The natural phosphoinositide derivative glycerophosphoinositol inhibits the lipopolysaccharide-induced inflammatory and thrombotic responses

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Inflammatory responses are elicited through lipid products of phospholipase A2 activity that acts on the membrane phospholipids, including the phosphoinositides, to form the proinflammatory arachidonic acid and, in parallel, the glycerophosphoinositols. Here, we investigate the role of the glycerophosphoinositol in the inflammatory response. We show that it is part of a negative feedback loop that limits proinflammatory and prothrombotic responses in human monocytes stimulated with lipopolysaccharide. This inhibition is exerted both on the signaling cascade initiated by the lipopolysaccharide with the glycerophosphoinositol-depen-
dent decrease in IκB kinase α/β, p38, JNK, and Erk1/2 kinase phosphorylation and at the nuclear level with decreased NF-κB translocation and binding to inflammatory gene promoters. In a model of endotoxemia in the mouse, treatment with glycerophosphoinositol reduced TNF-α synthesis, which supports the concept that glycerophosphoinositol inhibits the de novo synthesis of proinflammatory and prothrombotic compounds and might thus have a role as an endogenous mediator in the resolution of inflammation. As indicated, this effect of glycerophosphoinositol can also be exploited in the treatment of manifestations of severe inflammation by exogenous administration of the compound.

Inflammation is a beneficial host response to foreign pathogens and tissue injury that ultimately leads to bacterial clearance and restoration of tissue structure and function (1). The host response involves different cell types and signaling molecules, such as cytokines, chemokines, and bioactive lipids (2). These bioactive lipids include prostaglandins, leukotrienes, and endoperoxides, which originate enzymatically from the phospholipase A2/arachidonic acid cascade and are formed and act in a cell-specific fashion (3). More recently, other compounds that intervene in the resolution of inflammatory responses have been characterized; these include lipoxins, resolvins, protectins, and maresins (all of which are biosynthesized from essential polyunsaturated fatty acids), the receptors for the bioactive lipids mentioned above, and various microRNAs (4). It is now evident that the control of the initiation of inflammation and its natural resolution involve several essential components that need to be finely modulated in space and time to restore tissue homeostasis. Indeed, prolonged or uncontrolled inflammation represents the pathogenetic basis of chronic inflammatory diseases (5). As a prolonged activation of the cells of the immune system is the driving force behind inflammatory diseases, the identification of anti-inflammatory compounds that can switch off proinflammatory responses at one of the crucial steps and thus restore immunological homeostasis remains of great interest. Recent data obtained in our laboratory have addressed some aspects of this need as they show the potential of glycerophosphoinositol (GroPIns)6 as an anti-inflam-

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6 The abbreviations used are: GroPIns, glycerophosphoinositol; GroPIns4P, glycerophosphoinositol 4-phosphate; TLR, toll-like receptor; Mac-1, αMβ2 integrin; TF, tissue factor; COX, cyclooxygenase; IκBα, inhibitor of kB; IKK, IκB kinase; p38, p38 mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; PLA2, phospholipase A2; Tx, thromboxane.

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Results

GroPLns inhibits endotoxin-induced prothrombotic and inflammatory responses in human monocytes

Increases of the intracellular GroPLns levels have been observed in response to inflammatory stimuli, such as LPS, in macrophages (7). Based on this, we investigated the possibility that GroPLns can regulate the inflammatory reactions in a simple and well characterized in vitro model system: the production of inflammatory cytokines in human blood monocytes upon challenge with endotoxin.

Human monocytes have a major role in the innate immune response to bacterial infection. Bacterial LPS binds TLR4 on the monocyte membrane and triggers the expression of a variety of inflammatory mediators, which include cytokines such as TNF-α and interleukin (IL)-1β, and enzymes, such as cyclooxygenase (COX)-2. These in turn produce inflammatory prostanoids, such as prostaglandin E2 and thromboxane A2 (TXA2) (15), which act locally to elicit the cardinal signs of acute inflammation (16).

We first tested the effects of GroPLns on mRNA levels of some of the major inflammatory mediators in freshly isolated human monocytes challenged with LPS (0.1 µg/ml). As expected, LPS induced a pronounced increase in the expression of IL-1β, TNF-α, COX-2, and the anti-inflammatory mediator IL-10 (Fig. 1, A–D). Preincubation with 300 µM GroPLns reduced the levels of IL-1β, TNF-α, and COX-2 mRNAs although to different extents (by 50–75% at 24 h; Fig. 1, A–C). Of note, treatment with GroPLns considerably reduced the expression of the inflammatory genes at both early (1.5 h) and long times (24 h) of stimulation with very consistent effects at long times (24 h; Fig. 1, A–C). However, GroPLns did not affect the expression of the anti-inflammatory cytokine IL-10 (Fig. 1D) or the suppressor of cytokine signaling 3, a transcriptional target of the signal transducer and activator of transcription 3 (STAT3) that is downstream of IL-10 signaling (Ref. 17 and supplemental Fig. S1). In parallel experiments, monocytes preincubated with 300 µM GroPLns were challenged with TNF-α (50 ng/ml) for 1.5 h; no effect on gene expression was observed under these conditions (Fig. 1, E and F), indicating that GroPLns specifically interferes with the LPS signaling cascade. Accordingly, GroPLns also affected the expression of cytokines when monocytes were challenged with agonists of TLR7/8 and TLR9 that share a common downstream pathway with TLR4 (i.e. the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway (Ref. 18 and supplemental Fig. S1).

In line with the effects on gene expression, 300 µM GroPLns reduced the LPS-stimulated release of both TNF-α and IL-1β cytokines at 24 h (with up to 60% inhibition) with negligible effects at earlier times (Fig. 1G). Moreover, GroPLns reduced the accumulation of TxB2 by 60%. TxB2 is the stable metabolite of TXA2, which is a major product of the metabolic activity of COX-2 in monocytes (Fig. 1G), thus confirming that these decreased mRNA levels result in reduced metabolic activity.

Monocytes also have a major role in thrombotic events through the de novo synthesis of tissue factor (TF), a transmem-

matory compound that can prevent endotoxin shock in the mouse.

GroPLns is one of the naturally occurring phosphoinositide metabolites, the glycerophosphoinositols, that are produced through the activity of phospholipase A2 IVα (PLA2IVα), an enzyme that preferentially hydrolyzes phospholipids carrying arachidonic acid in position sn-2 of the glycerol backbone (6, 7). The formation of GroPLns requires two decylation steps, both carried out by PLA2IV. PLA2IVα has intrinsic phospholipase A2 and lysolipase activities; thus, it hydrolyzes the membrane phosphoinositide (carrying the arachidonic acid in the sn-2 position), producing in parallel arachidonic acid and the lysodervative lysophosphatidylinositol, and then in sequence, PLA2IVα forms GroPLns by decaying lysophosphatidylinositol (6, 8). Noticeably, this PLA2IVα-dependent metabolism gives rise to three biologically active metabolites that start independent cascades, i.e. G-protein-coupled receptor-dependent signaling (by the GPR55 ligand lysophosphatidylinositol), cyclooxygenase/lipoxygenase metabolism (by producing their substrate, arachidonic acid), and glycerophosphoinositol-modulated processes (which include cell proliferation and actin cytoskeleton organization) (6, 8–10).

The glycerophosphoinositols are ubiquitous, freely diffusible molecules, and they can be detected both within cells and in the extracellular space (11, 12). Their intracellular concentrations are cell type-dependent, and their production can vary upon oncogenic transformation, cell differentiation, and hormonal stimulation (6, 11). They are present in different forms that can be either unphosphorylated (GroPLns) or phosphorylated (e.g. glycerophosphoinositol 4-phosphate (GroPLns4P), glycerophosphoinositol 4,5-phosphate, and glycerophosphoinositol 3-phosphate) with different cellular activities (6, 13). Hematopoietic cells, such as monocytes and macrophages, have a strictly regulated PLA2IVα activity that provides a fine modulation of the intracellular glycerophosphoinositol levels in response to environmental stimuli, including proinflammatory agents such as bacterial lipopolysaccharide (LPS) (7). To further clarify the potential roles of these lipid-derived mediators in inflammation, we have investigated the effect of GroPLns in a well characterized in vitro model of inflammatory and proagulant responses mimicked by human blood monocytes challenged with LPS.

Here we show that GroPLns can inhibit the expression of prothrombotic and proinflammatory genes that are induced by Escherichia coli LPS through inhibition of the signaling cascade downstream of the toll-like receptor 4 (TLR4) and of the nuclear activity of nuclear factor-κB (NF-κB). Of note, the measurement of inflammatory markers in mice subjected to LPS-induced endotoxemia showed that treatment with GroPLns is associated with reduced plasma levels of tumor necrosis factor-α (TNF-α) and decreased expression of αMβ2 integrin (Mac-1) on the surface of circulating neutrophils, which strengthens the potential pharmacological relevance of this lipid-derived mediator. Thus, the present study provides evidence that GroPLns, which is produced endogenously by activated inflammatory cells, can act as a resolution signal to control the proinflammatory and prothrombotic responses associated with particularly exuberant inflammatory states (14).
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**Figure 1. GroPIns reduces the LPS-induced proinflammatory response in human monocytes.** A–C, GroPIns inhibits the LPS-mediated transcription of TNF-α, IL-1β, and COX-2 at 1.5 and 24 h. D, GroPIns does not inhibit the LPS-mediated expression of IL-10. E and F, GroPIns does not inhibit the TNF-α-mediated expression of proinflammatory genes (TNF-α and IL-1β). Human monocytes were purified from peripheral blood of healthy donors and incubated at 37 °C for 20 min without or with 300 μM GroPIns and for an additional 1.5 or 24 h in the absence or presence of 0.1 μg/ml LPS or 50 ng/ml TNF-α. At each time point, the RNA was extracted and converted to cDNA for real-time PCR analysis. Data were calculated using the ΔΔCT method (2^−ΔΔCT). Transcription of the housekeeping gene GAPDH was used to normalize the data. Measurements of mRNA levels are expressed as -fold increase over the respective control (unstimulated) cells, and are means ± S.D. of triplicates (n = 3) from two experiments performed with cells from two different donors (A–C) and means ± S.D. of three independent experiments (n = 3) performed in duplicate (D–F). Asterisks indicate statistically significant differences (*, p < 0.05; **, p < 0.01), ns, not significant. G, GroPIns inhibits LPS-induced protein expression levels of TNF-α and IL-1β and reduces COX-derived TxB2. Monocytes were preincubated for 20 min at 37 °C in the absence or presence of 300 μM GroPIns and then incubated for a further 5 and 24 h without or with 0.1 μg/ml LPS. At the end of this incubation, the supernatants were prepared, and the levels of TNF-α, IL-1β, and TxB2 in the medium were assessed using ELISA. In untreated monocytes, the protein concentrations were under the assay detection limits at both times. Data show the percentage of inhibition induced by GroPIns treatment on LPS-induced protein release. Data represent three independent experiments performed with cells from three different donors. Error bars represent S.D.

The action of GroPIns involves the inhibition of the extracellular signal-regulated kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) kinase activities

To investigate the mechanism through which GroPIns affects LPS-induced monocyte responses, we analyzed their effects on the phosphorylation of p38, JNK, and Erk1/2, three kinases that are known to transduce the LPS signal downstream of TLR4 (20). In these cells, LPS (0.1 μg/ml) activated Erk1/2, JNK, and p38, and treatment with GroPIns (300 μM) for different times reduced their phosphorylation by about 60% (Fig. 3A), indicating that GroPIns acts by modulating the activity of these kinases. Similar experiments were carried out in monocytes pretreated with 300 μM GroPIns4P, and no difference was observed between control and treated cells (Fig. 3A). This phosphorylated derivative, besides the already reported immunomodulatory activity in T lymphocytes (21), has also been shown to affect the LPS-induced transcription of proinflammatory genes in monocytes. Therefore, GroPIns and GroPIns4P are able to exert similar anti-inflammatory activity acting at different levels of the signaling cascade. These results are in line with current knowledge that both GroPIns and GroPIns4P act intra-

brane glycoprotein that initiates blood coagulation through its binding to factors VII and VIIa, which leads to fibrin clot formation (19). Treatment with 300 μM GroPIns reduced the LPS-induced expression of TF gene by 50% at 24 h of stimulation (Fig. 2A). Following these observations, we investigated whether modulation of TF gene expression also translates into a reduced procoagulant activity of LPS-challenged monocytes. Procoagulant activity is practically undetectable in freshly isolated monocytes, and it is strongly up-regulated by exposure to LPS (Fig. 2B). Treatment of monocytes with 50–300 μM GroPIns before LPS stimulation resulted in a dose-dependent reduction in TF activity with up to 60% inhibition (Fig. 2B). Similar to what was observed with the cytokines, the efficacy of GroPIns in reducing TF activity became significant after 24 h of stimulation (Fig. 2B). Moreover, a series of experiments were conducted at 24 h of GroPIns stimulation where the cells were challenged with 0.1–1000 ng/ml LPS after pretreatment with 300 μM GroPIns; the inhibitory effect of GroPIns on the procoagulant activity of TF was evident across this whole range of LPS concentrations (Fig. 2C).

Thus, GroPIns can suppress proinflammatory and prothrombotic responses in human monocytes stimulated with LPS. This activity was consistently observed in numerous assays with samples obtained from different donors.

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7 P. Zizza and D. Corda, unpublished observations.
Genes, including 5 acting DNA promoter elements have been characterized in the monocytes pretreated with 300 μg/ml LPS. The activation signaling to NF-κB is specific for the LPS signaling. The inhibitory effect of GroPIns on IKKα–β complex phosphorylation (see above). Following these observations, we performed an electrophoretic mobility shift assay (EMSA) on nuclear extracts of human monocytes treated with LPS (0.1 μg/ml) in the absence and presence of GroPIns (50 and 300 μM). Because the LPS-induced nuclear translocation of NF-κB became evident after 15 min of treatment and maximal after 30 min and the GroPIns inhibition was detected within this time frame, we analyzed these nuclear extracts after 30 min of stimulation. As shown in Fig. 5A, GroPIns consistently reduced the LPS-induced binding of NF-κB to a radiolabeled oligo probe containing the NF-κB-binding site sequence (see “Experimental procedures”); of note, GroPIns was effective at both 50 and 300 μM. Under the same experimental conditions, GroPIns did not affect the binding to DNA of either cAMP response element-binding protein or activator

cellularly as there is so far no evidence of a membrane receptor of specific activity at the membrane level (6, 11).

**GroPIns inhibits the LPS-dependent signaling leading to the nuclear translocation of NF-κB**

To evaluate the consequence of the reduced phosphorylation of Erk1/2, JNK, and p38, we have analyzed the well documented transcription factors that bind to these LPS-responsive elements include NF-κB, activator protein-1, and cAMP response element-binding protein, which all cooperate to produce various cytokines, the levels of which are barely detectable in resting cells. NF-κB, however, is the only transcription factor that is specific for the LPS signaling.

**GroPIns reduces LPS-induced nuclear translocation of NF-κB and the binding to the promoters of target genes**

As indicated above, IκBα determines the cell localization, and thus activity, of NF-κB. Because GroPIns was able to affect both the activation and degradation of IκBα, we evaluated the nuclear translocation of NF-κB in the presence of GroPIns by using antibodies specific for p65 and p50 proteins (the subunits forming the heterodimer mainly involved in transcriptional regulation; see also Ref. 24 and “Experimental procedures”). The LPS-induced nuclear translocation of NF-κB became evident after 15 min and maximal after 30 min (Fig. 4). Within 30 min of the GroPIns addition, the nuclear translocation of both p65 and p50 was reduced by 45 and 50%, respectively (Fig. 4B). This impairment in the nuclear translocation of NF-κB after LPS stimulation is in line with, and corroborates, the rapid effect of the GroPIns on IKKα–β complex phosphorylation (see above). Following these observations, we performed an electrophoretic mobility shift assay (EMSA) on nuclear extracts of human monocytes treated with LPS (0.1 μg/ml) in the absence and presence of GroPIns (50 and 300 μM). Because the LPS-induced nuclear translocation of NF-κB becomes evident after 15 min of treatment and maximal after 30 min and the GroPIns inhibition was detected within this time frame, we analyzed these nuclear extracts after 30 min of stimulation. As shown in Fig. 5A, GroPIns consistently reduced the LPS-induced binding of NF-κB to a radiolabeled oligo probe containing the NF-κB-binding site sequence (see “Experimental procedures”); of note, GroPIns was effective at both 50 and 300 μM. Under the same experimental conditions, GroPIns did not affect the binding to DNA of either cAMP response element-binding protein or activator

![Figure 2. GroPIns inhibits LPS-induced tissue factor expression and activity in human monocytes](https://example.com/image2.png)

**Figure 2. GroPIns inhibits LPS-induced tissue factor expression and activity in human monocytes.** A, human monocytes were purified from peripheral blood of healthy donors and incubated at 37 °C for 20 min without or with GroPIns 300 μM and for an additional 1.5 or 24 h in the absence or presence of 0.1 μg/ml LPS. The RNA was extracted and converted to cDNA for real-time PCR analysis. Each sample was measured in triplicate, and the data were calculated as -fold increase over the respective control (unstimulated) cells and are means ± S.D. of triplicates from two experiments performed with cells from two different donors. Asterisks indicate statistically significant differences (*, p < 0.05). B, human monocytes were purified from peripheral blood of healthy donors and incubated at 37 °C for 20 min without or with increasing concentrations (50–300 μM) of GroPIns and for a further 5 or 24 h in the presence of 0.1 μg/ml LPS. At these time points, the cells were lysed, and the procoagulant activity of tissue factor was assessed using one-stage clotting time (see “Experimental procedures”). For each experiment, monocytes that were not activated with LPS were analyzed as the negative control. The data are means ± S.D. of triplicates from two experiments performed on cells from two independent donors. Asterisks indicate statistically significant differences (**, p < 0.01; ***, p < 0.001). C, monocytes pretreated with 300 μM GroPIns or left untreated were stimulated for 24 h with increasing concentrations of LPS. At the end of incubation, the cells were lysed, and procoagulant activity of tissue factor was assessed using one-stage clotting time (see “Experimental procedures”). The data are representative of more than 10 experiments performed with cells from different donors. Error bars represent S.D.
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A

| TIME (min) | 0 | 5 | 10 | 20 | 30 | 5 | 10 | 20 | 30 | 5 | 10 | 20 | 30 |
|------------|---|---|----|----|----|---|----|----|----|---|----|----|----|
| LPS (0.1 μg/ml) |    |    |    |    |    |    |    |    |    |    |    |    |    |
| GroPIns 300 μM | - | - | - | - | - | + | + | + | + | - | - | - | - |
| GroPIns4P 300 μM | - | - | - | - | - | + | + | + | + | + | + | + | + |

- p-ERK
- ERK
- p-JNK
- JNK
- Vinculin
- p-p38
- p-38
- Vinculin

B

| TIME (min) | 0 | 5 | 10 | 20 | 30 | 5 | 10 | 20 | 30 | 5 | 10 | 20 | 30 |
|------------|---|---|----|----|----|---|----|----|----|---|----|----|----|
| LPS (0.1 μg/ml) |    |    |    |    |    |    |    |    |    |    |    |    |    |
| GroPIns 300 μM | - | - | - | - | - | + | + | + | + | - | - | - | - |
| GroPIns4P 300 μM | - | - | - | - | - | + | + | + | + | + | + | + | + |

- p-IKKα/β
- IKKα
- p-IκBα
- IκBα
- Vinculin

C

| TIME (min) | 0 | 5 | 10 | 20 | 30 | 5 | 10 | 20 | 30 |
|------------|---|---|----|----|----|---|----|----|----|
| TNFα (50 ng/ml) |    |    |    |    |    |    |    |    |    |
| GroPIns 300 μM | - | - | - | - | - | + | + | + | + |

- p-IKKα/β
- p-IκBα
- GAPDH

Bar graphs showing relative optical density for each time point.
Figure 3. GroPIns affects the LPS-induced activation of Erk1/2, JNK, p38, and the IKKα–β complex. A, GroPIns affects the phosphorylation of the IKK complex and the degradation of IκBα. B, GroPIns affects both the phosphorylation of the IKK complex and the degradation of IκBα. C, GroPIns does not affect the TNF-α-induced phosphorylation of the IKK complex and degradation of IκBα. A–C, blots, left side of the panel, monocytes were treated without or with either GroPIns or GroPIns4P (300 μM) for 20 min and for an additional 5, 10, 20, and 30 min in the presence of 0.1 μg/ml LPS (A and B) or 50 ng/ml TNF-α (C). Representative Western blots resolved using 10% SDS-PAGE and revealing phosphorylated (p-) Erk1/2 (Thr-202/Tyr-204), phosphorylated JNK (Thr-183/Tyr-185), phosphorylated p38 (Thr-180/Tyr-182), and total levels of Erk1/2, JNK, and p38 are shown. The phosphorylated IKKα–β complex (Ser-176/180 on IKKα and Ser-177/181 on IKKβ), the phosphorylated IκBα (Ser-32/36), and the total levels of IκBα are also shown. These blots are representative of three independent experiments performed with cells purified from three independent donors. A–C, bar graphs, right side of the panel, densitometric analysis of the bands was performed with ImageJ software. The protein abundance has been expressed relative to the housekeeping signal (vinculin or GAPDH).

Figure 4. GroPIns reduces the LPS-induced nuclear translocation of NF-κB. Representative confocal microscopy images of NF-κB intracellular localization in human peripheral blood monocytes are shown. A, cells were incubated at 37 °C for 20 min in the presence or absence of 300 μM GroPIns and then treated for 15 and 30 min (‘) with 0.1 μg/ml LPS. Cells were then fixed and processed for immunofluorescence (see “Experimental procedures”). The intracellular localization of NF-κB was detected using antibodies specific for p50 and p65 proteins revealed with Alexa Fluor 488-labeled (green) and Alexa Fluor 568-labeled (gray) secondary antibodies, respectively. Nuclei were stained with DAPI (blue). The samples were analyzed on a laser scanning confocal microscope (LSM710) equipped with a 63 × objective. B, the nuclear translocation of NF-κB on cells left untreated or treated with LPS in the presence or absence of GroPIns has been quantified by randomly counting 100 cells/sample and expressing the percentage of cells with nuclear staining of p50 or p65. Quantification of nuclear events is presented as the mean ± S.D. of three independent experiments. Statistical analysis was performed using Student’s t test (*, p < 0.05; **, p < 0.01). Error bars represent S.D. Scale bars, 5 μm.
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Figure 5. GroPlns reduces the LPS-induced binding of NF-κB to promoters of target genes. A, GroPlns reduces the LPS-triggered binding of NF-κB to DNA. For the EMSA, monocytes were incubated at 37 °C for 20 min without or with the indicated concentrations of GroPlns and then incubated for a further 30 min in the presence of 0.1 μg/ml LPS. Unstimulated monocytes were incubated in parallel as a control (T0). At the end of the incubation, the cells were lysed, and the nuclear extracts were incubated with radiolabeled oligonucleotide probes that contained the NF-κB-binding site (see “Experimental procedures”). Protein–DNA complexes were separated using 5% non-denaturing acrylamide gels and visualized by autoradiography. Data are representative of two independent experiments.

B, GroPlns directly displaces NF-κB from DNA. For the EMSA competition assay, GroPlns (at the indicated concentrations) was added to the binding mixture containing the radiolabeled probe and the nuclear lysates from monocytes treated with 0.1 μg/ml LPS for 60 min. The EMSA was performed as reported above. Data are representative of three independent experiments. C, left side of the blot, both p65–p50 heterodimer and p50–p50 homodimer participate in the binding to the radiolabeled probe. For the EMSA supershift, antibody specific for p65 or p50 protein was added to the binding mixture containing the radiolabeled probe and the nuclear lysates from monocytes treated with 0.1 μg/ml LPS for 60 min; an antibody specific for tubulin was used as a negative control. C, right side of the blot, HeLa cells treated with 20 ng/ml TNF-α for 20 min were used as a positive control. The EMSA was performed as reported above. D, GroPlns reduces the recruitment of p65 to promoters of tissue factor and TNF-α genes. For the ChIP assay, the binding of p65 on chromatin was evaluated by a specific rabbit polyclonal antibody to p65 protein; a normal rabbit IgG was used as a background control. The enriched promoters were quantified by real-time PCR using primers that specifically amplify a region containing the NF-κB site. The intensity of the PCR signal is proportional to the occupancy on the binding site. The amount of p65-immunoprecipitated chromatin is represented as signal relative to the total amount of input chromatin (percentage of binding relative to input). Data are mean ± S.D. of three independent experiments with cells from three independent donors. Significance was tested by analysis of variance with Dunnett post hoc analysis (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Error bars represent S.D. Untreated.

protein-1, implying that the GroPlns modulation is specific for the LPS-induced activation of NF-κB (supplemental Fig. S3).

To better clarify whether GroPlns could interfere with NF-κB activity by directly displacing it from the promoter, we performed in vitro competition assays. Nuclear extracts of LPS-treated monocytes were incubated with the radiolabeled probe and increasing concentrations of GroPlns; these were able to displace NF-κB from DNA (Fig. 5B).

Next, we examined which of the NF-κB subunits was involved in the binding to the radiolabeled probe by supershift assays (see “Experimental procedures”). The precubination of nuclear lysates with an antibody specific either for p65 or for p50 induced a supershift that was more pronounced in the case of the p50 antibody (Fig. 5C). This observation is in line with the reported high expression of p50 homodimer in fresh monocytes (25).

The specificity of the antibodies used was validated in nuclear extracts of HeLa cells treated with TNF-α (20 ng/ml) for 20 min. Both antibodies supershifted the same complex, indicating that in this cellular model the heterodimer p50–p65 is mainly involved in the binding.
According to the data obtained, GroPLns may participate in the regulation of transcription by modulating the nuclear-cytoplasmic trafficking of NF-κB as well as by affecting the binding to the promoters of target genes. Therefore, we investigated the recruitment of NF-κB to the promoters of those genes. To this end, we performed a chromatin immunoprecipitation (ChIP) assay (see “Experimental procedures”) using a p65-specific antibody. The binding of p65 to promoters was quantified by real-time PCR performed on immunoprecipitated chromatin using primers that specifically amplify a genomic region containing the sequence bound by NF-κB (κB site) (26–28). As shown in Fig. 5D, treatment of monocytes with LPS (0.1 μg/ml; 60 min) elicited a significant binding of p65 on the promoters of both TF and TNF-α genes, whereas the concomitant addition of GroPLns (300 μM) decreased the LPS-induced binding by about 60 and 40%, respectively. In summary, the GroPLns inhibitory activity may be exerted both at the cytosolic level by regulating the kinase activities necessary for NF-κB translocation and at the nuclear level by reducing the binding of the NF-κB subunits to the promoter region.

**GroPLns reduces LPS-induced endotoxin shock in the mouse**

The actions of GroPLns as anti-inflammatory agent indicated by the in vitro data reported above prompted us to analyze its potential efficacy in an in vivo model of endotoxin shock in mice (29). The efficacy of GroPLns on inflammatory responses triggered by LPS in vivo was evaluated by measuring the levels of the TNF-α cytokine in the plasma of placebo-treated and GroPLns (10 mg/kg)-treated mice 2 h after challenge with LPS (1 mg/kg). TNF-α was practically undetectable in healthy, placebo-treated mice (Fig. 6A). In contrast, TNF-α significantly increased in the plasma of the LPS-treated mice, and it was significantly reduced by GroPLns treatment (Fig. 6A).

As an additional marker of inflammatory cell activation, we evaluated the up-regulation of Mac-1 in circulating neutrophils using flow cytometry. LPS induces the delivery of the intracellularly stored Mac-1 to the plasma membrane of neutrophils. The increased cell surface expression favors leukocyte adhesion to the activated endothelium and crawling into blood vessels, contributing to transmigration toward inflamed tissues (30). Once there, Mac-1 also mediates inflammatory effector functions, such as phagocytosis and release of bactericidal products, that are responsible for tissue damage during acute inflammation (31). As shown in Fig. 6B, LPS significantly up-regulated Mac-1 expression, and this was significantly affected by the treatment with GroPLns.

**Discussion**

The present study provides new information on the physiological role and pharmacological potential of the PLA2IV metabolite GroPLns as a novel mediator in the resolution of inflammation. The data thus pertain to the line of studies that have addressed the possibility of using lipid derivatives for the treatment of immune/inflammatory diseases (4).

Although lipid mediators that originate from PLA2IV arachidonic acid metabolism, such as the prostaglandins and leukotrienes, are essential for mounting an inflammatory reaction, other lipid metabolites derived from arachidonic, eicosapentaenoic, and docosahexaenoic acids, such as lipoxins, resolvins, protectins, and maresins, mediate the resolution phase of inflammation (32). From a physiological point of view, GroPLns can be considered as the counterpart of the arachidonic acid cascade as it is formed in the same enzymatic pathway when the PLA2 acts preferentially on the membrane phosphoinositides (7, 8), and importantly, it parallels some of the anti-inflammatory effects of the arachidonic acid metabolites (see above). GroPLns can therefore be thought of as an adjuvant/cooperator in anti-inflammatory strategies.

The GroPLns anti-inflammatory potential is also involved in the ability of this compound to counteract blood-brain barrier failure, replicating in this way the effects of dexamethasone as analyzed in an in vitro model based on a co-culture of endothelial and glial cells (33). Indeed, GroPLns improved blood-brain barrier functions and repair in a dose-dependent manner without the cytotoxic effect that was observed with high doses of dexamethasone (33). The authors have thus proposed this natural compound as a powerful alternative to steroidal drugs to be further validated in in vivo studies (33).

As indicated, GroPLns counteracts the LPS-induced proinflammatory and prothrombotic responses in human blood monocytes, inhibiting the signaling of TLR4 and leading to a decrease in the nuclear translocation and binding of the transcription factor NF-κB to promoters. This reduces the transcription of inflammatory genes.

The activity of GroPLns is specific because it was ineffective when cells were treated with TNF-α. It is worth noting that both TNF-α and LPS share a common pathway downstream of the IKK complex, but they differ in regard to the components linking the receptor to the activation of the IKK complex (24). Thus, GroPLns appears to act on this early part of the LPS path-

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**Figure 6. GroPLns reduces the endotoxin shock in LPS-treated mice.** Effects on plasma levels of TNF-α cytokine and on Mac-1 expression in circulating neutrophils in LPS-treated mice are shown. The treatment protocols were described under “Experimental procedures.” Citrated whole blood from vehicle- or GroPLns-treated mice (nine mice per group) was collected 2 h after LPS challenge. A aliquots of whole blood were immediately centrifuged, and the platelet-poor plasma was stored at ~80°C for measurements of TNF-α cytokine using specific immunoassays. B aliquots were incubated for 15 min with phycoerythrin-conjugated rat anti-mouse Mac-1 antibody (clone M1/70). Nonspecific phycoerythrin-conjugated rat IgG was used as a negative control staining. After staining, the red blood cells were lysed by addition of lysis-fixing solution according to the manufacturer’s instructions. Flow cytometric analysis was performed with a FACStar flow cytometer as follows. Events showing side light scatter (granularity) and forward light scatter (dimension) characteristic of polymorphonuclear leukocytes were analyzed for Mac-1 expression levels. A and B, data are for single animals, and the horizontal lines indicate the median values. **, p < 0.01; ***, p < 0.001. MFI, mean fluorescence intensity.
way, which is independent of the TNF-α signaling. Accordingly, we observed a specific inhibitory effect of GroPIns in the TLR-activated pathway involving MyD88 but not in that involving toll/interleukin-1 receptor (TIR) domain-containing adapter inducing interferon-β (supplemental Figs. S1 and S2). This is also in line with our observation that GroPIns directly binds to a component of the TLR pathway, the protein-tyrosine phosphatase Shp1 (34), as indicated by our proteomic analysis of coimmunoprecipitated complexes, with consequences on its activity.8

The reported competition assays also suggest a direct nuclear activity of GroPIns. NF-κB binding to promoters and target gene expression rely on a number of nuclear events, such as the correct recruitment of remodeling complexes that act on the chromatin architecture (35), balance among the regulators of NF-κB stability (36), and relocalization of the monomer p65 from kB sites to specific nuclear compartments (37).

We have analyzed canonical and validated kB sites within the promoters (26) because it is common knowledge that functional NF-κB-binding sites are present inside enhancer/intronic regions (38). Indeed, NF-κB is a well recognized master regulator of the inflammatory response and a valuable therapeutical target. However, we cannot rule out an indirect effect of GroPIns involving its binding and/or action on other cofactors that cooperate with NF-κB in the transcriptional output (39). This possibility is supported by the observation that under the conditions tested there is no evidence of a direct binding of GroPIns to members belonging to the NF-κB family. At the same time, more than 50 different GroPIns potential targets with different activities have been identified in a proteomic study.9 These include several nuclear proteins that could play a role in the process reported, but their characterization and validation are still in progress.9

These actions of GroPIns have been studied using a pharmacological approach (i.e. exogenous addition); under physiological conditions, this process starts from the production and release of GroPIns upon activation of PLA2 IVα in monocytes (7). Indeed, the stimulated intracellular levels of GroPIns fall within the range of concentrations that can elicit the effects described (40); however, a direct demonstration that these phenomena can take place in co-culture of cells or tissue is hampered by the lack of a means to fully inhibit GroPIns production. Actually, GroPIns is still produced in PLA2 IVα−/− cells, potentially by a rescuing activity of other cytosolic PLA2s in the cells (11, 41).

A possible physiological role of endogenously formed GroPIns can be envisioned in the context of transcription. The nucleus is considered a functionally distinct compartment for inositol lipid metabolism (42), and because PLA2 IVα is able to translocate to the nuclear envelope and locally hydrolyze phosphoinositides (43), it could form GroPIns from the nuclear membrane phosphoinositides and be responsible for its reported nuclear activity. Alternatively, the GroPIns formed in the cytosol (40, 44) could diffuse into the nucleus to exert its function as also proposed for the inositol phosphates (45). The diverse nuclear roles of these compounds have long been known, but a clear quantification or visualization has been hampered by their ability to diffuse and by the lack of specific probes to follow their dynamic distribution (45). In either case, in parallel to the pharmacological applications reported in this study, endogenous GroPIns could exert a similar function intracellularly during the spontaneous resolution of inflammation. In support of this hypothesis, we have observed a pronounced increase (about 20-fold) in GroPIns levels (as measured by liquid chromatography-mass spectrometry (LC-MS/MS) (46)) in isolated human monocytes challenged with LPS; this may reflect the increase occurring in all cell compartments, including the nucleus (Fig. 7; the time course of the LPS-induced GroPIns production is also indicated; see supplemental Fig. S4).

In addition to the ability to modulate the inflammatory response, GroPIns and the other glycerophosphoinositols have been demonstrated to be active in different contexts (13, 41). For example, in human leukemia T-lymphocytes and in peripheral blood lymphocytes, GroPIns4P was shown to synergize with the chemotactic factor stromal cell-derived factor 1α (CXCL12) by increasing cytokine-dependent migration of T-lymphocytes, thus revealing an immunomodulatory effect of this compound (21). Although this effect of GroPIns4P was observed upon exogenous/pharmacological addition, its potential physiological relevance is consistent with the fact that a number of hematopoietic cells produce significant levels of glycerophosphoinositols upon proinflammatory stimuli (6, 11). In particular, antigen-presenting cells have been shown to produce and release glycerophosphoinositols in the extracellular medium where they could function as paracrine factors for lymphocytes (13, 21, 41).

It should be pointed out that, based on the information collected so far, the different glycerophosphoinositols exert separate functions based on the specific intracellular target/receptor to which they can bind.8 This is not surprising and in fact

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8 A. Varone, S. Mariggiò, A. Varriale, M. Vessichelli, F. Formiggini, N. Brancati, M. Frucci, S. D’Auria, P. Pucci, A. Flagiello, C. Iannuzzi, C. Valente, and D. Corda, submitted for publication.

9 A. Varone and D. Corda, unpublished observations.
parallels the behavior of the other inositol-containing molecules, such as the phosphoinositide and inositol phosphate families, which participate in different mechanisms according to the number of phosphate residues bound to the inositol ring (6). In addition, the metabolism of the inositol-containing molecules needs to be thought of as a whole in the context of cell homeostasis, and it depends on the available inositol pool in the different cell systems and conditions (resting versus receptor stimulation and transformation (40)).

Here we have focused on the pharmacological exploitation of GroPIns. The administration of GroPIns might act on monocytes to decrease the expression of cytokines and TF gene. This would favor the resolution of inflammation and control potentially harmful thrombotic activities. The same mechanism might be involved in the GroPIns-mediated blood-brain barrier repair mentioned above (33).

It is conceivable that the capability of GroPIns to regulate TF expression might be exploited to control disseminated intravascular coagulation, the most devastating complication of sepsis (14, 47), which remains untouched by specific therapeutic treatments. The advantages of using this compound in the resolution of inflammation are severalfold. Because of its chemical nature, GroPIns is easily manageable: it is water-soluble, and it crosses the plasma membrane, rapidly reaching its intracellular targets (48). As it is a natural compound that is present in virtually all cell types, it could be administered (e.g. intravenously) in cases of disseminated intravascular coagulation that do not respond to therapy (i.e. septic shock) (47). These are proposals that require further evaluation in vivo models of inflammatory disease. Nevertheless, GroPIns is a very promising compound as indicated by the reduced plasma levels of TNF-α it induces in a model of endotoxin shock in mice (see “Results”). Moreover, GroPIns reduces the surface expression of Mac-1, which is involved in both chemokine-guided leukocyte infiltration and detrimental inflammatory effector functions; indeed, the inhibition of Mac-1 has been shown to reduce both neutrophil transmigration and tissue injury in vivo (49). Although it is widely accepted to consider leukocyte infiltration as a common marker of inflammation, it is worth mentioning that neutrophils can also perform active healing functions during resolution (50); indeed, the inhibition of their trafficking toward injured sites is a common and effective mechanism shared by several proresolving lipid mediators (4).

We conclude that GroPIns has the potential to regulate the inflammatory response. When produced by monocytes/macrophages, GroPIns can act in a potential negative feedback loop that signals the switching off of the inflammatory response and, in parallel, the inhibition of both expression10 and activity (51) of PLA2. In summary, by targeting proinflammatory and prothrombotic mediators and thereby affecting inflammatory-related transcription factors and deactivated inflammatory cells, GroPIns would shape an anti-inflammatory microenvironment favoring the restoration of tissue homeostasis. In view of this scenario, our data supportive the pharmacological exploitation of this compound (55).

Experimental procedures

Cell culture

Monocytes were isolated from whole blood obtained from healthy donors who gave their informed consent to participate in the study and who did not take any form of medication for at least 10 days before blood donation. Approval was obtained from the independent ethics committee, and buffy coats were provided by Istituto Nazionale per lo Studio e il Trattamento dei Tumori, Fondazione Giovanni Pascale (Naples, Italy). Initial experiments were carried out on human monocytes obtained with approval from the Consorzio Mario Negri Sud Review Board for these studies. The human monocytes were taken to more than 95% purity through two centrifugation steps on Lymphoprep (Axis-Shield, Oslo, Norway) followed by a Percoll (GE Healthcare) gradient as described previously (52) and finally resuspended in serum-free RPMI 1640 medium (Gibco, Life Technologies). These monocytes were left unstimulated or were challenged with 0.1 μg/ml bacterial LPS (Escherichia coli serotype 055:B5; Sigma-Aldrich) or TNF-α (210-TA-010/CF, R&D Systems, Minneapolis, MN) with incubations for different times at 37 °C in 5% CO2. GroPIns and GroPIns4P were provided by Echelon Biosciences Inc. HeLa cells were from American Tissue Type Collection (ATCC) and were grown in minimum Eagle’s medium supplemented with 10% fetal calf serum (Biochrom, Cambridge, UK). Cells were grown under a controlled atmosphere in the presence of 5% CO2 at 37 °C.

Procoagulant activity

The procoagulant activity of TF was measured in cell lysates according to one-stage clotting time. Briefly, 100 μl of cell lysate was added to a tube containing 100 μl of prewarmed, pooled normal human plasma. After addition of 100 μl (20 ml) CaCl2, the clotting time was determined using a KC4A Amellung coagulometer (Mascia Brunelli, Milan, Italy). The clotting times were converted to arbitrary units by interpolation with a standard curve generated with serial dilutions of human recombinant thromboplastin (Hemoliance RecombiPlasTin, Instrumentation Laboratory Co., Pleasantville, NY). As the concentration of recombinant TF (Hemoliance RecombiPlasTin) used to produce the standard curve of procoagulant activity is not reported by the manufacturer, we measured the TF concentrations in the serial dilutions used to produce the standard activity curve using an IMUBIND enzyme-linked immunosorbent assay (ELISA) (International Laboratory Co.). The correlations between TF activity and the concentration of the TF antigen in the recombinant TF preparations have been reported (52).

Western blotting

The levels of phosphorylation of p38, Erk1/2, and JNK were analyzed by Western blotting using the following phosphospecific antibodies: phospho-p38 MAPK (Thr-180/Tyr-182, catalog number 9211, rabbit polyclonal, Cell Signaling Technology, Beverly, MA; 1:3000), phospho-JNK (Thr-183/Tyr-185, catalog number 9211, rabbit polyclonal, Cell Signaling Technology; 1:3000), and phospho-p44/p42 MAPK (Erk1/2) (Thr-202/Tyr-204, catalog number 4370, rabbit monoclonal, Cell Signaling Technology; 1:3000). The level of phosphorylated IκBα was

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10 M. Vessichelli, A. Varone, and D. Corda, unpublished observations.
analyzed using an antibody that specifically recognizes phosphoserine 32/36 on IkBa (phospho-IkBa, catalog number 9246, mouse monoclonal, Cell Signaling Technology; 1:3000). The levels of IKKα–β phosphorylation were analyzed using an antibody that recognizes phosphoserine 176/180 on IKKα and phosphoserine 177/181 on IKKβ (phospho-IKKα-β, catalog number 2697, rabbit monoclonal, Cell Signaling Technology; 1:3000). In parallel, total proteins were monitored with the following specific antibodies: p38 (p38α mitogen-activated protein kinase, catalog number 9218, rabbit polyclonal, Cell Signaling Technology; 1:3000), JNK (SAPK/JNK, catalog number 9258, rabbit monoclonal, Cell Signaling Technology; 1:3000), Erk1/2 (Erk1, sc-94, rabbit polyclonal, Santa Cruz Biotechnology, Inc., San Diego, CA; 1:3000), IKKα (catalog number 11930, mouse monoclonal, Cell Signaling Technology; 1:2000), and IkBα (catalog number 4812, rabbit polyclonal, Cell Signaling Technology; 1:3000). The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (catalog number 4699-9555, mouse monoclonal, AbD Serotec, Kidlington, Oxford, UK; 1:10,000) and vinculin (hVin-1, catalog number v9131, Sigma-Aldrich) were used as loading controls.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from the cells using the thiocyanate/cesium chloride method. One microgram of total RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase (Applied Biosystems). Real-time PCR was performed using 10 ng of cDNA, 50 nM concentration of reverse transcriptase (Applied Biosystems) and vinculin (hVin-1, catalog number v9131, Sigma-Aldrich) were used as loading controls.

**ELISA**

Conditioned medium from monocytes was sedimented by centrifugation at 300 × g at room temperature for 10 min, and the supernatant was recovered. The concentrations of TNF-α, IL-1β, and TxB2 were assessed according to the manufacturer’s protocol (Amersham Biosciences).

**NF-κB DNA-binding assay**

Nuclear extracts for EMSA were prepared as reported previously (53) with modifications. Monocytes, pretreated with GroPlns (300 μM; 20 min) and challenged with LPS (0.1 μg/ml), were harvested in PBS and centrifuged 5 min at 300 × g. Pellets were lysed in buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) supplemented with protease and phosphatase inhibitors (Roche Applied Science) and incubated for 15 min at 4 °C. 10% Triton X-100 was added, and extracts were centrifuged for 1 min at 12,000 × g. The supernatants were recovered as the cytosolic fraction, and the pellets containing intact nuclei were lysed in buffer C (20 mM Hepes, pH 7.9, 25% (v/v%) glycerol, 0.4 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) supplemented with protease and phosphatase inhibitors and incubated for 30 min at 4 °C on a shaker. Lysates were centrifuged for 10 min at maximum speed, and the supernatants were recovered as the nuclear fraction. Nuclear extracts were incubated with 32P-radiolabeled (Amersham Biosciences) double-stranded oligonucleotide probes containing the NF-κB-binding site, 5′-AgTTgAggggATTTC-CCAggC-3′ (Sigma-Aldrich). Protein–DNA complexes were separated on a 5% non-denaturing acrylamide gel in 0.5 × Tris borate-EDTA. Autoradiography was performed on Kodak XAR film. For competition assays, monocytes were treated with LPS (0.1 μg/ml) or left untreated as a control, and nuclear lysates were obtained as reported above. Different concentrations of GroPlns were added to the nuclear lysates together with the radiolabeled probe. For supershift assays, p65 antibody (NF-κB p65, catalog number 8242, rabbit monoclonal, Cell Signaling Technology), p50 antibody (NF-κB1 p105/p50, catalog number 12540, rabbit monoclonal, Cell Signaling Technology), or tubulin (sc-5286, mouse monoclonal, Santa Cruz Biotechnology, Inc.) was added together with the radiolabeled probe to the nuclear lysates obtained from untreated or LPS-treated monocytes.

**ChIP assay**

Monocytes were treated with GroPlns (300 μM; 20 min) and then challenged with LPS (0.1 μg/ml) for 60 min. Chromatin for the immunoprecipitation was prepared by fixing the cells by adding 37% formaldehyde (Sigma-Aldrich) into the medium at a final concentration of 1% for 10 min and quenching with 125 mM glycine (Sigma-Aldrich) for 5 min. Cells were collected and lysed in SDS buffer (1.1% SDS, 10 mM EDTA, pH 8, 50 mM Tris, pH 8) with protease inhibitors. Chromatin was sonicated for three cycles of 10-s pulse at 30% of maximum power (Branson Sonifier) to obtain fragments 500–1000 kb in size. Samples were then centrifuged and diluted by 10-fold with dilution buffer (1% Triton X-100, 1.2 mM EDTA, pH 8, 16.7 mM Tris, pH 8, 167 mM NaCl) with protease inhibitors and precleared with 25 μl of 50% salmon sperm/protein A-agarose slurry (EMD Millipore) for 30 min at 4 °C. Precleared chromatin was incu-
bated overnight with 2 µg of p65 polyclonal antibody (7970, chip grade, Abcam, Cambridge, UK) or normal rabbit IgG (Santa Cruz Biotechnology, Inc.). A portion of the precleared chromatin (10% of the sample) was stored at 4 °C as input to further quantitate the amount of immunoprecipitated DNA. The antibody–protein–chromatin complex was isolated with 25 µl of 50% salmon sperm/protein A-agarose slurry (Millipore) for 2 h at 4 °C. After washing, pellets were eluted (1% SDS, 0.1 M NaHCO3), reverse cross-linked with 5 M NaCl at 65 °C overnight, and treated with RNase A (Thermo Fisher Scientific, Waltham, MA) and Proteinase K (Thermo Fisher Scientific). Samples were purified with a commercially available purification kit (Qiagen, Hilden, Germany) and used as templates in real-time PCR analysis. The intensity of the PCR signal is proportional to the occupancy on the binding site. Normalization of data has been performed by using the percent input method: signals obtained by ChIP/PCR have been divided by signals obtained from input/PCR. The validated primers used were: TNF-α forward, GCGATGGA-GAAGAAACCGAG; TNF-α reverse, GAGGCGGGGAAAG-AATCA; TF forward, GCAACTAGACCCGCCTG; and TF reverse, CTCCCTCCGGTAGGAAACTC.

**Immunofluorescence**

For nuclear trafficking of NF-κB, immunofluorescence experiments were performed as follows. Cells (3 × 10^5) were seeded on glass coverslips in culture medium and treated the day after according to the experimental protocol. Cells were rinsed with PBS and fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature and permeabilized with blocking solution containing 0.05% (w/v) saponin for 20 min. The intracellular localization of NF-κB was revealed using antibodies specific to p65 (catalog number 7970, Abcam; dilution, 1:30) or p50 (catalog number 12540, Cell Signaling Technology; dilution, 1:50) for 1 h at room temperature. After washes, cells were stained with Alexa Fluor 568-labeled (A10042, Molecular Probes) or Alexa Fluor 488-labeled (A21206, Molecular Probes) secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) dye for 1 h at room temperature. Coverslips were mounted in Mowiol (Calbi- 

**Flow cytometric analysis of Mac-1 expression in neutrophils from mouse whole blood**—Citrated whole blood was diluted 1:1 with citrated saline and incubated for 15 min with phycoerythrin-conjugated rat anti-mouse Mac-1 (clone M1/70, BD Pharmingen TM). Nonspecific phycoerythrin-conjugated rat IgG was used as the staining negative control. After staining, the red blood cells were lysed by addition of lysing-fixing solution (BD Biosciences) according to the manufacturer’s instructions. Flow cytometry analysis was performed with a FACStar flow cytometer as follows. Events showing side light scatter (granularity) and forward light scatter (dimension) characteristic of polymorphonuclear leukocytes were analyzed for Mac-1 expression levels. The data are shown as mean fluorescence intensities for each of the nine mice analyzed.

**Statistical analysis**

The data are expressed as means ± S.E. Statistical differences between two groups were determined by paired t tests. To test for differences across different treatment groups, repeated-measures analysis of variance and Dunnett tests were used. Statistical significance was defined as p < 0.05.

**Author contributions**—M. V. and P. Z. designed, performed, and analyzed all the experiments and co-wrote the manuscript. A. V. contributed to the in vitro experiments and to the discussion. S. M. and V. E. designed and analyzed the initial experiments; V. E. also co-wrote the manuscript. M. Z and T. d. C. performed and discussed the EMSA. A. D. S., C. A, and G. D.’ E. performed all the in vivo experiments and part of the in vitro experiments. A. C. and A. F. performed and discussed LC-MS/MS analysis. C. C. and G. D. C. provided buffy coats. D. C. conceived and supervised the project, discussed and analyzed the data, and co-wrote the manuscript.

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