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*J Immunol* 2008; 181:3495-3502; doi: 10.4049/jimmunol.181.5.3495

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Lipopolysaccharide Is a Direct Agonist for Platelet RNA Splicing

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Platelets express TLR4 receptors, but its ligand LPS does not directly activate thrombotic functions nor, obviously, transcription by these nucleate cells. Platelets, however, store information that changes their phenotype over a few hours in the form of unprocessed RNA transcripts. We show even low concentrations of LPS in the presence of soluble CD14 initiated splicing of unprocessed IL-1β RNA, with translation and accumulation of IL-1β protein. LPS was a more robust agonist for this response than thrombin. Platelets also contained cyclooxygenase-2 pre-mRNA, which also was spliced and translated after LPS stimulation. Flow cytometry and immunocytochemistry of platelets extensively purified by negative immunodepletion showed platelets contained IL-1β, and quantitative assessment of white blood cell contamination by CD14 real time PCR confirms that leukocytes were not the IL-1β source, nor were they required for platelet stimulation. LPS did not initiate rapid platelet responses, but over time did prime platelet aggregation to soluble agonists, induced actin rearrangement, and initiated granule secretion with P-selectin expression that resulted the coating of quiescent leukocytes with activated platelets. LPS is a direct agonist for platelets that allows these cells to directly participate in the innate immune response to bacteria.

1 Abbreviations used in this paper: LBP, LPS-binding protein; DAPI, 4′,6′-diamidino-2-phenylindole.

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potent agonist for prolonged platelet responses. We generated highly purified platelets by negative immunoselection and then quantified monocyte contamination by quantitative RT-PCR for CD14 mRNA. This allowed us to show that LPS acts directly on platelets to induce RNA processing and translation, including cyclooxygenase-2 RNA processing and expression, functionally altered platelet physiology, and initiated platelet coating of quiescent leukocytes.

Materials and Methods

Cell isolation

Human blood in a protocol approved by the Cleveland Clinic IRB was drawn into acid-citrate-dextrose and centrifuged (200 × g, 20 min) to obtain platelet-rich plasma. All centrifugations were without braking. Platelet-rich plasma was filtered through two layers of 50 μm mesh to remove nucleated cells and then centrifuged (500 × g, 20 min) in the presence of 100 mM prostaglandin E1. The pellet was resuspended in 50 ml PBS/saline/glucose (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 mM Na2HPO4, 1 mM MgCl2, and 5.5 mM glucose) containing 100 nM of 10 ng LPS/ml, respectively) was added with 0.1 U/ml thrombin or LPS for the stated time. These cells were centrifuged (500 × g, 20 min), resuspended in AutoMACS sample buffer, 5 μl anti-CD45-, anti-CD15-, anti-CD14 and anti-glycoprocin-coated magnetic beads (Miltenyi Biotec) per 10^6 cells for 25 min with constant rotation before purification in an AutoMACS magnetic separator (Miltenyi Biotec). For some experiments, this negative microbead selection was repeated. Light microscopy was used to confirm the cells had a discoidal, unactivated shape. Recovered platelets were centrifuged (500 × g, 20 min) and resuspended in HBSS/A (0.5% human serum albumin in HBSS) at 4 × 10^8 cells/ml for confocal microscopy, aggregation and FACS analysis, and at 8 × 10^8 platelets/ml for all other uses. Platelet activation was induced by 100 ng/ml LPS or KDO2-Lipid A, 10 μg/ml Pam2Cys-SK4, or 0.1 U of thrombin for the stated time. Autologous serum (0.5%) or recombinant soluble CD14 and LBP (final concentration 0.15 μg/ml and 0.1 μg/ml, respectively) was added with TLR4 agonists. Density purified monocytes were isolated as before (36).
**Materials and Methods**

**A**

RNA isolation and real time RT-PCR

Total RNA from 2.4 × 10^8 platelets or 10^7 monocytes or PMNs was isolated using RNEasy Mini Kit (Qiagen) and treated with RNase-free DNase (Qiagen). Total RNA was quantitated by NanoDrop and used to normalize PCR samples. Real time reverse transcriptase-PCR primers for IL-1β mRNA, sense 5′-GTATAGGCCCACATCCGCAAAG-3′ (exon 1), antisense 5′-GGTTAGGCCCACATCCGCAAAG-3′ (exon 3); COX-2 mRNA, sense 5′-TGAAACCTCAAAACACCA-3′ (exon 3) and antisense 5′-GAGAAGGCTTCCCCAGCTTTT-3′ (exon 4); CD14 mRNA, sense 5′-GGGTTCACAGAGGAGGGAAC-3′ (exon 1), antisense 5′-CCGGCTCCATGTCGTTGTA-3′ (exon 3), data collection (80°C, 15′), 40–45 cycles with SYBR Green I in either an Applied Biosystems ABI Prism 7700 Sequence Detection System or a MyCycler. This amplification across intron 2 does not detect unprocessed IL-1β RNA. Conditions for COX-2 and CD14 were the same, with the exception that annealing temperature was 60°C and 61.9°C, respectively, and data collection for COX-2 was at 75.5°C. Products were analyzed by melting curve, gel electrophoresis, and sequencing. DNase I treatment did not affect IL-1β mRNA expression, while RNase I and no RT abolished amplification.

**Confocal microscopy**

Platelets were incubated for the stated times in gelatin-coated 4-well borosilicate chambered coverslips. Adherent cells were washed with endotoxin-free PBS and fixed with 2% paraformaldehyde for 30 min. Globular G-actin was detected using Alexa488-conjugated goat-anti-mouse IgG, and polymerized F-actin was stained with Alexa594-wheat germ agglutinin. White blood cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Microscopy used a Plan Apochromat ×63, N.A.1.4 oil objective and Leica confocal software.

**Flow cytometry**

FACScan analysis used the same anti-IL-1β antibody as above and PerCP-conjugated secondary goat-anti-mouse Abs. Cells were washed and then permeabilized using BD Pharmingen Cytofix/Cytoperm kits. Platelets were gated by forward and side scatter that defined a CD42b+ population.

**Aggregometry**

Acid-citrate-dextrose initiated aggregation in platelet-rich plasma, assessed by transmittance (Chrono-Log). Platelets from different donors demonstrated broad variation in their agonist sensitivity, ranging from 2 to 10 mN/g/ml collagen (n = 13), so a threshold concentration was determined for every donor.

**Western blotting and mass spectrometry**

Monocytes or platelets were incubated with buffer or LPS and MG132, lysed in RIPA buffer, and the proteins were resolved by SDS-PAGE before the resulting blot was stained with anti-cyclooxygenase-2 mAb and goat-anti-mouse HRP. Western blotting and mass spectrometry demonstrated broad variation in their agonist sensitivity, ranging from 2 to 10 mN/g/ml collagen (n = 13), so a threshold concentration was determined for every donor.
Purified platelets were stimulated with 100 ng of Alexa594-wheat germ agglutinin, Alexa594-DNase I, and DAPI, Molecular Lipids; Pam3Cys-SK4, and Cdc2-like kinase (Clk-1) inhibitor TG003, CalBiochem; 4-well Lab-Tek II Chamber Slide System, Nalge Nunc International; Alexa488-phalloidin, Alexa488-chicken anti-rabbit IgG (H+L), Alexa488-Phallolidin, Alexa594-wheat germ agglutinin, Alexa647-DSNase 1, and DAPI, Molecular Probes; recombinant soluble CD14, LBP, IL-1β ELISA kit, ELISPOT kit, IL-1β Ab, R&D Systems; PerCP-conjugated goat-anti-mouse, FITC-mouse IgG1, PE-anti-human CD42b, and PE-mouse IgG1k, BD Pharmingen; anti-cyclooxygenase-2 Western blot using Ab preincubated with its cognate peptide.

Chemicals and reagents
Chemicals and reagents were purchased from the following sources: sterile filtered HBSS and M199, BioWhittaker; sterile tissue culture plates, Falcon Labware; human serum albumin, Baxter Healthcare; endotoxin-free PBS, phenol-extracted LPS (Escherichia coli O111:B4) that is free of lipoprotein contamination, List Biological Laboratories; KDO2-Lipid A, Avanti Polar Lipids; Pam3Cys-SK4, and Cdc2-like kinase (Clk-1) inhibitor TG003, Calbiochem; 4-well Lab-Tek II Chamber Slide System, Nalge Nunc International; Alexa94 chicken anti-rabbit IgG (H+L), Alexa94-Phallolidin, Alexa94-wheat germ agglutinin, Alexa94-DSNase 1, and DAPI, Molecular Probes; recombinant soluble CD14, LBP, IL-1β ELISA kit, ELISPOT kit, IL-1β Ab, R&D Systems; PerCP-conjugated goat-anti-mouse, FITC-mouse IgG1, PE-anti-human CD42b, and PE-mouse IgG1k, BD Pharmingen; anti-cyclooxygenase-2 and cognate peptide, Cayman Chemical; rhodamine-labeled G-actin, Cytoskeleton. Other chemicals were from Sigma-Aldrich or Biomol Research Laboratories.

Expression of data and statistics
Experiments were performed at least three times with cells from different donors, and all assays were performed in triplicate. The mean ± SE from all experiments are presented. Representative experiments are shown in some cases because we observed interassay variation in IL-1β expression from different donors. ANOVA was used to determine differences among the groups. If significant differences were found, a Newman-Keuls post hoc procedure was used to determine the location of the difference. A value of $p < 0.05$ was considered statistically significant.

Results
LPS stimulates IL-1β transcript processing
Thrombin rapidly stimulates platelet processing of IL-1β pre-mRNA to functional, intronless mRNA (18), which we confirmed using real time PCR with primers anchored in exons 1 and 3 that do not amplify unprocessed message (Fig. 1A). The progression curves of the real time PCR show that E. coli LPS also induced platelet IL-1β RNA processing, and was significantly better at this than thrombin. The thousand-fold increase in spliced IL-1β RNA after LPS exposure was time dependent (Fig. 1B); maximal accumulation of processed IL-1β RNA occurred 3 h after stimulation, but with significant accumulation of spliced message 1 h after LPS exposure. Accumulation of processed IL-1β RNA in response to LPS developed more slowly than in response to the complete platelet agonist thrombin, but was both more effective and more prolonged (Fig. 1B). The amount of processed RNA after stimulation with either agonist gradually declined, although not to background levels, by 20 h post stimulation. The loss of processed RNA was not due to a loss of cell viability or functionality because platelets, even after 20 h of continuous LPS stimulation, remained metabolically competent with functional, polarized mitochondria that could be depolarized with the protonophore CCCP (Fig. 1C).

The response to E. coli LPS was receptor mediated because it was enhanced by a source of soluble CD14, which is needed to present LPS to TLR4, that we supplied as recombinant CD14 along with LBP (Fig. 1D). Additionally, the response to LPS was blocked by the competitive TLR4 antagonist (37) Rhodobacter sphaeroides LPS (Fig. 1D). The invariant lipid core of LPS was all that was required to stimulate platelet splicing because the homogeneous lipid ligand KDO2-lipid A that lacks the outer carbohydrate chain of LPS was a highly effective agonist. Platelets express (4) the TLR2 receptor for endotoxic bacterial lipopolysaccharides (38), and we found (Fig. 1D) that the synthetic TLR2 agonist Pam3Cys-SK4, which contains the bioactive lipid modification of Gram-negative and Gram-positive bacterial lipopolysaccharides,
stimulated IL-1β RNA splicing. This synthetic lipopeptide was less effective than LPS, being about as effective as thrombin.

**LPS stimulates IL-1β protein accumulation in platelets**

Newly synthesized IL-1β primarily remains cell-associated in purified preparations of platelets stimulated by thrombin (16). Because platelet preparations contain contaminating monocytes, we used flow cytometry to define the population(s) of cells that synthesized and accumulated IL-1β in response to LPS stimulation. We gated the platelet population by forward and side scatter and then for cells that stained for surface CD42b (data not shown). Staining this population for IL-1β after permeabilization revealed no staining beyond background levels in unstimulated cells, with an increase in IL-1β over time after LPS stimulation (Fig. 2A). This occurred in a way that varied with the concentration of agonist, with as little as 10 ng of *E. coli* LPS increasing the content of immunoreactive IL-1β (Fig. 2B).

**Monocytes are not required in the platelet response to LPS**

Monocytes may directly, or indirectly through cytokine production, contribute to platelet IL-1β accumulation, and quantification of small numbers of contaminating monocytes is problematic. We took advantage of the abundant expression of CD14 by monocytes and the paucity of this component of LPS signaling on platelets to establish a quantitative definition of monocyte contamination by real time PCR for CD14 mRNA. This mRNA was abundant in unstimulated monocytes (Fig. 3A), but was present in platelet-rich plasma even after filtration through a 5 μm mesh. We immunodepleted monocytes with a combination of anti-CD14, anti-CD15, and anti-CD45 conjugated to magnetic beads, while a second immunodepletion after addition of additional beads further reduced the CD14 RNA content. Based on RNA content, we calculate that 106 filtered, immunodepleted, and doubly immunodepleted platelets could contain up to 17 ± 16, 7.4 ± 3.6, and 2.5 ± 0.9 monocytes, respectively.

Monocyte-depleted platelet preparations still produced IL-1β after LPS stimulation when analyzed by ELISPOT (Fig. 3B), and the frequency of cells expressing IL-1β after LPS stimulation was significantly greater than the 25 ± 9 monocytes that could have been present in the well. We visualized the cells that contained IL-1β by confocal microscopy and used DAPI to stain any contaminating nucleated cells in the platelet preparation. Confocal microscopy showed that LPS increased the number and intensity of cells that stained for IL-1β protein (Fig. 3C), which was maximal by 6 h and then declined modestly after 18 h of LPS exposure. DAPI labeling of cell nuclei showed the platelet preparation was depleted of nucleated cells, and that all of the newly generated IL-1β protein was associated with anucleate platelets. We also found (data not shown) that inhibition of transcription by actinomycin D suppressed monocyte production of IL-1β, but not that associated with anucleate platelets. These data show stimulation of monocyte cytokine production has no part in the platelet response to LPS.

**LPS stimulates platelet cyclooxygenase-2 RNA splicing**

Quiescent platelets are reported (33, 34) to contain no cyclooxygenase-2, but to test whether LPS-stimulated platelets might do so, we designed primers that hybridized within exon 3 of cyclooxygenase-2 and at the junction formed by the splicing of exons 3 and 4. Little processed cyclooxygenase-2 RNA was present in unstimulated platelets, but we found that purified platelets stimulated by either LPS or the homogenous endotoxin KDO2-Lipid A now contained spliced cyclooxygenase-2 RNA (Fig. 4A). Amplification across exons 9 and 10 confirmed that LPS initiated cyclooxygenase-2 splicing (data not shown). The synthetic TLR2 agonist (Fig. 4A) and thrombin (data not shown) also stimulated splicing, but again were not as effective as the TLR4 agonists. Two-color flow cytometry (Fig. 4B) showed the platelets that accumulated IL-1β protein after LPS stimulation also accumulated cyclooxygenase-2 protein. Highly purified platelet preparations expressed immunoreactive cyclooxygenase-2 at the appropriate m.w. after 17 h of LPS treatment (Fig. 4C). Staining of this band was abolished by

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**FIGURE 5.** LPS primes platelet aggregation and alters platelet structure. A, LPS primes ADP-induced aggregation in platelet-rich plasma (PRP). LPS (100 ng), KDO2-Lipid A (100 ng), or Pam3Cys-SK4 (100 μg) was added to 1 ml of PRP and incubated for 1 h before addition to a stirred aggregometer cuvette. As shown by the arrow, 20 μM ADP was added and light transmittance was assessed over the subsequent 6 min. LPS did not induce aggregation without the addition of ADP (data not shown). B, Polymor ized actin staining of adherent platelets. Confocal microscopy images of control (left) and platelets stimulated with 100 ng/ml *E. coli* LPS (right) in the presence of autologous serum or soluble CD14 and LPS binding protein for 3 h. Green staining is polymerized (F-actin) stained by FITC-phalloidin and red fluorescence is unpolymerized G-actin. The bar is 20 μm. Lower insets: ×2 magnified fields of respective images. Upper inset: pretreatment with cytochalasin B abrogated actin polymerization. C, LPS affects platelet structure. Platelets were treated or not with LPS for 3 h as in the preceding panel and then analyzed by flow cytometry for forward scatter (size) and side scatter (granularity). The area of the gate imposed on the dot blot stained for the platelet protein CD42b. This experiment has been repeated more than twenty times.
the cognate peptide, and peptide sequencing of this band by mass spectrometry demonstrated unique cyclooxygenase-2 peptides (data not shown).

**LPS alters platelet physiology over time**

LPS was not a typical platelet agonist, and did not induce rapid platelet shape change or aggregation (data not shown), as previously reported (10). However, both LPS and KDO2-Lipid A augmented ADP-induced aggregation in platelet rich plasma (Fig. 5A). In contrast to splicing, the synthetic TLR2 agonist Pam3Cys-SK4 was not an agonist for this response. Changes in platelet physiology in response to LPS included a rearrangement of their cytoskeleton with an increase in F-actin at the expense of monomeric G-actin (Fig. 5B). Direct quantification of actin nucleation in platelet lysates by incorporation of rhodamine-labeled G-actin showed 3 h of LPS treatment increased actin polymerization 3.8 ± 0.8-fold. Changes in platelet physical structure after LPS exposure was also reflected in the change in both forward and side scatter during flow cytometry (Fig. 5C) reflecting changes in granularity and microparticle formation.

Surface expression of granule-stored proteins was increased after 3 h of LPS exposure. Flow cytometry showed that LPS increased expression of CD40L in some cells with the formation of a CD40L high population (Fig. 6A). LPS also initiated a time-dependent increase in surface P-selectin expression that was evident by 1 h of exposure encompassing most platelets, with one population becoming particularly bright (Fig. 6B). Neutrophils constitutively express P-selectin glycoprotein ligand-1, which serves as a ligand for the P-selectin displayed by activated platelets. Platelets treated with LPS for 2.5 h to increase surface P-selectin readily associated with quiescent neutrophils (Fig. 6C). Blockade of this ligand-receptor interaction with anti-P-selectin mAb suppressed platelet-induced neutrophil interaction (data not shown).

**Discussion**

These data show platelets are components of the innate immune system that recognize and then respond to endotoxin. Over time, LPS sensitized platelets to soluble agonists, altered the platelet cytoskeleton, altered platelet size and granularity, generated microparticles, promoted degranulation, induced surface expression of P-selectin and CD40L, and promoted leukocyte binding. LPS also proved to be a robust agonist of platelet RNA processing and translation that eclipsed thrombin as an agonist. This occurred in the virtual absence of contaminating monocytes, which we could document by quantifying CD14 mRNA. Therefore, while LPS is a slowly acting secondary agonist for many of the responses typical of platelets, it now becomes the most robust agonist that alters the platelet transcriptome and proteome.

Whether LPS directly affects platelet function has been controversial (9, 10), but in vivo experiments are definitive and show the TLR4 receptor of platelets has a role at least in the systemic response to endotoxin (2, 4, 5). A critical issue to be answered in experiments examining LPS as an ex vivo platelet agonist is the
potential role of monocytes remaining in the preparation because monocytes abundantly produce IL-1β that might then be incorrectly ascribed to activated platelets (24). Moreover, it is possible that LPS acts solely on monocytes to produce an agonist that then acts on platelets. Quantification of contaminating leukocytes at less than one per hundred thousand platelets (24) is problematic by flow cytometry (25), and platelet-monocyte aggregates will be excluded from the analysis. We approached this issue by first creating a protocol that produced highly purified platelet populations and then devising a method to quantitatively assess monocyctic cell content in this preparation. We filtered platelet-rich plasma through 5 μm mesh, and then negatively purified platelets by removing cells that expressed CD14, CD15, and CD45, along with anti-glycophorin to remove residual erythrocytes, by magnetic bead separation. Although we found this reduced leukocyte contamination, we also found that a second pass through the AutoMax column after addition of additional Abs and magnetic beads further reduced nucleated cell contamination, perhaps because the sample overloaded the capacity of the column.

Platelets are not known to express CD14 and, accordingly, we found it necessary to provide exogenous CD14, provided either as a recombinant protein or as the constitutively expressed protein in human serum (data not shown), to aid their response to LPS. This enhancement varied among donors, but most often the response was greatly enhanced by exogenous CD14. Platelets, then, are analogous to endothelial cells (39, 40) that are aided by circulating soluble CD14 and LBP. In contrast, CD14 is a prominent mRNA and protein of monocytes, and so quantifying CD14 mRNA by real time PCR provides a quantifiable measure of the number of monocytes that could be present in any given platelet preparation. We used this value to show that the number of cells expressing IL-1β in an ELISPOT assay was significantly larger than the maximum number of monocytes that could be present, and used this approach to show that an equivalent number of monocytes fail to produce detectable IL-1β (data not shown). We found that reducing the number of contaminating monocytes to a few per million platelets did not diminish the responsiveness of the platelet population to LPS, and, as a complementary direct test, we found that blocking transcription with actinomycin D blocked IL-1β production by purified monocytes but not purified platelets. All these data show that LPS is a direct platelet agonist.

LPS stimulation of platelets initiated splicing of cyclooxygenase-2 RNA and ultimately, production of the corresponding protein. This occurred in highly purified platelet populations and in a population of cells defined as platelets during flow cytometry by and protein of monocytes, and so quantifying CD14 mRNA by real time PCR provides a quantifiable measure of the number of monocytes that could be present, and used this approach to show that an equivalent number of monocytes fail to produce detectable IL-1β (data not shown). We found that reducing the number of contaminating monocytes to a few per million platelets did not diminish the responsiveness of the platelet population to LPS, and, as a complementary direct test, we found that blocking transcription with actinomycin D blocked IL-1β production by purified monocytes but not purified platelets. All these data show that LPS is a direct platelet agonist.

LPS stimulation of platelets initiated splicing of cyclooxygenase-2 RNA and ultimately, production of the corresponding protein. This occurred in highly purified platelet populations and in a population of cells defined as platelets during flow cytometry by forward, scatter, side scatter, and CD42b staining. Western blotting and mass spectrometry confirmed the presence of the protein in LPS-stimulated platelets. We emphasize, however, the extreme variability of this response, even when compared with IL-1β expression; the protein was present in some preparations without activation, while in others it could not be up-regulated by LPS stimulation. The basis for this variability may lie in donor variability. More importantly, we could not demonstrate increased product formation nor sensitivity to the cyclooxygenase-2 inhibitor NS398 (data not shown). Unstimulated platelets are not a source of cyclooxygenase-2 products (34), and it appears LPS stimulated platelets either generate protein that is not active, or it is present in insufficient quantities to affect arachidonate metabolism.

These data show that LPS synergizes with rapidly acting stimuli, but over time develops an independent capacity as a direct platelet agonist. Platelets also express TLR1, TLR2, TLR6, and TLR9 (4, 41, 42), and a synthetic TLR2 ligand, the only other TLR ligand we tested, proved to be an agonist, although a weak one, for splicing. Agonist stimulation of platelet splicing and activation-depen-

dent translation of platelet mRNA includes IL-1β (18), Bcl-3 that aids clot retraction (43), tissue factor (19), now cyclooxygenase-2, and indeed many other currently unidentified proteins (11). LPS promoted platelet granule release accompanied by surface P-selectin expression that then promoted leukocyte interaction, so in vivo endotoxin exposure has the potential to directly alter platelet reactivity. Indeed, in vivo exposure to LPS shows platelet TLR4 to be a critical component of the response to endotoxemia (2).

A key distinction between the findings reported in this study and other experiments examining the effect of LPS on platelets that requires emphasis is that LPS is not a typical rapidly acting, soluble agonist. Thus, the response of platelets to LPS developed over time and, for example, the binding of human neutrophils to LPS-stimulated platelets was apparent after several hours (Fig. 6C) and not after several minutes (data not shown) of incubation. Platelet responses are typically analyzed as immediate responses to introduced agonists, and LPS modulation of platelet function does not fit the paradigm of established platelet agonists. The measured rate of change in platelet phenotype after LPS stimulation indicates that the effect of LPS on platelets will manifest in systemic changes, rather than local ones, as indeed has been observed in in vivo models of endotoxemia (2).

Acknowledgments

The technical aid of Mark Calabro, Manisha Sharma, Jessica Cemate, and Stacy Haucek is greatly appreciated, as was the helpful advice of Drs. Andrew Weyrich and Guy Zimmerman. This experiment performed by R. Chen is greatly appreciated. The significant aid of Dr. Michael Kinter and the Mass Spectrometry core facility and the aid of Dr. Josephine Adams with the nucleation assay are gratefully acknowledged. We thank Dr. D. Zeldin (National Institutes of Health/NIEHS, Research Triangle Park, NC) for the gift of recombinant cyclooxygenase-1. Figure preparation by Diana Lim is, again, greatly appreciated. We appreciate the support provided by the Flow Cytometry and Imaging core laboratories of the Lerner Research Institute. We have no commercial conflicts.

Disclosures

The authors have no financial conflict of interest.

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