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*J Immunol* published online 30 September 2013

http://www.jimmunol.org/content/early/2013/09/30/jimmunol.1300354

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2013/11/06/jimmunol.1300354.DC1

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Lipopolysaccharide Stimulates Platelets through an IL-1β Autocrine Loop

G. Thomas Brown,*†,1 Padmini Narayanan,*† Wei Li,* Roy L. Silverstein,*† and Thomas M. McIntyre*†

LPS activates platelets through TLR4, aiding productive sepsis, with stimulated splicing and translation of stored heteronuclear pro–IL-1β RNA. Although the IL-1R type 1 (IL-1R1) receptor for IL-1 shares downstream components with the TLR4 receptor, platelets are not known to express IL-1R1, nor are they known to respond to this cytokine. We show by flow cytometry and Western blotting that platelets express IL-1R1, and that IL-1β and IL-1α stimulate heteronuclear 1β splicing and translation of the newly made mRNA in platelets. Platelets also respond to the IL-1β they make, which is exclusively associated with shed microparticles. Specific blockade of IL-1R1 with IL-1R antagonist suppressed platelet stimulation by IL-1, so IL-1β stimulates its own synthesis in an autocrine signaling loop. Strikingly, IL-1R antagonist inhibition, pharmacologic or genetic suppression of pro–IL-1β processing to active cytokine by caspase-1, or blockade of de novo protein synthesis also blocked LPS-induced IL-1β mRNA production. Robust stimulation of platelets by LPS therefore also required IL-1β amplification. Activated platelets made IL-1β in vivo as IL-1β rapidly accumulated in occluded murine carotid arteries by posttranscriptional RNA splicing unique to platelets. We conclude that IL-1β is a platelet agonist, that IL-1β acts through an autocrine stimulatory loop, that an IL-1β autocrine loop is required to amplify platelet activation by LPS, and that platelets immobilized in occlusive thrombi are activated over time to produce IL-1β. IL-1β is a new platelet agonist that promotes its own synthesis, connecting thrombosis with immunity. The Journal of Immunology, 2013, 191: 000–000.

Interleukin-1β initiates the inflammatory program of endothelium and circulating innate immune cells in a range of acute and chronic inflammatory events (1, 2). Indeed, autoinflammatory disease is defined as a chronic inflammation ameliorated by blockade of IL-1 stimulation (1). IL-1β is synthesized as a proprotein precursor by stimulated inflammatory and immune cells, which is then cleaved to the active, mature cytokine by activated caspase-1. This protease is itself the proteolytic product of stimulated inflammasome digestion (1). The resulting IL-1β is a leaderless protein, so the way it is released from activated cells is opaque, but it can include exosome release (3, 4) and microvesicle shedding (5, 6). IL-1β is produced in nucleated cells in response to LPS stimulation of TLR4 (1), but it is also produced in these cells in response to activation of its own IL-1R type 1 (IL-1R1) signaling receptor (7, 8). LPS and IL-1β receptors share a common Toll/IL-1R (TIR) domain and so share major downstream signaling components, including MyD88, TNFR-associated factor (TRAF)6, and MAPKs (9, 10), to induce similar responses.

Platelets express TLR4 (11–13) that binds LPS (14), and LPS from enterohemorrhagic Escherichia coli is a potent platelet agonist (14). LPS promotes platelet action (11, 13, 15–17), induces thrombocytopenia (13), and expands the inflammatory reaction through TNF-α expression (13). LPS, however, is not a typical platelet agonist because it neither increases the intracellular Ca²⁺ concentration nor enhances rapid homotypic platelet aggregation, nor does LPS mobilize P-selectin from α-granules to the platelet surface (18). Also in contrast to the rapidity of typical platelet agonists that act through a Ca²⁺ flux, the response to LPS occurs over minutes to hours (19). MyD88, common to both TLR and IL-1 signaling, is an essential component of platelet LPS signaling (19, 20).

Platelets modify their proteome by translating stored mRNAs encoding an array of proteins (21), including plasma plasminogen activator inhibitor-1 (22) and Bcl-3, that aids clot retraction (23). Platelets also store untranslatable, intron-containing heteronuclear (hn)RNA that is spliced upon appropriate activation (24, 25) to functional mRNA. This occurs in a unique posttranscriptional process that, for human platelets, generates tissue factor mRNA (26) and produces functional IL-1β mRNA in stimulated human and mouse platelets (19). Thrombin (24), although not ADP or collagen, and LPS acting through TLR4 (19, 27) stimulate pro–IL-1β hnRNA splicing, whereas LPS additionally stimulates robust translation to functional IL-1β cytokine (27).

Isolated platelets produce IL-1β, but their potential contribution in vivo is difficult to ascertain because activated platelets rapidly disappear from the circulation, although circulating platelets from septic patients do show that their transcriptome has been altered

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Received for publication February 7, 2013. Accepted for publication September 5, 2013.

This work was supported by National Institutes of Health Grants P50 HL081011 and 1 R01 HL092747 and by Case Western Reserve University/Cleveland Clinic Clinical and Translational Science Award UL1 RR024989 from the National Institutes of Health/National Center for Research Resources.

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The online version of this article contains supplemental material.

Abbreviations used in this article: hn, heteronuclear; IL-1R1, IL-1R type 1; IL-1Ra, IL-1R antagonist; LPB, LPS binding protein; TIR, Toll/IL-1R; TRAF, TNFR-associated factor.

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(28). Activated platelets do accumulate for prolonged times in occlusive thrombi of damaged arteries (29), but the transcriptome of platelets emmeshed in thrombi is unexplored.

We show that platelets express the IL-1R1 receptor, that this receptor is functional, and that platelets respond to the IL-1β they make to form an autocrine stimulatory loop. Moreover, amplification through this IL-1β loop is essential for LPS stimulation through TLR4; absent IL-1R1 signaling, TLR4 is an ineffective platelet agonist. Platelets retained in maturing thrombi rapidly accumulate IL-1β, so platelets are activated in this novel way in vivo, connecting sterile vascular thrombosis to inflammatory cytokine production.

Materials and Methods

Cell isolation

Human blood was obtained in a protocol approved by the Cleveland Clinic Institutional Review Board using heparin as the anticoagulant. Platelet-rich plasma was filtered through two layers of 5-μm mesh (BioDesign) to remove nucleated cells. These cells were centrifuged, resuspended in autoMACS sample buffer containing anti-CD45-, anti-CD15-, anti-CD14-, and anti-glycoporphin-coated magnetic beads (Miltenyi Biotec; 5 μL/107 cells) for 25 min with constant rotation before purification in an autoMACS magnetic separator (Miltenyi Biotec). For some experiments, this microbead selection was repeated. This negative selection resulted in a platelet population containing just one monocyte per 2 × 106 platelets based on CD14 mRNA content (27). Platelet activation was induced for the stated time at 37°C with 100 ng/ml LPS with addition of 100 ng/ml each of human rCD14 and recombinant LPS binding protein (LPB). Detection of surface Ag by flow cytometry was as before (19) using goat anti–IL-1R1.

Human monocytes were recovered from platelet-rich plasma and purified by density centrifugation as previously described (30). Mouse blood from sacrificed animals was a generous gift from the laboratory of Dr. Ariel Feldstein provided by Laura Dickson (Cleveland Clinic Foundation, Cleveland, OH).

Mouse thrombosis

Occlusive thrombosis was induced as described (31, 32). Briefly, mice were anesthetized before carotid arterial injury through topical application of 7.5% FeCl3 for 1 min. Animals were maintained on a 37°C heated stage for the stated times before sacrifice and the vessels containing murine thrombi excised. Some mice were treated with actinomycin D (0.6 mg/kg) by i.p. injection (33) 30 min prior to FeCl3 application. Whole blood of mice, injected or not with actinomycin D, was isolated 90 min after injection and stimulated with LPS for 60 min before leukocytes were isolated, and their ability to synthesize IL-1β mRNA relative to constitutive GAPDH was assessed by RT-PCR.

Confocal microscopy

Murine vessels containing thrombi were fixed in formalin and embedded in paraffin. Sections (6 μm) were blocked with 1% BSA and 10% goat serum, endogenous peroxidase inhibited (Thermo Scientific), and IL-1β stained with rabbit polyclonal (1:250; Abcam, ab9722) or isotype IgG1 non-immune (Santa Cruz Biotechnology) primary Ab. The primary Ab was detected using a Pierce peroxidase immunohistochemistry detection kit with metal-enhanced diaminobenzidine generation (Thermo Scientific) and hematoxylin staining. IL-1β was also detected with rabbit Ab (1:250; Abcam, ab9722) or nonimmune isotype Ab in 10% goat serum with 1% BSA and then detected with Alexa 488–conjugated goat anti-rabbit Ab (1:1000; Invitrogen, CA11098). Platelet membranes were visualized with 5 μg/ml Alexa Fluor 594–conjugated wheat germ agglutinin or anti–P-selectin and then with Vectashield (Vector Laboratories) mounting media containing DAPI for confocal microscopy. A composite image was formed in Image-Pro Plus 6.1. Some sections were similarly stained with anti–P-selectin (1:200 dilution) and Alexa Flour 488 secondary Ab and anti–IL-1β (1:200) and Alexa Flour 568 secondary Ab.

Microparticle quantification

Human platelet microparticles derived from washed platelets were collected by centrifugation (20,000 × g) (34) and counted by flow analysis using defined amounts of 3.0-μm polystyrene latex beads (Sigma-Aldrich) as an internal standard. Microparticle number was determined by lowering the threshold to 200 with forward scatter and side scatter gates defined by 1-μm beads (Sigma-Aldrich) and 1 μm and smaller events were counted until 50,000 (3-μm) events were recorded.

RNA isolation and real-time RT-PCR

Total RNA from 2.0 × 106 platelets was isolated using RNeasy Mini kits (Qiagen) and treated with RNase-free DNase (Qiagen) before quantitation by NanoDrop to normalize aliquots. Primers for RT-PCR amplification were: human IL-1β mRNA, sense, 5′-AAACCTCTCTGAGGAGCAAG-3′; exon 3, antisense, 5′-GTTTGAGGCATCCAGCCTCA-3′ (exon 3); mouse IL-1β, sense, 5′-CGAGGCTAATGGCTCATCT-3′; antisense, 5′-GGTGGT-GAAGGAGCTCCTCAT-3′. Amplification across intron 2 in this way does not detect unprocessed heteronuclear pro–IL-1β RNA. Real-time RT-PCR examined 40–45 cycles of reverse transcription (50°C, 30 min), PCR (94°C, 15 s; 61°C, 30 s; 72°C, 30 s), and data collection (80°C, 15 s) with SYBR Green I in a Bio-Rad MyiQ Cycler. Results were normalized with real-time PCR of 18S rRNA using Ambion primers. Products were analyzed by melting curve, gel electrophoresis, and sequencing. DNase I treatment did not affect IL-1β mRNA expression, whereas RNase I treatment and reverse transcriptase removal abolished amplification.

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 and phenol-extracted LPS (E. coli 0111:B4) that is free of lipopolysaccharide composition (List Biological Laboratories); recombinant soluble CD14, rLPB, and IL-1β ELISA kits (R&D Systems); Caspase-1 inhibitors z-WEHD-FMK (FMK002) and z-YVAD-FMK (FMK005) (R&D Systems); Alexa Fluor 594–conjugated wheat germ agglutinin (Invitrogen); Alexa Fluor 488–conjugated goat anti-rabbit (Invitrogen, A11034); rabbit polyclonal anti–IL-1β (Abcam, ab59995); isotype allophycocyanin-conjugated mouse IgG1 (BD Pharamingen, 555751); rabbit anti-mouse IL-1β (Abcam, Ab9722); anti-CD14 (eBioscience, 16-0149-82); anti–β-actin (Santa Cruz Biotechnology); goat polyclonal anti–P-selectin (C-20, SC-6941); and recombinant human IL-1R antagonist (IL-1Ra; R&D Systems). Anti-caspase-1 Ab (sc-515) was from Santa Cruz Biotechnology. Other chemicals were from Sigma-Aldrich or Biomatik Research Laboratories. The chimeric peptides (Imgenex) are MyD88, DROIKIWFQNRRMKWKKRDVLPGT, and Traf6 decoy peptide, DROIKIWFQNRRMKWKKRPVTEDEY. Underlined amino acids represent antennapedia sequence.

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 SEMs from all experiments are presented as error bars except for multiple comparisons that used one-way ANOVA. Graphing of figures and statistical analyses were generated with Prism 4 (GraphPad Software). A p value < 0.05 was considered statistically significant.

Results

Platelets express type I IL-1 receptors

Platelets express and respond to LPS by activating the TLR4 receptor that couples to MyD88 (19) through the TIR domain of this receptor. Platelets therefore should be able respond to the related IL-1 receptor were they express the signaling IL-1R1. We explored the possibility that platelets sense the IL-1β they produce by quantifying IL-1R1 receptors on the platelet surface by flow cytometry. We found that human platelets abundantly expressed IL-1R1, but not the receptor IL-1R2 (Fig. 1A). We confirmed the presence of IL-1R1 by resolving proteins in lysates of platelets and monocytes as a positive control by SDS-PAGE and then Western blotting for IL-1R1. The resulting immunoblots showed the appropriate 65-kDa band of IL-1R1 in both cells (Fig. 1B). Reprobing platelet lysates showed that the platelet-specific marker CD42b was uniformly present in platelet lysates, whereas the monocyte marker CD14, which platelets lack, was not detectable in platelet lysates. Conversely, the monocytic cell preparation was largely free of platelet CD42b, but did express CD14. IL-1β stimulates its own formation in platelets

We determined whether IL-1β stimulates its own production in platelets, as in nuclelated cells (7, 8). In platelets, however, this occurs through the splicing introns from stored IL-1β hnRNA and translating the mRNA this creates. Freshly isolated (t = 0) platelets
did not contain spliced pro–IL-1β mRNA, but processed mRNA for this cytokine precursor had accumulated after 3 h in culture even in the absence of overt stimulation (Fig. 2A). rIL-1b significantly increased pro–IL-1β hnRNA processing to spliced mRNA over this background, as did rIL-1a that also stimulates IL-1R1. The dominant-negative family member IL-1Ra (anakinra) that specifically inhibits IL-1R1 activity abolished pro–IL-1β mRNA accumulation after IL-1b or IL-1a stimulation. IL-1Ra also reduced IL-1β production by incubated, but unstimulated, control cells, showing over time that IL-1β production created an autocrine loop that enhanced its own production.

IL-1R1 and TLR4 receptors both recruit MyD88 to their TIR domains, and the cell-permeable antennapedia-MyD88 peptide that interferes with Myd88 homodimerization during LPS stimulation (19) also abolished IL-1β induction of IL-1β RNA splicing (Fig. 2B). As expected, then, both receptors stimulate platelets through MyD88 engagement. LPS is an atypical platelet agonist that fails to induce either a rapid increase in intracellular Ca²⁺ or homotypic platelet aggregation (Supplemental Fig. 1A). E. coli LPS and Salmonella minnesota LPS both activate platelet TLR4 (35), and both forms of LPS activate AKT phosphorylation (Supplemental Fig. 1B) that transduces TLR4 signaling in platelets (19). IL-1β, similar to LPS, also is an atypical agonist that failed to promote platelet degranulation that increases surface P-selectin expression (Supplemental Fig. 1C), nor did LPS stimulate formation of the activated epitope of gpIIb/IIIa recognized by the Ab PAC-1.
Platelet-derived microparticles display active IL-1

We determined whether the IL-1β that platelets generated was available to activate surrounding cells. IL-1β associates with microvesicles shed from nucleated cells (5, 6), and microparticles released from platelets also displayed functional cytokine. We found particles (μ in size) released from platelets maintained in buffer stimulated splicing of the pro–IL-1β hnRNA of quiescent target platelets (Fig. 3A). Activation by these microparticles was abolished when IL-1Ra was added, so activation was through IL-1R1 and so reflects particle-associated IL-1β. Platelets activated with the combination of LPS, rCD14, and rLPB shed microparticles that were more effective in stimulating pro–IL-1β mRNA splicing in naive platelets, and this too was fully inhibited by IL-1Ra. The stimulatory principle in these microparticles was IL-1, and not residual LPS, because the target platelets lacked soluble CD14 and could not respond to LPS carryover.

( Supplemental Fig. 1D), showing that IL-1β and LPS share downstream signaling and responses.

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**FIGURE 3.** Platelet stimulation by LPS or LPS-derived microparticles requires IL-1R1 signaling. (A) Microparticles from LPS-stimulated platelets stimulate naive platelets through IL-1R1. Microparticles from LPS-treated (μLPS) or buffer-treated (μCtrl) platelets were recovered and washed by centrifugation before addition to naive purified platelets before spliced pro–IL-1β mRNA levels were quantified 3 h later by quantitative PCR (n = 3). Significance was attained using a one-way ANOVA. *p < 0.05 for the shown comparison and from control cells. (B) IL-1Ra blockade of IL-1R1 prevents platelet stimulation by LPS. Washed human platelets were treated with buffer, rIL-1β (100 pg/ml), the combination of phenol-extracted E. coli LPS (100 ng/ml) along with rCD14 (100 ng/ml) and LPB (100 ng/ml), or a stimulatory TRAF6 ligand-antennapedia chimeric peptide (5 μg/ml) for 3 h before total RNA was collected and analyzed for spliced pro–IL-1β mRNA by quantitative RT-PCR. Some cells were exposed to the inhibitor IL-1Ra at 150 ng/ml for 30 min before stimulation (n = 5). *p < 0.05.

**FIGURE 4.** LPS platelet stimulation requires IL-1β production. (A) LPS induction of pro–IL-1β hnRNA splicing requires de novo protein synthesis. Purified platelets were treated for 3 h with LPS, rCD14, and rLPB (100 ng/ml each) in the presence or absence of cycloheximide (CHX, 100 μg/ml) prior to quantifying spliced pro–IL-1β RNA by quantitative PCR (n = 3). *p < 0.05 for the shown comparison. (B) Caspase inhibition blocks platelet LPS stimulation. Purified platelets were treated for 3 h with LPS, rCD14, and rLPB (100 ng/ml each, 3 h) in the absence or after 30 min preincubation with caspase-1 inhibitors (z-WEHD-fmk or z-YVAD-fmk, 100 μM) before spliced pro–IL-1β RNA was quantified (n = 3) *p < 0.05 for the shown comparison. (C) IL-1β hnRNA splicing in murine platelets requires caspase-1. Platelets of wild-type or caspase-1−/− animals were stimulated with LPS along with LPB and soluble CD14 (100 ng/ml each) for 3 h, treated with 100 pg/ml human IL-1β, or left unstimulated for 3 h before spliced pro–IL-1β mRNA was quantitated by quantitative PCR (n = 2). Significance was attained using one-way ANOVA. *p < 0.05 for the shown comparison, as well as from control cells.
LPS stimulation requires autocrine IL-1β signaling
Soluble IL-1β was a robust agonist for pro–IL-1β hnRNA splicing and was as effective as LPS (Figs. 2A, 3). Signaling by these agonists, in fact, interacts because these experiments show blockade of the IL-1 receptor with IL-1Ra not only blocked IL-1β stimulation, IL-1Ra also blocked LPS-stimulated pro–IL-1β hnRNA splicing. This surprising outcome elucidates an unexpected role for IL-1R1 in LPS signaling. IL-1Ra was selective in this inhibition of pro–IL-1β hnRNA splicing after TLR4 stimulation because IL-1Ra did not reduce splicing stimulated by a TRAF6 interacting antennapedia peptide that initiates signaling below ligation of TIR domain receptors (Fig. 3B).

LPS activation requires pro–IL-1β processing
Other observations support an essential role for IL-1β in LPS stimulation of platelets. LPS signaling in platelets depends on activation of existing kinases (19), not the stimulated protein synthesis of nucleated cells, yet we found that cycloheximide blockade of de novo protein production abolished splicing induced by LPS that itself does not require de novo protein synthesis (Fig. 4A). Additionally, suppressing active cytokine formation by inhibiting caspase-1 cleavage of pro–IL-1β at Asp116 with either the selective caspase-1 inhibitor z-WEHD-fmk or the less selective z-YVAD-fmk inhibitor also blocked LPS stimulation of platelet hnRNA splicing (Fig. 4B) Caspase inhibition did not suppress IL-1R1 signaling itself because IR-1RA did not block stimulation by rIL-1β. Caspase-1 itself must be activated through the action of a stimulated inflammasome (36) aided by P2X7 signaling. Inhibition of this purinergic receptor with A438079 also reduced pro–IL-1β splicing in a concentration-dependent fashion (Supplemental Fig. 2A).

We confirmed the participation of caspase-1 as an essential component of LPS signaling using platelets isolated from control and caspase-1–null mice. Pro–IL-1β hnRNA splicing in cells maintained in buffer for 3 h moderately increased in both strains, whereas stimulated splicing in response to LPS was absent in platelets

FIGURE 5. IL-1β accumulates in platelets during thrombus maturation. (A) IL-1β accumulates within arterial thrombi over time. Occlusive thrombi were induced by a brief (1 min) ectopic application of 7.5% FeCl3 to carotid arteries followed by sacrifice at the stated times as described in Materials and Methods. Fixed arterial sections were stained with hematoxylin and probed with rabbit nonimmune or rabbit anti-mouse IL-1β (1:250) Ab. Primary Abs were detected with HRP-conjugated anti-rabbit secondary Ab and visualized through formation of brown peroxidase reaction product. (B) Thrombus IL-1β associates with anuclear platelet deposition. Thrombi were induced in carotid arteries as above and harvested at the stated times, fixed, and sectioned before staining platelet membranes with Alexa Fluor 594–conjugated wheat germ agglutinin (red), IL-1β with rabbit anti-mouse IL-1β and Alexa Fluor 488–conjugated goat anti-rabbit (green), or nuclei with DAPI (blue). The control was isotype nonimmune rabbit Ab. Sections were imaged by confocal microscopy, and the images were pseudocolored and overlaid. Original magnification ×63. *p < 0.05.
lacking caspase-1 (Fig. 4C). Conversely, murine platelets of both strains responded to human rIL-1β with stimulated splicing, and this response to IL-1β was insensitive to the loss of caspase-1. We conclude that LPS signaling in platelets depends on the IL-1β it generates to greatly augment the initial TLR4 stimulus. This IL-1β amplification loop is present in both human and murine platelets.

**IL-1β accumulates within sterile thrombi**

We sought to determine whether platelets make IL-1β in vivo. Activated platelets are adherent platelets, but because individual platelets are not easily visualized we examined the large concentration of activated platelets that form thrombi. LPS does not induce thrombosis, so we initiated thrombosis in murine carotid arteries by oxidative damage subsequent to a brief exposure to ectopic FeCl₃. This is the standard model of arterial thrombosis (32) initiated from sterile FeCl₃-induced oxidative damage to the arterial wall. Immunohistochemistry confirmed that IL-1β was absent in early platelet plugs, but it appeared after several hours with a significant increase 3–6 h after thrombosis (Fig. 5A). This cytokine was not uniformly distributed, with strong staining in a central portion of the thrombus, accompanied by lesser reactivity at the periphery of the clot. DAPI staining of nucleated cells revealed few nucleated cells at early times after thrombosis that were not associated with the core of IL-1β deposition. At later times, mononuclear cells infiltrating the thrombus coalesced within areas of IL-1β deposition.

We determined whether the IL-1β within occlusive thrombi was associated with enmeshed platelets by labeling platelets with Alexa Fluor 594–conjugated wheat germ agglutinin and IL-1β with Alexa Fluor 488–labeled Ab. We found (Fig. 5B) focal IL-1β deposition by 1 h after thrombosis, a time when DAPI fluorescence showed that few nucleated cells were present, and even these were not allied with IL-1β positivity. Nucleated cells organized into leukocyte-rich areas 6 h after thrombus formation, which then strongly stained with anti–IL-1β. Platelets stimulated ex vivo translate existing mRNA encoding Bcl3, and we found Bcl3 accumulated within thrombi in parallel with IL-1β accumulation (Supplemental Fig. 2B).

We determined whether thrombus IL-1β was derived from the processing of hnRNA stored in platelets, or from stimulated translation in infiltrating mononuclear cells or vascular cells, by globally inhibiting DNA transcription with actinomycin D prior to thrombus formation. We found that IL-1β protein was present within thrombi 3 h after vascular damage in mice unable to synthesize new RNA by transcription in nucleated cells, and that this IL-1β was associated with platelet P-selectin (Fig. 6). We confirmed that nucleated cells were unable to contribute to IL-1β accumulation in thrombi in mice injected with actinomycin D injection by isolating circulating nucleated cells from these animals and exposing them to LPS ex vivo. We found WBCs from these animals were no longer able to respond to LPS by synthesizing new mRNA for IL-1β (Supplemental Fig. 3). These results show platelets are activated in unconventional ways in vivo, and that IL-1β accumulation marks this activation state.

**Discussion**

We establish that platelets express functional receptors for IL-1, that platelets are stimulated by recombinant soluble IL-1α and IL-1β, and that they are stimulated by IL-1β–containing platelet-derived microparticles. These results establish a new, and functional, platelet receptor that enables platelets to respond to a key inflammatory mediator. We also show that IL-1β synthesis and its subsequent stimulation of IL-1R1 are essential components for platelet activation by LPS. Platelet activation by LPS, in turn, is essential to the septic response (11, 16, 17), indicating this newly identified IL-1β autocrine stimulatory loop underlies platelet participation in sepsis. We also establish that platelets themselves generate IL-1β in recently formed thrombi in close juxtaposition to the vascular wall prior to infiltration of mononuclear cells. Platelets therefore contribute to inflammatory signaling in a sterile setting over times well past their immediate activation by establishing and amplifying an IL-1β signaling loop.

IL-1 is a paradigmatic inflammatory cytokine that activates cells of the innate immune system and cells of the vascular wall through the signaling receptor IL-1R1 (1, 37) that promotes inflammation, angiogenesis, and differentiation of myeloid cell progenitor cells (1). IL-1 also promotes remodeling of atherosclerotic vessels and plaque stability (38). mRNA for this cytokine is not normally present in circulating cells, but it becomes detectable after coagulation (39), connecting thrombosis and inflammation. The source of this cytokine is not defined, although monocytes require hours for mRNA production and then protein synthesis (40) whereas platelet expression from existing RNA is more rapid (27).

We show that platelet IL-1β stimulates its own production, as in nucleated cells (7, 8), but in platelets this results from the unique process of stimulated posttranscriptional splicing and translation of hnRNA in a currently unique process that has been retained during evolutionary times (19, 23, 41). This hnRNA splicing does not require de novo RNA synthesis, and accordingly whereas actinomycin D blocked IL-1β production in cells of the vascular wall and infiltrating monocytes, inhibition of de novo RNA synthesis did not inhibit IL-1β accumulation within thrombi. In addition to local signaling within and immediately adjacent to thrombi, IL-1–activated platelets may also extend inflammatory signaling

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**FIGURE 6.** Accumulation of thrombus IL-1β is independent of transcription. Mice were injected with actinomycin D 30 min prior to initiation of arterial injury by application of 7.5% FeCl₃ to an exposed carotid artery. Anesthetized mice with surgical windows maintained at 37˚C were sacrificed 3 h later, arterial segments were fixed with OTC, and sections were permeabilized by 0.5% Triton X-100 and stained with high concentrations of Alexa Fluor 568 secondary Ab that fluoresces in the green channel (bottom left) and anti–IL-1β detected with Alexa Fluor 488 secondary Ab that fluoresces in the red channel (top right) as nuclei were stained with DAPI mounting media that produce blue nuclear fluorescence (bottom left). These images were then merged (bottom right). Original magnification ×63.
through microparticle shedding. Platelet microparticles can circulate 10-fold longer (42) than the few minutes (43) of soluble IL-1b, and these particles stimulate an inflammatory response in endothelial cells (19, 25). Microparticles activate naïve platelets through the IL-1R1 they express, and IL-1–containing microparticles activate endothelium (19, 25), so platelet microparticles carrying IL-1 have the potential to extend immune signaling throughout the vasculature.

Unexpectedly, blocking the platelet IL-1 receptor with IL-1Ra blocked most, but not all, LPS stimulation. Because IL-1Ra is a natural and specific inhibitor of IL-1b and IL-1α receptor signaling (44), this outcome indicates that LPS signaling in platelets employs the IL-1 receptor. Flow cytometry shows that IL-1R1 is abundant on platelets relative to monocytes, where IL-1 is an established agonist, so potentially IL-1R1 abundance on platelets augments signaling from less abundant TLR4. Confirmation of the contribution of IL-1 signaling to LPS signaling in platelets came from the requirement of de novo protein synthesis, which is not required for TLR4 signaling in platelets (19), from caspase 1 inhibition of pro–IL-1β proteolysis, and from loss of LPS signaling in caspase-1−/− mice.

We show that IL-1α and IL-1β can masquerade as endogenous TLR4 ligands (45, 46), producing sterile inflammation by entering and greatly activating the pathway below LPS ligation of TLR4. These observations in combination define a new route from the rapid thrombotic response induced by acute vascular damage to a chronic inflammatory stimulation promoted by IL-1β.

Acknowledgments

We are grateful to Dr. Ariel Feldstein and Laura Dickson for the gift of caspase-1−/− murine blood and to Dr. George Dubyk (Case Western Reserve University) for reagents and cogent discussions. We are especially grateful to Judy Drzaba and the Digital Imaging Core for efforts. We also appreciate the advice of Anne Cotule and Sage O’Bryant of the Lerner Research Institute Flow Cytometry Core, the efforts of Rui Chen and Erin Brady, and our many blood donors.

Disclosures

The authors have no financial conflicts of interest.

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