mobilization of AnxA1 in human PMNs. Human PMNs were pretreated with CsH (10 $\mu$M, 10 min), prior to addition of vehicle (CTR), Ac2-26 (10 $\mu$M), or fMLF (1 $\mu$M) for an additional 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). Data, mean ± SEM, are calculated as percentage of vehicle-treated cytosolic AnxA1 expression taken as 100. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus CTR (i.e., no CsH), $^{p} p < 0.05$, $^{b} p < 0.01$, $^{b b} p < 0.001$ versus vehicle (i.e., in presence of CsH). fMLP, formyl-methionyl-leucyl-phenylalanine.

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intracellular AnxA1 apparently replenished. The FPR1 agonist fMLF mobilized AnxA1 with kinetics similar to those produced by peptide Ac2-26: At 30 min significant reduction of intracellular AnxA1 was observed, which was partially replenished by 60 min, possibly consequent to a reduction in the membrane pool by this time point (Fig. 1B).

Changes in AnxA1 concentrations in cell supernatants were detected only after 30 min of 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 PMNs (data not shown). From this set of experiments, we selected the 30-min time point and the two FPR2/ALX agonists AF2 and LXA₄ for further studies.

Characterization of AF2- and LXA₄-induced AnxA1 mobilization

Incubation of human PMNs with AF2 (1–10 μM) provoked a reduction of cytosolic AnxA1 and a simultaneous increase in either membrane fraction and cell supernatant (Fig. 2A) AnxA1. This effect was evident at 1 μM AF2 and was robustly observed at concentrations of 3–10 μM. Treatment of cells with LXA₄ (10–100 nM) induced changes in AnxA1 location similar to those made by AF2; notably, these effects were concentration dependent and optimal at ~10 nM (Fig. 2B).

Pretreatment of human PMNs with the antagonist WRW₄ (10 μM, 10-min preincubation) affected both LXA₄- and AF2-induced AnxA1 mobilization. In particular, the marked mobilization of AnxA1 on the cell surface induced by either FPR2/ALX agonist was essentially abolished by WRW₄; representative blots are shown (Fig. 3A), with cumulative data for cytosolic, membrane, and supernatant pools of AnxA1, respectively (Fig. 3B–D). Peptide Ac2-26 was also tested in these experiments; however, its modulation of PMN AnxA1 localization was not affected by WRW₄ (Fig. 3B–D).

Experiments performed in the presence of the selective FPR1 antagonist CsH showed that AnxA1 mobilization induced by fMLF was genuinely due to engagement of this receptor (Fig. 4). A different scenario emerged for peptide Ac2-6, which retained its ability to mobilize AnxA1 (Fig. 4). Of interest, even when a combination of CsH and WRW₄ was tested, peptide Ac2-26, used at 10 μM, yielded marked mobilization of AnxA1 (Supplemental Fig. 1).

To determine whether AnxA1 mobilization was secondary to, or accompanied by, granule mobilization, Abs 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 of incubation, unlike PMA (positive control), neither LXA₄ nor AF2 induced changes in the PMN cell surface expression of any of these markers (Fig. 5A). Interestingly, both agonists caused internalization of FPR2/ALX over the 30-min incubation time, with no effect on FPR1 (Fig. 5B).

Because membrane AnxA1 did not appear to derive from intracellular granules, we monitored AnxA1 phosphorylation status in the cytosolic pool (25, 24), using an Ab against phospho-Ser²⁷-AnxA1 (25). Shorter time points were tested in this study, with Fig. 6 reporting data obtained at 10 min. In resting cells, there was little membrane phosphorylated AnxA1; addition of LXA₄ and AF2 caused a rapid increase in this species (Fig. 6A). Incubation
with fMLF augmented AnxA1 on the membrane surface (Fig. 6B), but this did not appear to be phosphorylated.

Cell surface AnxA1 in stimulated PMNs could also be quantified by flow cytometry. At the optimal 30-min incubation time, both LXA4 and AF2 augmented AnxA1 expression at the single-cell level (2-fold increase in MFI units; Fig. 7A, 7B) and a marked increase in AnxA-positive PMNs was noted, with values close to 70% versus control (resting PMN) levels of ∼15%. A similar pattern was seen following incubation with fMLF.

Anti-inflammatory properties of LXA4 and AF2 are tightly coupled to AnxA1

Next, we tested whether the rapid nongenomic modulation of PMN AnxA1 might be of relevance to PMN trafficking in inflammation. To this end, we used a validated protocol whereby LXA4 (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 ischemia/reperfusion procedure, allowing the selection of postcapillary venules with approximately six to eight adherent leukocytes, in contrast to the minimal leukocyte interaction with the postcapillary venule endothelium observed in sham-operated animals (zero to two cells per vessel) (see Supplemental Fig. 2).

Injection of a single bolus of LXA4 (1 μg i.v., corresponding to 2.8 nmol) to AnxA1+/+ mice provoked a marked (>50%) detachment of PMNs from the endothelium within the 10-min observation period (Fig. 8A). This effect was drastically reduced in AnxA1−/− mice, with no significant alteration in the fate of the adherent leukocytes (∼15%; Fig. 8C). A similar result was obtained with AF2 (1 μg i.v., corresponding to 1 nmol), which afforded significant detachment of adherent leukocytes in AnxA1+/+, but not AnxA1−/−, mice (Fig. 8B, 8C).

It should be noted that, in line with the higher inflammatory reactivity observed within the microcirculation of AnxA1−/− mice (43), absence of AnxA1 was associated with a slightly higher degree of adherent cells in both sets of experiments (e.g., Fig. 8A, 8B).

Finally, we investigated whether the crucial role of endogenous AnxA1 in LXA4-induced effects was also evident in a more severe type of inflammation. IL-1β injection into dorsal air-pouches of mice provoked an intense recruitment of Gr1+ cells at a 4-h time point, which was inhibited by treatment with LXA4 in AnxA1+/+, but not AnxA1−/− mice (Fig. 8D).

**FIGURE 7.** Detection of cell surface AnxA1 by flow cytometry. Human PMNs were treated with vehicle (CTR), AF2 (10 μM), LXA4 (100 nM), or fMLF (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 units, are mean ± SEM from three different experiments. ***p < 0.001 versus CTR. fMLP, formyl-methionyl-leucyl-phenylalanine.

**FIGURE 8.** LXA4 and AF2 provoke leukocyte detachment by mobilizing endogenous AnxA1. Intravital microscopy analyses of AnxA1+/+ and AnxA1−/− mouse mesenteric microcirculation inflamed by an ischemia-reperfusion procedure. A and B, Administration of LXA4 and AF2 [1 μg i.v. at time 0 (i.e., once the preparation is on stage and the post-capillary venule is selected)] induced detachment of adherent leukocytes in AnxA1+/+, but not in AnxA1−/−, mice.

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but not AnxA1−/−, mice (Fig. 8D). Fig. 9 summarizes the modes of AnxA1 mobilization in activated PMNs.

Discussion
In this study we provide strong evidence that engagement of FPR2/ALX by selective agonists would induce AnxA1 phosphorylation and mobilization in human PMNs. We propose that this cellular response operates to control PMN recruitment to postcapillary venules and migration to inflamed sites, as demonstrated with proof-of-concept experiments in AnxA1−/− mice, in which LXA4 and AF2 lost their antimigratory properties.

An effective inflammatory reaction relies on the concerted action of several pathways that attract WBCs from the vasculature into specific tissue sites. A classical example is that of the synovial joint; at this site, 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; this approach aims to capitalize on the action of endogenous anti-inflammatory and proresolving 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, proinflammatory prostanoids induce LXA4 synthesis in, what has been proposed to be, a “class switching” from the proinflammatory to the proresolving phase of an acute experimental inflammation (49). However, the option that a network might be operative within anti-inflammatory mediators themselves has been inadequately explored: In this article, we show a functional link between LXA4 and endogenous AnxA1. Incubation of human PMNs with LXA4 induced a rapid translocation of AnxA1 to the cell surface, with a time profile different from that provoked by peptide Ac2-26 or fMLF. The effect of LXA4 was mimicked by the anti-inflammatory synthetic nonapeptide AF2.

It is intriguing that both LXA4 and AF2 inhibit neutrophil activation in vitro as well as recruitment of these cells in vivo. AF2 reduces HL-60 cell 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 in this article 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 LXA4 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 a few examples of crosstalk in resolution are beginning to emerge, with resolvin D1 promoting exudate expression of LXA4, although the specific cell targets of this mediator were not identified (52). In addition, there is an indication that endogenous epi-LXA4, synthesized upon aspirin’s unique blockade of cyclo-oxygenase 2, can mobilize NO from the endothelium (constitutive NO synthase activation) and macrophages (inducible NO 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 NO might also be occurring in our settings, although the experiments of leukocyte detachment reported in this article took place within 10 min. In any case, the recent involvement of rapidly produced endothelial NO and prostacyclin in the anti-inflammatory effects of resolvins D2 (54) would suggest also keeping this option viable upon the acute application of LXA4 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 nongenomic loops in resolution; however, evidence for genomic associations is also emerging. One classical example is that linking glucocorticoids to overexpression of AnxA1, with increments in gene promoter activity being measured with a reporter gene (14). In addition, LXA4 could induce two specific signaling molecules, NGFI-A binding protein 1 (56) and suppressor of cytokine signaling 2 (57), to reprogram the phenotype of PMNs and dendritic cells, respectively. Among the biological effects elicited by LXA4, its analogs, and other proresolving lipid mediators, it is worthwhile highlighting the augmented expression of CCR5 on apoptotic leukocytes, a response that facilitates 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 LXA4 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 interrelationship 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 rearrangement 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). In contrast, the typical mobilization induced by glucocorticoids using a receptor-dependent nongenomic pathway (25, 62, 63) seems to be replicated by other classes of drugs, including camromes (23, 24) and, as described in this paper, LXA4 and AF2. Mobilization of this cytosolic pool of AnxA1 is downstream protein kinase C activation and phosphorylation at Ser27 on the AnxA1 N terminus (25) (Fig. 9). It is unclear whether protein kinase C isoforms—advocated for dexamethasone and nedocromil action (23, 25)—also operate downstream FPR2/ALX activation by LXA4 and AF2. In any case, the involvement of this specific G-protein–coupled receptor 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 LXA4 as a prerequisite for stimulation of phagocytosis by this anti-inflammatory lipid (64).

As stated above, LXA4 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, although 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 intravital microscopy protocol, which we reported being sensitive to LXA4 treatment (42). The results we present in this paper 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 in our study, 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 contains its antigalactocytic 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 LXA4 and AF2 (and fMLF) because 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 with 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 LXA4 in the air-pouch model were lost in AnxA1−/− mice. With data from the recent observation of lack of effect of LXA4 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 in our study, would bring about AnxA1 mobilization. This event would, in turn, act in a juxtacrine/autocrine fashion (6) to reinforce the overall attenuation of the process of leukocyte recruitment. Clearly, other intracellular pathways (69, 70) and mediators might also act downstream of LXA4 activation (5, 66); nonetheless, the data presented and discussed in this article point to the nongenomic mobilization of AnxA1 as one of the major downstream effectors of LXA4, and AF2, actions that negatively modulate the cell-trafficking process. Networks of proresolving 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.

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Disclosures

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