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# Frontline Science: Anthrax lethal toxin-induced, NLRP1-mediated IL-1$\beta$ release is a neutrophil and PAD4-dependent event

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## Abstract

Anthrax lethal toxin (LT) is a protease that activates the NLRP1b inflammasome sensor in certain rodent strains. Unlike better-studied sensors, relatively little is known about the priming requirements for NLRP1b. In this study, we investigate the rapid and striking priming-independent LT-induced release of IL-1$\beta$ in mice within hours of toxin challenge. We find IL-1$\beta$ release to be a NLRP1b- and caspase-1-dependent, NLRP3 and caspase-11-independent event that requires both neutrophils and peptidyl arginine deiminiase-4 (PAD4) activity. The simultaneous LT-induced IL-18 response is neutrophil-independent. Bone marrow reconstitution experiments in mice show toxin-induced IL-1$\beta$ originates from hematopoietic cells. LT treatment of neutrophils in vitro did not induce IL-1$\beta$, neutrophil extracellular traps (NETs), or pyroptosis. Although platelets interact closely with neutrophils and are also a potential source of IL-1$\beta$, they were unable to bind or endocytose LT and did not secrete IL-1$\beta$ in response to the toxin. LT-treated mice had higher levels of cell-free DNA and HMGB1 in circulation than PBS-treated controls, and treatment of mice with recombinant DNase reduced the neutrophil- and NLRP1-dependent IL-1$\beta$ release. DNA sensor AIM2 deficiency, however, did not impact IL-1$\beta$ release. These data, in combination with the findings on PAD4, suggest a possible role for in vivo NETs or cell-free DNA in cytokine induction in response to LT challenge. Our findings suggest a complex interaction of events and/or mediators in LT-treated mice with the neutrophil as a central player in induction of a profound and rapid inflammatory response to toxin.

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AUTHORSHIP

A.J.G. and M.M. designed studies, performed experiments, analyzed data, and wrote the paper. M.K.P., N.K.M., and M.A.M. designed studies, performed experiments, analyzed data, and edited the paper. D.O. and D.C. performed animal studies. S.H.L. contributed vital reagents, revised and edited the paper.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

**Keywords**

inflammasome; IL-18; pyroptosis; NETs; IL-1β

## 1 | INTRODUCTION

Inflammasomes are large, multiprotein complexes that form in the cytosol as a platform for activation and release of proinflammatory cytokines in response to pathogen- or damage-associated molecular patterns (PAMPs, DAMPs). This cytokine release is often accompanied by a rapid cell death (pyroptosis). Inflammasome sensors oligomerize to recruit and activate the caspase-1 or caspase-11 cell death proteases, which in turn cleave pro-IL-1β and pro-IL-18 to their biologically active forms (for reviews see [1, 2]). PAMP activation for release of cytokines by inflammasomes is a two-step process. It usually requires a priming "signal 1," often a TLR/NF-κB-activating signal, leading to upregulation of pro-IL-1β and inflammasome components, followed by a sensor-activating "signal 2." Signal 2 can vary in nature among inflammasome sensors, from direct binding to post-translational modification. The NAIP/NLRC4 inflammasome, for example, can receive both a TLR-activating signal 1 and a subsequent direct-binding activation (signal 2) from the same flagellin molecule.[3] Alternatively, the NLRP3 inflammasome can be activated by pore-forming toxins, noninfectious danger signals, particulate/crystalline matter, or agents inducing ion flux events, leading to a "sterile inflammation" that is important in diseases such as atherosclerosis or neuroinflammatory conditions. This activation still requires priming, which in the absence of classic TLR signals has been suggested to occur through reactive oxygen species, hypoxia, metabolites, complement, or other pathways. In some inflammatory conditions, activation may occur by mechanisms independent of transcriptional priming (for review see [4]).

NLRP1b is a sensor of pathogen effector proteins that induce degradation of its N-terminus by the proteasome and release of an autoproteolytically-generated, noncovalently associated C-terminal CARD domain. The best-studied activator of this inflammasome, the anthrax lethal factor protease (LF), cleaves the N-terminus of NLRP1b to initiate destabilization of the protein through ubiquitylation by an N-end rule E3 ubiquitin ligase.[5–8] Lethal toxin (LT), a major virulence factor of *Bacillus anthracis*, is an AB toxin comprised of two proteins, the receptor-binding protective antigen (PA), which binds receptors and transports the second component, LF, to the host cell cytosol, where it cleaves its substrates. Anthrax toxin receptors and LF's mitogen-activated protein kinase kinase (MEK) substrates are ubiquitously expressed in most mammalian cell types. While the cleavage of NLRP1b by LF is limited to select rat strains[9] and all tested mouse strains, MEK cleavage appears to occur in any cell harboring PA receptors. Interestingly, in some mouse strains, LF cleavage of NLRP1b does not activate the sensor, likely due to other polymorphisms in the sensor protein or possible negative regulatory components unique to those strains (for review see [10, 11]).

Little is known about the priming requirements for NLRP1b. In contrast to activation of the NLRP3 sensor,[12] LT-induced, NLRP1b-mediated pyroptosis of macrophages and dendritic

cells requires no priming or sensor up-regulation. If IL-1$\beta$ and IL-18 are present, LT can activate their release from rodent macrophages that express LT-responsive (sensitive) NLRP1b (designated NLRP1b$^S$ or NLRP1$^S$), whereas other macrophages expressing resistant sensors (NLRP1b$^R$ or NLRP1$^R$) only succumb to the consequences of LT cleavage of MEKs and other unknown substrates. Mouse peripheral blood monocytes, macrophages and bone marrow-derived macrophages (BMDMs), unlike human monocytes, only express pro-IL-18 at steady-state, and do not express pro-IL-1$\beta$. Thus, in mice IL-1$\beta$ release by these cells generally requires a priming event.[13] We and others have shown LT will not induce any IL-1$\beta$ from macrophages without TLR priming in vitro, whereas IL-18 activation and release can be mediated by signal 2 alone.[14,15]

In this report, we investigate a rapid and striking LT-induced release of IL-1$\beta$ in vivo within hours of toxin challenge. This “sterile” inflammation in the absence of classic TLR signaling was found to be NLRP1b- and caspase-1-dependent, caspase-11-independent, and surprisingly, also dependent on neutrophils and PAD4 activity. Importantly, we show that while the in vivo cytokine response originates from a hematopoietic compartment, LT is not capable of upregulating or activating IL-1$\beta$ release or inducing formation of NETs from murine neutrophils in vitro. LT also does not cause neutrophil pyroptosis, despite its ability to bind and enter neutrophils and cleave its MEK cytosolic substrates. These findings suggest a complex interplay among different cells, events, and mediators in vivo that cannot be recapitulated in a reductionist system ex vivo. This study highlights the importance of understanding downstream consequences of inflammasome activation in a variety of cell types in vivo, and describes a unique, rapid series of inflammasome-dependent events in which the neutrophil plays a central, nonredundant role in mice in the initiation of a very rapid, yet potentially multicellular complex “sterile” inflammatory response to a bacterial toxin.

## 2 | METHODS

### 2.1 | Toxins, spore, and irradiated TB

Endotoxin-free LF, active-site nonproteolytic mutant LF (E687C), PA, and FP59 (AGG variant) were purified from *B. anthracis* as previously described.[16,17] FP59 is a fusion of the receptor-binding N-terminus of LF to the enzymatic domain of *Pseudomonas* exotoxin A and inhibits protein synthesis upon entry into the mammalian cytosol.[17] LF used in all studies has an amino acid sequence of “HMAGG” at its N-terminus, except where indicated as LF-OS or LT-OS, where the sequence is “AGG.” These LF preparations have different potencies in vivo,[18] which are reflected by different doses used in some experiments, as the final HMAGG preparations in this laboratory have been depleted. LF and PA batches were tested for TLR activation by overnight incubation on RAW264.7 macrophage-like cells and assessment of TNF$\alpha$ protein levels or induction of IL-1$\beta$ transcript. PA was labeled for flow cytometry with the DyLight-650 Fast Conjugation Kit (Abcam, Cambridge, MA), using 100 μg fluorophore per 100 μg PA, following manufacturer protocols. Concentrations of LT refer to equal concentrations of PA and LF (i.e., 1 μg/ml LT is a mixture of LF and PA, each at 1 μg/ml).

The nonencapsulated toxigenic *B. anthracis* Sterne-like strain A35 was used for mouse infections.[19] To prepare spores, A35 bacteria were grown on nutrient broth-yeast (NBY) sporulation agar at 37°C for 24 h, followed by 4–6 days at either room temperature or 30°C, with inspection by microscopy on days 4–6 to verify >90% sporulation. Plates were washed with 5–10 ml of cold sterile water to dislodge spores. After 4 washes in 30 ml cold water per plate, spores were heat-treated at 75°C for 1 h to kill any remaining vegetative bacteria. Spore quantification was performed using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) and verified by dilution plating. Irradiated, nonviable *Mycobacterium tuberculosis* H37Rv (TB) was sonicated using a cup-horn sonicator and used at 50 μg/ml.

## 2.2 | Mice

Inbred mice were purchased from Jackson Laboratories (Bar Harbor, ME). $C57^{Nlrp1b\ R/R}$ and $C57^{Nlrp1b\ S/S}$ are congenic mice created by backcrossing of previously described $C57BL/6NTac^{R/R}$ and C57 BL/6 $NTac^{S/S}$ mice[20] to C57BL/6J mice for 11 generations, with genotyping to maintain the $Nlrp1b^{R}$ (C57BL/6J origin) or $Nlrp1b^{S}$ (129 origin) loci. $129^{Nlrp1-/-}$ and $129^{Nlrp1+/+}$ mice were a gift of Seth Masters, created similarly to previously described *Nlrp1* knockout mice.[21] These mice lack all 3 *Nlrp1* alleles ($Nlrp1a^{-/-}$, $Nlrp1b^{-/-}$, $Nlrp1c^{-/-}$). RAG and SCID knockout mice on the BALB/cJ background and *Caspase-1/11, IL1R, Nlrp3, Aim2, Elane (Neutrophil elastase* [NE]), *Pad4*, and *Ccr2* knockout mice on the C57BL/6J background were purchased from Jackson Laboratories, crossed to $C57^{Nlrp1b\ S/S}$ mice, and intercrossed to establish colonies of mice homozygous for knockout of the gene of interest and for $Nlrp1b^{S}$ alleles. $Il1b^{-/-}$ and $Il1a^{-/-}/Il1b^{-/-}$ mice were originally derived by Y. Iwakura (Tokyo University) and crossed to the $C57^{Nlrp1b\ S/S}$ background. For Jackson Labs, mice all genotyping was performed using primers and protocols published at www.jax.org. For all other mice, primers used for genotyping in crosses are shown in Supplemental Table S1.

For irradiations, mice 9–14 weeks old were subjected to a lethal irradiation dose of 946–1000 Rads over 6–9 min, injected i.v. with $3–4 \times 10^7$ donor bone marrow cells 4–6 h post-irradiation, and maintained on trimethoprim sulfamethoxazole (TMS) antibiotics for 6 weeks prior to toxin injections at week 8–10. For antibody depletion studies, anti-mouse Ly6G clone 1A8, anti-mouse Ly6G/Ly6C (Gr-1) clone RB6-8C5, anti-mouse platelet CD42b clone R300, and control antibody anti-keyhole limpet hemocyanin, clone LTF-2 (BioXCell, West Lebanon, NH; Emfret Analytics , Wurzburg, Germany; or BD Biosciences, Franklin Lakes, NJ) were injected at 50–100 μg (R300) or 100–200 μg (all others)/mouse by i.p. route, 24 h prior to toxin treatments or studies as indicated in figure legends. Dornase Alfa (Pulmozyme®) (Genentech, San Francisco, CA) was a kind gift from Dr. Daniel Barber (NIAID) and injected at 400 μg/mouse at 18 and 2 h before toxin injections. Cardiac puncture or submandibular terminal bleeds in mice for cytokine analyses were performed into serum micro-tainer tubes (BD Life Sciences, Franklin Lakes, NJ). ForCBC analyses or circulating DNA assessment, cell-free plasma EDTA bleeds were used (EDTA vacutainer tubes; BD Life Sciences). For DNA measurements, 400 μl plasma/mouse/group ($n = 3$/group) were pooled before processing using the DNeasy blood kit (Qiagen, Germantown, MD) according to the manufacturer's protocol, with elution in 40 μl of elution buffer/

column. For peripheral blood cell isolation or whole blood treatments, mice were bled with 4% citrate anticoagulant or using heparin coated syringes into heparin vacutainer tubes (BD Life Sciences). Animal studies were performed in accordance with guidelines from the National Institutes of Health and the Animal Welfare Act, on protocols approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

### 2.3 | ELISAs

Cytokine ELISAs were performed using murine IL-1$\beta$, IL-18, or TNF$\alpha$ kits from R&D Systems (Minneapolis, MN) or Invitrogen/eBioscience kits from Thermo Fisher (Waltham, MA) according to manufacturers' protocols. High Mobility Group Box-1 (HMGB1) was measured with a kit from G-biosciences (St. Louis, MO).

### 2.4 | Cells, Western blot, and flow cytometry

RAW264.7 and BMDMs were cultured as previously described.[14] For isolation of highly purified neutrophils from peripheral blood, groups of 5–6 mice per treatment were bled in 4% citrate, and erythrocytes removed by hypotonic lysis in ACK buffer. Cells were subjected to magnetic separation by negative selection using a MACS® mouse neutrophil isolation kit (Milteny Biotec, Auburn, CA) according to manufacturer protocols, achieving purity of 98% as assessed by flow cytometry. Bone marrow neutrophils (BMNs) were isolated either using the same kit, to >98% purity by flow cytometry, or by density gradient using Histopaque 1119/Histoapque 1077 as previously described, to 94% purity.[22] Platelet-rich plasma was isolated by bleeding 8 mice into 4% sodium citrate buffer containing indomethacin (10 μg/ml) (Sigma, St. Louis, MO), and prostaglandin E1 (PGE1, 1 μM) (Cayman Chemicals, Ann Arbor, MI) at roughly 1:9 (bleed:buffer) ratio, followed by 100x$g$ spin for 10 min to remove plasma, and 400x$g$ to pellet platelets and resuspend as needed.

Viability assays were performed by flow cytometry using viability dyes described in the next section, by MTT (Sigma) staining as previously described,[14] or using NucGreen Dead 488 ReadyProbes® (ThermoFisher/Invitrogen, Pittsburgh, PA) to test for membrane integrity. NucGreen Dead staining was performed using dye diluted 1:50 in PBS added to cells for 5 min, followed by fluorescent microscope image collection on a Nikon Eclipse TE-2000U (Nikon USA, Melville, NY). For assessment of NET formation, purified neutrophils were plated on 10 mm sterile poly-L-lysine treated coverslips and the same NucGreen 488 staining method was utilized following various treatments. The following antibodies and reagents were utilized with standard flow cytometry staining practices: BD CytoFix/CytoPerm (BD Biosciences) FluroFix Buffer (BioLegend, Deadham, MA), Ultra-Comp eBeads (eBioscience/ThermoFisher), CD16/CD32 (mouse Fc Block) (BD Pharmingen; 1:100), PacBlue-anti-Ly6G (BioLegend; 1:500), PE-anti-Ly6G (Biolegend; 1:2000), Alexa488-anti-CD11b (Biolegend; 1:100), PE-anti-CD41 (MWReg30) (Biolegend; 1:100), PECy7 Anti-CD68 (eBioscience/ThermoFisher; 1:300), APC-eFLuor®780 anti-mouse pro-IL-1$\beta$ (eBiosceince/ThermoFisher; 1:500). Live/dead staining was done using Fixable Viability Dye eFLuor 780 (eBioscience/ThermoFisher, 1:1000). For Western blots (WBs), BMDMs, BMNs, or RAW264.7 cells were treated with or without LPS as indicated in figure legends, followed by LT (1 μg/ml) for various times and lysis in RIPA buffer

containing complete, EDTA-free protease inhibitor (Roche Diagnostics, Indianapolis, IN) and 5 ng/ml LF inhibitor PT-168541-1 (gift of Alan Johnson, Hawaii Biotech). Primary antibodies against mouse IL-1$\beta$ (R&D Systems), $\beta$-Actin (Cell Signaling, Danvers, MA), MEK3 (Santa Cruz Biotech, Dallas, TX) and MEK1 N-terminus (United States Biological, Salem, MA) were purchased and used at 1:1000 dilution. Infrared (IR)-dye conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE) were used at 1:20,000 and gel imaging was performed on LI-COR Odyssey or CLx Infrared Imaging Systems (LI-COR Biosciences). Blots were analyzed using Image Studio software (LI-COR Biosciences).

## 3 | RESULTS

### 3.1 | Anthrax LT induces a caspase-1-dependent, caspase-11-independent IL-1$\beta$ response that requires LT-sensitive NLRP1b in a hematopoietic compartment and controls resistance to spore infection

All sequenced mouse NLRP1b proteins contain an N-terminal LF cleavage site[23,24] and all previously tested variants are cleaved by the toxin.[25,26] Only some NLRP1b variants, such as those found in BALB/c or 129 inbred strains, are LT-sensitive (NLRP1b$^S$), such that their cleavage by LF leads to activation of the inflammasome and rapid pyroptosis of macrophages and dendritic cells. Other NLRP1b variants, like those found in C57BL/6J or NOD/ShiLtJ mice, are also cleaved by LF but this does not activate the inflammasome, so these LT-resistant variants (NLRP1b$^R$) do not support macrophage pyroptosis. The *Nlrp1b* allele S/R status does not have a significant impact on animal susceptibility to LT, as congenic C57BL/6 mice expressing either NLRP1b$^S$ or NLRP1b$^R$ succumb similarly to challenge with LT within 1 week ([27, 28] and Fig. 1A). Furthermore, deletion of the *Nlrp1$^{abc}$* locus does not result in a significant change in susceptibility to purified toxin (Fig. 1B). This is likely due to the fact that LT-induced lethality in mice is primarily mediated by the toxin targeting of smooth muscle cells and cardiomyocytes through unknown mechanisms, as knockout of the PA receptor on myeloid cells has little impact on LT lethality.[29,30] In congenic mice that differ in NLRP1b responsiveness, the slightly more rapid death of NLRP1b$^S$ mice (Fig. 1A) is possibly linked to inflammatory mediators released following macrophage pyroptosis,[31] but there are also NLRP1b$^S$-expressing inbred strains that succumb more slowly to toxin than NLRP1b$^R$-expressing strains, suggesting other genetic loci impact toxin susceptibility.[32]

Sensitivity to infection with *B. anthracis* spore, on the other hand, shows significantly different outcomes in congenic mice expressing NLRP1b$^S$ vs. NLRP1b$^R$ proteins ([20] and Fig. 1C) as well as when the *Nlrp1$^{abc}$ locus* is knocked out (Fig. 1D). Unlike for challenge with purified LT, NLRP1b$^S$ confers resistance against lethal spore challenge. The resistance associated with the LT-responsive NLRP1b$^S$ proteins is likely due to a rapid cytokine response associated with activation of the inflammasome sensor.[20,33] The earliest cytokine released following toxin challenge is IL-1$\beta$, and knockout of IL-1 receptor or caspase-1/11 in mice expressing NLRP1b$^S$ proteins results in increased sensitivity to spore infection (Supplemental Fig. 1 and [20]). NLRP1b$^S$-expressing mice show high levels of IL-1$\beta$ release within 2 h of toxin challenge and levels continue to increase through 6 h, whereas mice epxressing NLRP1b$^R$ have no IL-1$\beta$ response (Fig. 2A). Similarly, IL-18

release is strictly correlated to NLRP1b status (Fig. 2F). The cytokine response is dependent on LF entry into the cytosol and requires its enzymatic activity, as catalytically inactive toxin and PA or LF alone do not induce the response (Fig. 2B). This also indicates that the toxin components do not contain impurities that cause independent induction of the response in vivo. The response is dependent on NLRP1b and caspase-1 (Figs. 2C and G) but not on caspase-11, as *caspase-1/11* knockout mice lose the cytokine response (Figs. 2C and G), whereas the naturally caspase-11-deficient NLRP1b$^{S}$-expressing 129 strain[34] has robust IL-1$\beta$ and IL-18 responses to toxin challenge (Figs. 2C–E and H). NLRP1b sensor response in a hematopoietic compartment is required for cytokine release, demonstrated by bone marrow reconstitutions between MHC-compatible 129 and C57 mice (Figs. 2D, H, left panels and E), as well as between congenic C57$^{Nlrp1b\ S/S}$ and C57$^{Nlrp1b\ R/R}$ mice (Figs. 2D and H, middle panels) or *Nlrp1* knockout and wild-type (WT) littermates on a 129 background (Figs. 2D and H right panels). Interestingly, the 129 to 129 reconstituted mice always had a higher level of IL-1$\beta$ release than nonirradiated 129 mice after toxin challenge (Figs. 2D and E), with no baseline IL-1$\beta$ release as a result of irradiation alone (data not shown). This suggested an increase in pro-IL-1$\beta$ levels or in sensitivity of a cellular compartment post-irradiation in this strain that allows for a higher LT-induced cytokine response. The IL-1$\beta$ response was robust by 2 h for both i.p. and i.v. routes of toxin administration (Fig. 2E), suggesting a rapid, unknown signal 1-like event or release of transcription/translation independent stores of IL-1$\beta$ in a caspase-1-dependent manner.

### 3.2 | Neutrophils are required for the LT-induced IL-1$\beta$ response in mice

Our previous studies demonstrated that resistance to anthrax spore infections requires a rapid IL-1R signaling-dependent neutrophil infiltration after spore germination in mouse skin.[20] It was previously thought that the IL-1$\beta$ cytokine response to LT is a direct result of monocyte or macrophage activation and pyroptosis. However, we and others have found that mouse monocytes and BMDMs require TLR priming for upregulation of pro-IL-1$\beta$ and LT is not able to induce pro-IL-1$\beta$ expression in these cells[14,15] (Supplemental Fig. 2A) although it can activate the inflammasome to induce cleavage of LPS-induced IL-1$\beta$ (Supplemental Fig. 2B). In the case of the *B. anthracis* bacterium, a variety of TLR activators may act as signal 1 during infection, but LT itself does not activate TLR signaling. In fact, LT's robust inhibition of multiple MEK pathways blocks NF-$\kappa$B-mediated upregulation of IL-1$\beta$ in BMDMs, unless TLR priming occurs prior to LT treatment (Supplemental Figs. 2C and D). As BMDMs are uniquely sensitive to TLR activators, and LT is unable to induce IL-1$\beta$ in these cells, we hypothesized that the toxin also acts on another, heretofore-unidentified cell type that contains steady-state pro-IL-1$\beta$, ready to be released upon NLRP1b inflammasome activation. Because LT is known to induce a strong NLRP1b$^{S}$-dependent neutrophilic recruitment, we depleted mice of neutrophils and were surprised to find the LT-induced IL-1$\beta$ response, but not the IL-18 response, was almost completely abrogated (Figs. 3A and B). However, treatment of mouse whole blood in vitro with the toxin for up to 24 h never resulted in any IL-1$\beta$ release (Supplemental Fig. 2E), suggesting toxin components could not provide both priming and activation signals to a circulatory cell in vitro. Thus, neutrophils, while necessary in vivo, were not sufficient for IL-1$\beta$ release from circulatory cells ex vivo.

### 3.3 | LF is translocated into neutrophils but does not activate the inflammasome or induce pyroptosis

The results in Fig. 3 suggested that LT induces IL-1$\beta$, activates the inflammasome, and results in IL-1$\beta$ release from neutrophils, whereas in parallel, premade stores of IL-18 may be released from pyroptosing monocytes, as treatment of mice with antibody RB6-8C5 (anti Ly6G/Ly6C), which can deplete neutrophils (Ly6G+) but also subpopulations of Ly6-expressing dendritic cells, lymphocytes, and monocytes, resulted in a 20–25% decrease in IL-18 levels (data not shown). When we first observed this neutrophilic requirement for IL-1$\beta$ release, few reports of inflammasome activation in, or IL-1$\beta$ release from, neutrophils existed. There have since been many studies on inflammasome activation in human neutrophils. However, in our hands, and as shown by others, highly purified mouse neutrophils have little-to-no detectable caspase-1 (data not shown and [35]), and very low intracellular or released IL-1$\beta$ levels, even after stimulation with various inflammasome activators.[36,37] We tested if LT creates in vivo conditions for neutrophils to up-regulate IL-1$\beta$ by intracellular staining for the cytokine in Ly6G+ circulating cells from mice treated with LT or LPS and compared the response to highly pure bone marrow-derived neutrophils (BMNs) treated similarly. Staining showed that LT treatment in animals increased neither the percentage of circulating IL-1$\beta$+ cells nor mean fluorescence intensity (MFI) when compared with LPS-treated animals (Fig. 4A). While purified BMNs had a high baseline level of *IL-1b* mRNA (Fig. 4B), treatment of these cells with LT did not induce IL-1$\beta$ protein expression (Supplemental Fig. 2F). Treatment of NLRP1b$^S$-expressing RAW264.7 macrophages or BMDMs with LT induced significant IL-1$\beta$ protein upregulation and cleavage only after LPS priming ([14] and Fig. 4C, Supplemental Figs 2A, B, F, and I). IL-1$\beta$ protein was often difficult to detect in similarly treated purified BMNs by WB, even after LPS treatment (Supplemental Fig. 2F). On rare instances where the cytokine was detectible by WB, levels were at <2–10% relative to same number of similarly stimulated BMDMs (Fig. 4C). The lower level of pro-IL-1$\beta$ in LT-treated BMNs relative to LPS-treated BMNs (Fig. 4C) could be interpreted as resulting from a more efficient cleavage following inflammasome activation. However, secreted IL-1$\beta$ was also not detectible by ELISA following BMN treatment with LPS, LT, or LPS followed by LT (Supplemental Figs. 2G–I). Treatment with irradiated Mtb extract resulted in a modest IL-1$\beta$ response, but 20-fold less than that released by LPS-primed BMDMs (Supplemental Figs. 2G–I). Unlike for macrophages, LT treatment of NLRP1b$^S$-expressing neutrophils did not result in pyroptosis and death within 2 h, and even by 6 h only very low levels of cell death were observed relative to PA-treated controls (Figs. 4D, E, F, and D). To ensure that resistance to LT in neutrophils was not due to any receptor or uptake issues, we treated neutrophils with PA and FP59, a toxin made by fusion of LF's N-terminus and the catalytic domain of *Pseudomonas* Exotoxin A. FP59 requires PA binding for its uptake and delivery to the cytosol in order to kill cells by apoptosis following inhibition of protein synthesis. Cell death occurs over 18–40 h, depending on the cell type. We found BMNs were highly sensitive to PA+FP59, although under the same conditions they did not respond to LT (Fig. 4G). The translocation of proteolytically active LF to the neutrophil cytoplasm was also demonstrable by its ability to cleave the MEK1 substrate (Fig. 4H). Thus, we found that despite high IL-1$\beta$ transcript levels in neutrophils, LT cannot induce and activate IL-1$\beta$ protein in neutrophils as it can in BMDMs. These in vitro findings led us to hypothesize that neutrophils, while required for

in vivo events to systemically induce and release IL-1$\beta$, are unlikely to be the direct cellular source of IL-1$\beta$ in LT-treated animals.

### 3.4 | Platelets cannot take up LF and their depletion does not inhibit toxin-mediated IL-1$\beta$ release

Platelets have been reported to have very high levels of unprocessed IL-1$\beta$ RNA transcripts and are uniquely able to splice and rapidly produce mRNA and secrete the cytokine.[38–40] They have also recently been shown to have functional inflammasome components.[41,42] Inflammatory conditions and changes in vasculature, a hallmark of LT treatment in mice, which has been associated with neutrophil margination at the endothelium[43] results in close association of neutrophils and platelets. We hypothesized that the 1A8 antibody binding and removal of neutrophils could induce conditions that alter platelet levels or their ability to respond to LT. Previous conflicting reports exist on LT effects on platelets.[44–46] We first tested whether the PA receptor is expressed on platelets and if LF can enter platelets. We found no mRNA for PA receptors in platelets (data not shown) and, accordingly, that the toxin is unable to bind to platelets, as measured with fluorophore-labeled PA by flow cytometry (Fig. 5A). Furthermore, the MEK3 LF substrate, which is highly expressed in platelets, is not cleaved upon LT treatment, supporting the conclusion that despite some reports that LT can act directly on platelets, LF cannot enter these cells (Fig. 5B). Platelets treated with LPS and LT did not show induction in IL-1$\beta$ protein (Fig. 5B). Finally, depletion of platelets from mice also did not impact the LT-induced IL-1$\beta$ (Fig. 5C) or IL-18 response (data not shown) in vivo. However, despite the inability of LT to target platelets in vitro, LT treatment of NLRP1b$^S$ mice rapidly altered circulatory neutrophil and platelet numbers, suggesting possible in vivo events that involve both cells. Interestingly, while LT induces a robust neutrophilia at 6 h in NLRP1b$^S$ mice that is not seen in NLRP1b$^R$ mice, we see an inverse effect on platelets, such that platelets are decreased in LT-challenged NLRP1b$^S$ mice (Figs. 5D and E). This suggested a possibility of toxin induction of platelet-neutrophil aggregates over time, although such aggregates were not easily noted in plasma by flow cytometry. Because LT targets endothelial and smooth muscle cells, major changes to the vasculature following toxin challenge could contribute to the observed platelet loss, but the event also requires mediators released in an NLRP1b$^S$-dependent manner.

### 3.5 | LT-induced IL-1$\beta$ originates from the hematopoietic compartment independently of NLRP3, inflammatory monocytes, and lymphocytes

We next hypothesized that LT-induced monocyte pyroptosis generates DAMPs such as ATP, which can act as a signal 2 for other inflammasome sensors, leading to “sterile” IL-1$\beta$ release. Under this hypothesis, LT-activated neutrophils in vivo could play a role in generating signal 1 for such sensors, such as NLRP3. We found that *Nlrp3* knockout in a *Nlrp1b*$^S$ background did not result in significantly different cytokine responses compared with WT mice (Fig. 6A and Supplemental Fig. 3A). While there was a minordecrease in response when NLRP3 was absent in the nonhematopoietic compartment, it was not comparable to the effects of loss of NLRP1b in the hematopoietic compartment (Fig. 6A). Interestingly, testing of *IL-1a*$\beta$ double-knockout mice showed that IL-1$\beta$ was primarily originating from the hematopoietic compartment, eliminating the possibility that NLRP3 activation in epithelial or endothelial cells results in IL-1$\beta$ release from the

nonhematopoietic compartment (Fig. 6B). IL-18 release was primarily but not completely from the hematopoietic compartment and absolutely required hematopoietic NLRP1b$^S$ and caspase-1 (Supplemental Fig. 3B). Finally, to test whether inflammatory monocytes recruited in response to an LT-induced, neutrophil-generated stimulus were possibly the source of IL-1$\beta$, we crossed CCR2 knockout mice deficient in monocyte chemotaxis to the *Nlrp1b*$^S$ background and found no significant impact on IL-1$\beta$ release when challenged with LT (Fig. 6C) and only a slight reduction in IL-18 release (Supplemental Fig. 3C). Similarly, commercially available RAG and SCID mice on the BALB/c (*Nlrp1b*$^S$) background eliminated a role for cells of the lymphocytic lineage (Fig. 6D).

### 3.6 | LT-induced IL-1$\beta$ production is independent of NE but reduced in PAD4-deficient mice and after in vivo treatment

We hypothesized that although neutrophils appear not to be the in vivo source of IL-1$\beta$, they may be a key mediator providing a sterile “alarm” signal for other hematopoietic cells. Along these lines, we considered the ability of neutrophils to produce neutrophil extracellular traps (NETs). NETs are large, web-like DNA structures coated with histones and neutrophilic enzymes expelled by neutrophils in response to many stimuli. They have been shown to play a role in many aspects of the innate immune response (for recent reviews, see [47, 48]). Recently, the noncanonical caspase-11 inflammasome and gasdermin D (the caspase-1/11 substrate required for membrane pore formation and pyroptosis) were implicated in NET formation in response to inflammasome activation.[35,49]

Both neutrophil elastase (NE) and peptidyl arginine deiminiase-4 (PAD4) have been implicated in NET formation,[50–53] although there is disagreement on the conditions and requirements for their involvement (for reviews, see [47, 48, 54]). PAD4 is extensively studied in the context of NET formation, where it mediates citrullination of histones, disrupts nucleosomes and allows chromatin unspooling and NET formation (for review see [55]). NE is also known to directly cleave extracellular IL-1$\beta$ at tissue damage sites[56,57] although reports show this processing may actually inactivate the cytokine.[58] We tested NE and PAD4 knockout mice on the *Nlrp1b*$^S$ background and found NE knockouts had similar LT-induced IL-1$\beta$ release relative to WT littermates (Fig. 7A). PAD4 knockout mice had a significantly reduced response, and the requirement for PAD4 was in the myeloid compartment (Fig. 7B). LT-treated animals also had higher levels of circulating DNA in plasma (Fig. 7C), possibly due to the rapid pyroptosis of monocytic and dendritic cells and not necessarily accompanied by neutrophil NET generation or death. Supporting the idea that DNA release is mediated by pyroptosis, we found high levels of the nonhistone protein HMGB1 in LT-treated NLRP1b$^S$ mice (Fig. 7D). We asked if cell-free circulating DNA was acting as a sterile alarmin DAMP by activating the AIM2 inflammasome, a DNA sensor that can sense dsDNA released from necrotic cells[59] and if this inflammasome was responsible for the robust cytokine activation and release. AIM2 knockout mice on the *Nlrp1b*$^S$ background had no change in their cytokine response compared to WT mice (Fig. 7B). We next treated mice with a DNase to test if depletion of cell-free DNA could reduce the IL-1$\beta$ response. Dornase Alfa (Pulmozyme®), a clinical grade DNase used for treatment of cystic fibrosis patients led to a reduction of IL-1$\beta$ upon LT treatment, with IL-1$\beta$ levels approaching that of 1A8 neutrophil-depleted mice (Fig. 7E). Interestingly, all

other tested nonclinical commercial grade DNases increased the cytokine response to LT (data not shown), suggesting care should be taken in selection of these reagents for in vivo studies. Exhaustive attempts to observe NET formation following LT treatment of purified BMNs in vitro showed the neutrophil response to LT was not different than that to PA up to 6 h post-stimulation with various buffer conditions (data not shown and Fig. 7F). The images in Fig. 7F represent the highest level (25%) of background BMN death at 6 h of toxin treatment, in a manner independent of LF activity, with a similar response in PA-treated cells. In almost all experimental variations, neutrophil death at 6 h of LT treatment did not exceed levels seen for 3 h of treatment in Fig. 4F and was similar to levels in untreated BMNs. In contrast to the absence of NETs in LT- and PA-treated cells, nigericin treatment consistently showed significant elongated NET generation (Fig. 7F). Interestingly, the MEK 1/2 pathway has been reported to be required for NET formation,[60] so LT should be an inhibitor of this process, although this may be specific to certain activating conditions. While LT does not induced NETs in vitro, it remains to be seen if LT-induced NETs can be observed in vivo, or if the effects of DNase treatment and PAD4 are possibly independent of neutrophil-generated NETs. It is also unclear if alarmins such as HMGB1 act as the primary signal 1 in a cascade leading to cytokine induction and if neutrophils themselves are the source of these signals. We conclude that the inflammasome- and neutrophil-dependent IL-1$\beta$ response induced by anthrax LT in mice is dependent on PAD4 activity and the presence of circulatory DNA generated after toxin challenge, despite the inability of the toxin to generate NETs from purified neutrophils.

## 4 | DISCUSSION

Inflammasome activation is an important first line of defense for the innate immune system's recognition of pathogens, tissue damage or sterile "danger signals." Cytokine release is a "two-hit" process: signal 1 leads to sensor and cytokine precursor upregulation and signal 2 activates sensor oligomerization, caspase-1 recruitment and cytokine processing. Signal 1 is not required if cells have steady-state levels of sensor or substrate. Almost all signal 1 priming reports focus on the NLRP3 inflammasome[4] and priming requirements for other sensors are not as well-studied. Mouse macrophages, BMDMs and most monocytic cells do not have steady-state pro-IL-1$\beta$ protein and thus require upregulation via TLR signaling. While signal 1 alone is not sufficient for cytokine release from mouse cells, human monocytes have an "alternative" inflammasome pathway in response to LPS, with no signal 2 or gasdermin requirement[61] resulting in an uncoupling of pyroptosis from IL-1$\beta$ release, similar to what has been described for neutrophils.[62]

In this study, we report a neutrophil-dependent IL-1$\beta$ release in mice following challenge with anthrax LT. This response to the toxin is an example of a bacterial virulence factor able to initiate events in vivo that lead to inflammasome-dependent inflammatory responses in the absence of a classic bacterium-supplied signal 1 activation. LT activates the NLRP1b inflammasome in mouse macrophages but is not capable of providing a signal 1 for up-regulation of IL-1$\beta$.[14,15] The NLRP1- and caspase-1-dependent IL-1$\beta$ response in mice originates from a hematopoietic compartment. While LT can bind and enter neutrophils, we find it is unable to up-regulate IL-1$\beta$, induce neutrophil pyroptosis, or produce NETs in vitro. Knockout of PAD4, which has been closely associated with NET formation, and

DNase treatment both resulted in a reduced IL-1$\beta$ response, suggesting the possibility of in vivo NET formation downstream of other toxin-dependent events or mediators.

LT may induce the rapid NLRP1-dependent pyroptosis of macrophages and dendritic cells, resulting in release of a wide range of mediators, including eicosanoids,[31] the TLR-ligating DAMP HMGB1, cell death-derived ATP, other alarmins or DNA, which act as signal 1 for neutrophils. We hypothesize that the neutrophil-independent IL-18 release induced by the toxin may also be a result of monocytic and dendritic cell lysis. Unlike IL-1$\beta$ that must be induced in mouse monocytes and dendritic cells, these cells harbor IL-18 in significant translated quantity, ready for release following inflammasome activation or lysis. The Ly6G/Ly6C reactive antibody RB6-8C5, which inefficiently depletes Ly6C-expressing monocytes and dendritic cells resulted in a 20–25% decrease in toxin-induced IL-18 levels (data not shown), compared to an almost complete loss of IL-1$\beta$, through its efficient depletion of neutrophils (Fig. 7E).

These initial pyroptosis and linked priming events may be independent of NET release, but neutrophils may also later contribute to alarmin or DNA release in a positive feedback loop as a second amplifying signal for IL-1$\beta$ up-regulation in multiple cell types. The neutrophil likely requires in vivo activation and/or interaction with the endothelium to initiate the cytokine response and it is unclear if monocyte pyroptosis alone provides the mediators for neutrophil activation, or if toxin actions on neutrophils and the endothelium are also required. Certainly, inflammatory conditions are known to activate neutrophil NET formation following histone hypercitrullination[52,53] and NETs have been shown to induce cytokine production from macrophages in atherosclerosis and in response to LPS.[63,64] The LT-induced IL-1$\beta$ response, however, is independent of any DNA uptake and sensing by the intracellular DNA sensor AIM2. It is possible that both AIM2 and Nlrp3 are required in combination for the sensing event in vivo. IL-18 or HMGB1, both released after LT challenge, may also directly prime for IL-1$\beta$ production.[65–69] Similarly, tissue damage in nonhematopoietic compartments may release alarmins as signal 1 for neutrophils, explaining the in vivo requirement for cytokine release. Finally, neutrophil efferocytosis of lysing monocytes could activate them to produce IL-1$\beta$, an event we may not be able to replicate in vitro. The identification of the exact host-dependent responses, which supply signal 1 for NLRP1-mediated cytokine response in mice requires further investigation.

It is important to note that the PAD4 knockout data presented here are not sufficient to suggest that LT induces NETs in vivo, as substantial questions remain on PAD4's role in NET formation.[47,48,54,70] PAD4 impacts NLRP3 inflammasome function,[71] and could thus also possibly alter NLRP1 responses. Furthermore, citrullination of NF-$\kappa$B has recently been shown to control TLR-mediated expression of cytokines, suggesting a direct role for PAD4 activity in upregulation of cytokines by a variety of signals, possibly including signal 1 in our studies.[72] The fact that we have not found neutrophils to produce either IL-1$\beta$ or form NETs in response to LT in vitro complicates the hypothesis that PAD4-mediated NET formation plays a role in the IL-1$\beta$ response, although the heterogeneity of neutrophils in both blood and marrow cannot be underestimated.[73] Tissue resident neutrophils or smaller subset of Ly6G+ cells might be responsible for the response in vivo, thus not captured by our experiments in vitro.

Although platelets were not found to bind PA and their depletion did not impact the cytokine response to LT, we are fascinated by the LT-induced neutrophilia that is accompanied by decreases in platelet levels. Platelets have been implicated in neutrophil NET generation and are capable of de novo cytokine synthesis. Given the interaction between platelets and neutrophils, platelets may modulate the neutrophil response to toxin.[74,75] The neutrophilia seen upon LT challenge is also intriguing, given that LF is known to inactivate all the MEK pathways normally associated with neutrophil recruitment. Interestingly, an *Nlrp1a* activating mutation in mice was associated with 15-fold higher neutrophilia and a loss of peritoneal macrophages, where the sensor drove a systemic inflammatory phenotype dependent on caspase-1 and IL-1$\beta$ signaling.[76] Thus, NLRP1a, a protein with high sequence homology to NLRP1b, but not activated in the same manner, controlled an IL-1 signaling-dependent myelopoiesis with a granulocyte expansion.

Our study demonstrates the surprising requirement for neutrophils in a robust system-wide IL-1$\beta$ response in mice, despite neutrophils themselves being resistant to toxin-induced pyroptosis or cytokine induction ex vivo. This is a unique example of a “sterile” inflammation induced by a bacterial toxin not requiring the bacterium to provide a classic signal 1 priming signal, and instead relying on the host response to provide the required activation signals. The study attests to the complicated events in live hosts, which may not always be easily recapitulated in isolated cell populations in vitro. The sequence of events and the precise in vivo molecular mechanisms and signaling for neutrophil, DNA and PAD4 requirements in LT-induced IL-1$\beta$ response need further study.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

| | |
|---|---|
| **AIM2** | absent in melanoma 2 |
| **BMDM** | bone marrow-derived macrophage |
| **BMN** | bone marrow-derived neutrophil |
| **DAMP** | danger/damage associated molecular pattern |
| **HMGB1** | high mobility group-box 1 protein |
| **LF** | lethal factor |
| **LT** | lethal toxin |
| **MEK** | mitogen activated kinase |

| | |
|---|---|
| **NE** | neutrophil elastase |
| **NET** | nuclear extracellular trap |
| **NLRP** | nucleotide-binding oligomerization domain leucine rich repeat protein |
| **PA** | protective antigen |
| **PAD4** | peptidyl arginine deiminase-4 |
| **PAMP** | pathogen-associated molecular pattern |
| **WT** | wild-type |

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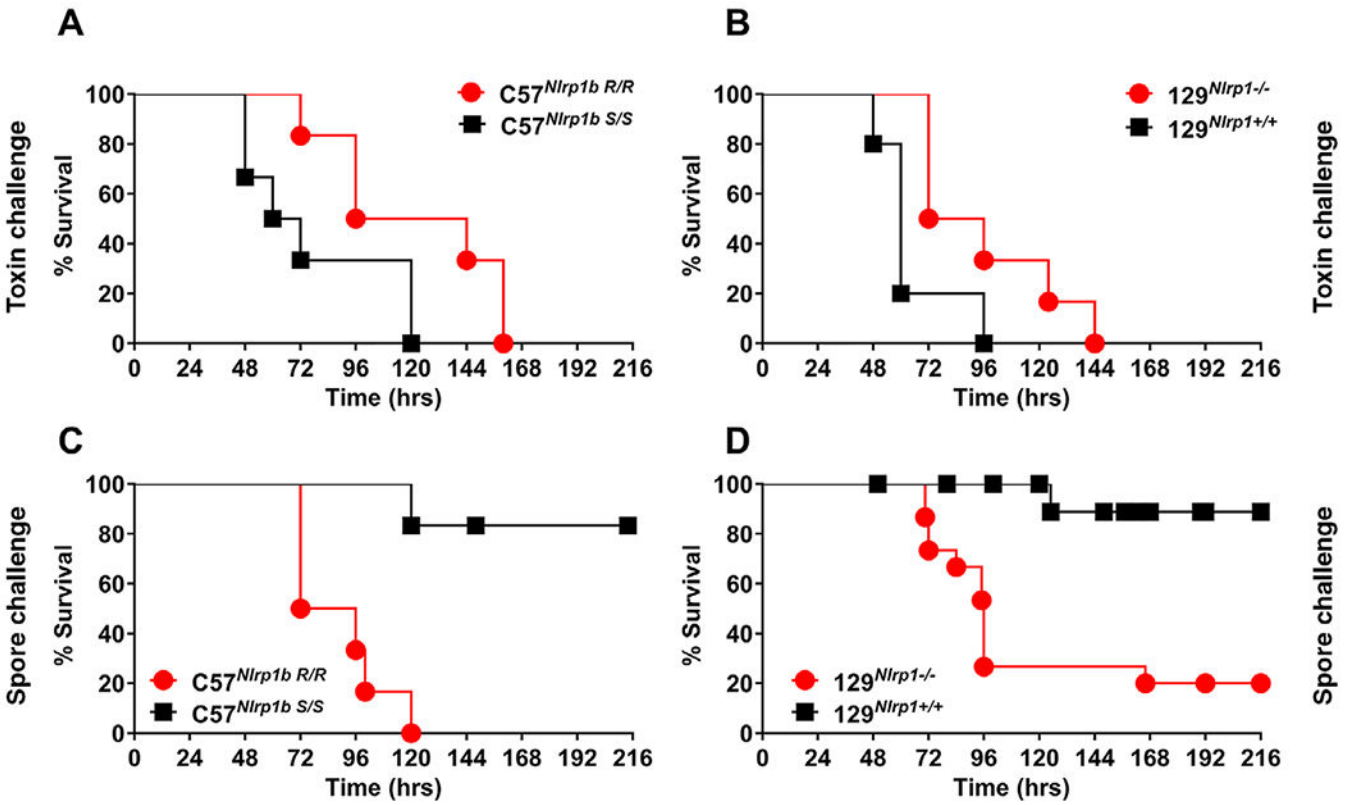


**FIGURE 1. NLRP1b status controls murine susceptibility to anthrax spore infection but not to LT challenge.**

$C57^{Nlrp1b\ R/R}$, $C57^{Nlrp1b\ S/S}$, $129^{Nlrp1-/-}$, and $129^{Nlrp1+/+}$ mice were challenged with (**A** and **B**) LT (100 μg, i.p.) or (**C** and **D**) A35 spore ($1 \times 10^8$, s.c., for C57 strains and $2 \times 10^8$, s.c. for 129 strains) and monitored for survival. For all C57BL/6 challenges, $n = 6$ per group. For 129 strains, LT challenge: $129^{Nlrp1-/-}$ ($n = 6$), $129^{Nlrp1+/+}$ ($n = 5$) and spore challenge: $129^{Nlrp1-/-}$ ($n = 15$), $129^{Nlrp1+/+}$ ($n = 13$). *P* values by log-rank (Mantel–Cox) test for comparison of curves are (**A**) $P = 0.0551$, (**B**) $P = 0.0342$, (**C**) $P = 0.0010$, (**D**) $P = 0.0006$

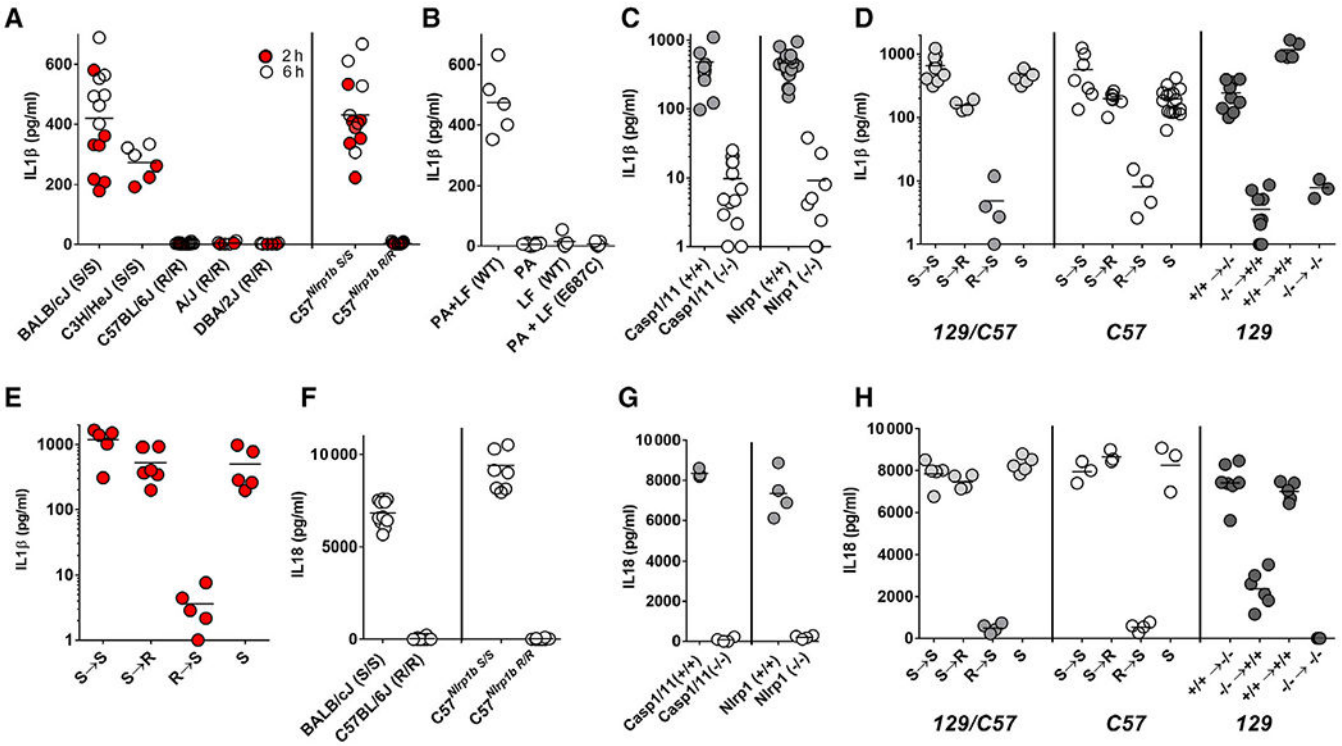


**FIGURE 2. Anthrax LT induces a signal 1-independent, caspase-11-independent but caspase-1-dependent IL-1*β* response that requires LT-responsive NLRP1b expressed in hematopoietic cells.**

(**A** and **F**) Inbred mouse strains with either *Nlrp1b*$^{S}$ or *Nlrp1b*$^{R}$ alleles as well as congenic C57$^{Nlrp1b\ R/R}$ and C57$^{Nlrp1b\ S/S}$ mice were injected with LT (100 μg, i.p.) and bled at 2 h (red) or 6 h (white) and IL-1*β* (2 h, 6 h) (**A**) or IL-18 levels (6 h) were assessed (**F**). Group sizes range from $n = 6$ to $n = 17$. (**B**) BALB/cJ (NLRP1b$^{S}$) mice were injected with WT LT ($n = 5$), PA ($n = 8$), LF ($n = 5$) or PA combined with an enzymatically inactive LF mutant ($n = 5$) (100 μg each, i.p.), and bled at 5–6 h to assess cytokine levels (**C** and **G**). Caspase-1/11-and NLRP1-deficient mice were compared to WT controls for IL-1*β* (**C**) and IL-18 (**G**) levels at 6 h post-LT injection (100 μg, i.p., group sizes range from $n = 4$ to $n = 17$). (**D, E,** and **H**) Cytokine levels were assessed in irradiated mice reconstituted with donor marrow as shown (arrow indicates donor → recipient), after toxin challenge at 8–10 weeks post-reconstitution. In panels **D** and **H**, "S" indicates 129S1/SvlmJ mice in the left panel, and C57$^{Nlrp1b\ S/S}$ mice in the middle panel. In panel **E** "S" indicates 129S1/SvlmJ mice. "R" stands for C57$^{Nlrp1b\ R/R}$ mice in all panels. In panels **D** and **H**, *Nlrp1*$^{+/+}$ and *Nlrp1*$^{-/-}$ mice are abbreviated as "+/+" or "−/−." LT treatments were either (100 μg, i.p.) with bleeds at 5–6 h post-toxin injection (**D** and **H**) or with 50 μg LT, i.v. with bleeds at 2 h post injection (**E**). Each circle in graphs represents a single mouse, with IL-18 levels assessed for a smaller subset of mice. In panels **D–H** group sizes range from $n = 4$ to $n = 16$. *P* values are as follows: Any panel where every mouse within a particular treatment group did not exceed IL-1*β* levels of 100 pg/ml or IL-18 levels of 1000 pg/ml, the low-cytokine group differed from all other treatment groups in that panel by $P < 0.000.1$. In panel **A**, C3H/HeJ versus BALB/cJ, *P* value is 0.0379. In panel **D**, left panel, S → S compared with S (nonirradiated), $P = 0.0047$; S → S compared with S → R, $P = 0.0006$; S → R

compared with S (nonirradiated), $P = 0.0017$. In panel **D** center, S → S compared with S (nonirradiated), $P = 0.0026$; S → S to S → R, $P = 0.0401$; S → R to S (nonirradiated), $P = 0.9958$. In panel **D**, right panel, ((+/+) → (−/−)) compared with ((+/+) → (+/+)) has a $P$ value of <0.0001 whereas for ((−/−) → (−/−)) compared with ((−/−) → (+/+)) $P$ is 0.0380. In panel **E** S → S compared with S → R has $P = 0.0332$ and when compared to S (nonirradiated), $P = 0.0469$

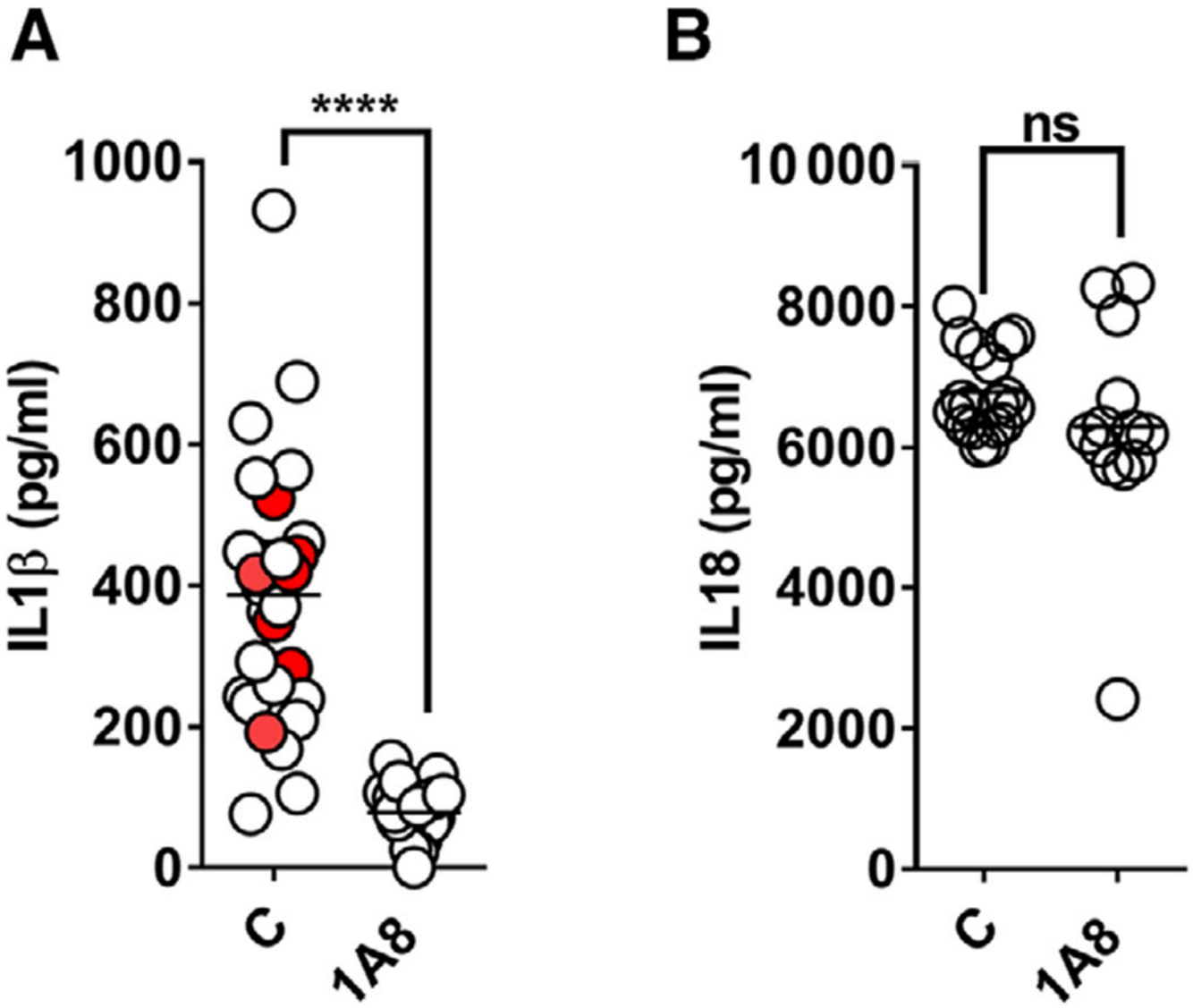


**FIGURE 3. Neutrophils are required for the LT-induced IL-1*β* response but dispensable for IL-18 production.**

BALB/cJ or C57$^{Nlrp1b\ S/S}$ mice were injected with 1A8 antibody (100 or 200 μg, i.p.), or control treatments (“C”, either LTF2 control antibody used at same dose, shown by red fill circles, or PBS, black open circles) at 24 h prior to injection with LT (100 μg, i.p.) and bled at 5–6 h for cytokine assessment. Neutrophil depletion was verified in two preliminary experiments by flow cytometry, as per reference 29. Data represents pool of 5 separate experiments. “C” ($n$ = 30) and 1A8 ($n$ = 25) in panel (**A**) and “C” ($n$ = 18) and 1A8 ($n$ = 13) in panel (**B**). $P$ value comparing two groups in panel (**A**) by 2-way ANOVA or by $t$-test is <0.0001. The two groups in panel B are not significantly different

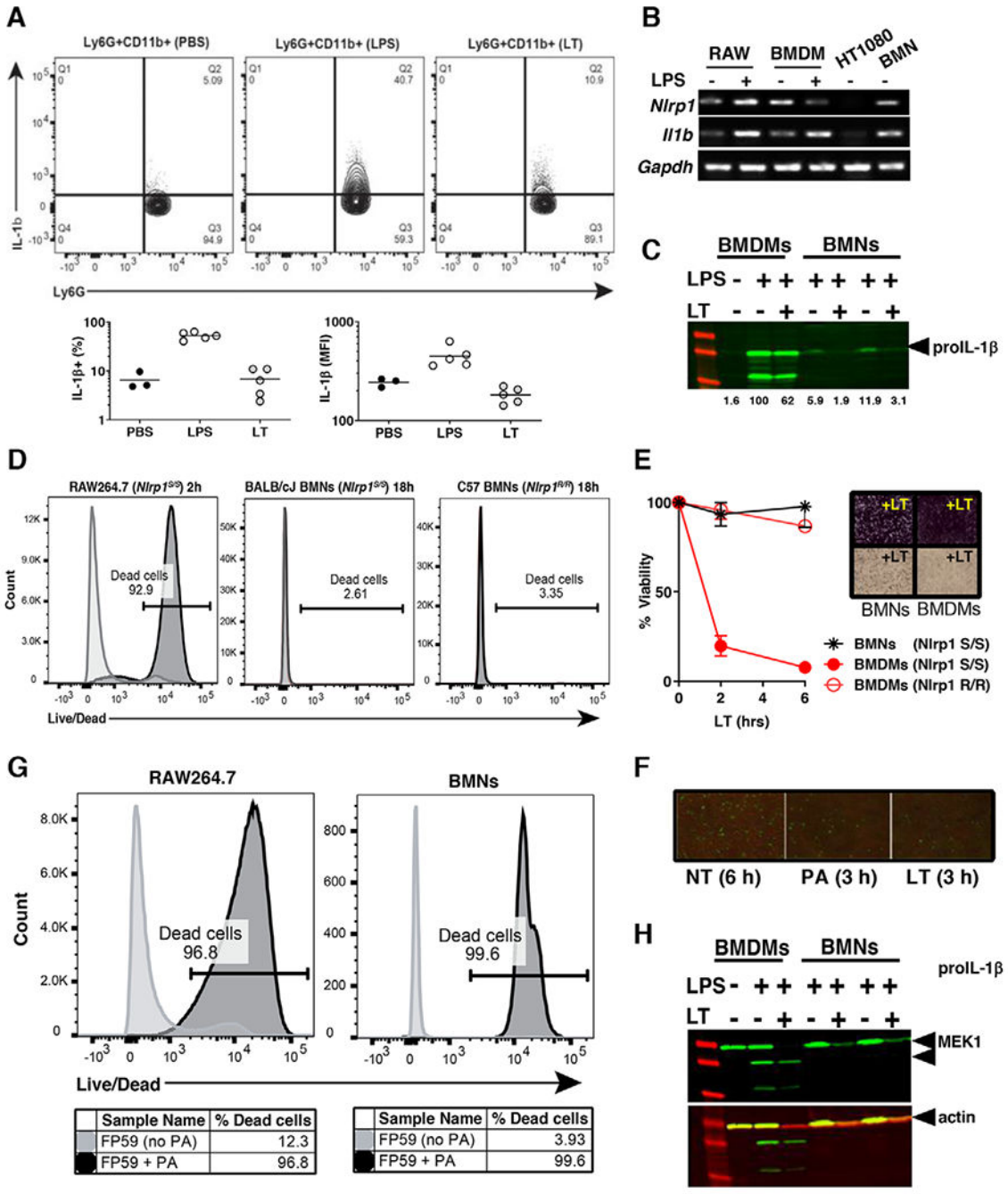


**FIGURE 4. LF translocation into neutrophils does not activate the inflammasome or induce pyroptosis.**

(**A**) BALB/cJ (NLRP1b$^S$) mice were injected with PBS, LPS (5 μg, i.p.) or LT (100 μg, i.p.) for 3 h and CD11b+Ly6G+ neutrophils from peripheral blood were assessed for intracellular IL-1$\beta$ staining. Representative flow cytometry plots from one experiment are shown in panel (**A**), upper, with quantifications for percentage of positive staining and mean fluorescence for individual mice shown in the lower two graphs. (**B**) BMNs and BMDMs from BALB/cJ (NLRP1b$^S$) mice, RAW264.7 (NLRP1b$^S$) macrophages and HT1080 (nonmyeloid) cells

were treated with or without LPS (1 μg/ml, 2 h) and analyzed for *Nlrp1b, Il1b*, and *Gapdh* transcript levels. Data represent three independent experiments. (**C**) BMNs or BMDMs from $C57^{Nlrp1b\ S/S}$ were treated with or without LPS (1 μg/ml, 2 h) followed by LT (1 μg/ml, 70 min) and lysates analyzed for IL-1$\beta$ levels. Lysates for two separate BMN isolations are shown in last 4 lanes. Visualization of LT-cleaved mature IL-1$\beta$ or reduction of pro-IL-1$\beta$ in BMDMs is limited here by scan settings that allow demonstration of the small difference between LPS and LT-treated BMN samples. Densitometry readings for pro-IL-1$\beta$ levels (normalized relative to +LPS/+LT lane) are shown below the gel as an indirect measure of cleavage. (**D**) RAW264.7 ($NLRP1b^{S}$) macrophages, C57BL/6J ($NLRP1b^{R}$) or BALB/cJ ($NLRP1b^{S}$) BMNs were treated with PA (1 μg/ml) or LT (1 μg/ml) for 2 or 18 h, and cell viability (to measure pyroptosis) was assessed by flow cytometry. Light gray fill represents PA alone, darker gray fill shows LT treatments. (**E** and **F**) $C57^{Nlrp1b\ S/S}$ BMDMs and BMNs were treated with LT (1 μg/ml) for 2–6 h and viability assessed by MTT assay (**E**) or NucGreen Dead staining (**F**) and visualization by light (**E**) or fluorescent (**F**) microscopy. The upper images in panel **E** are inverted pairs for the MTT staining shown in the lower images. Data represent >5 independent experiments. (**G**) RAW264.7 ($NLRP1b^{S}$) macrophages and BALB/cJ ($NLRP1b^{S}$) BMNs were treated with FP59 (50 ng/ml), a toxin comprising LFn fused to the catalytic domain of *Pseudomonas aeruginosa* exotoxin A, with or without PA (100 ng/ml). FP59 requires PA and PA receptors for translocation to the cytosol to induce cell death in 18–24 h in myeloid cells. Cell death was assessed by flow cytometry after 18 h of treatment. Light gray fill represents FP59 treatment without PA, and dark grey fill represents FP59 + PA. (**H**) To assess LF entry into BMNs, WB in C was probed for MEK1 cleavage by LT, using an antibody that binds to an epitope in the first 25 amino acids of the kinase and spans the LT cleavage site. Reactivity to this antibody is lost upon cleavage of MEK1 by LT, indicating cytoplasmic translocation of the toxin. The same WB is shown probed with an antibody against beta-actin, detected with a secondary antibody conjugated to a different IR-dye. Actin, which runs at the same molecular weight as MEK1, is seen in yellow when MEK1 is uncleaved, and red/orange in lanes where MEK1 is fully or mostly cleaved

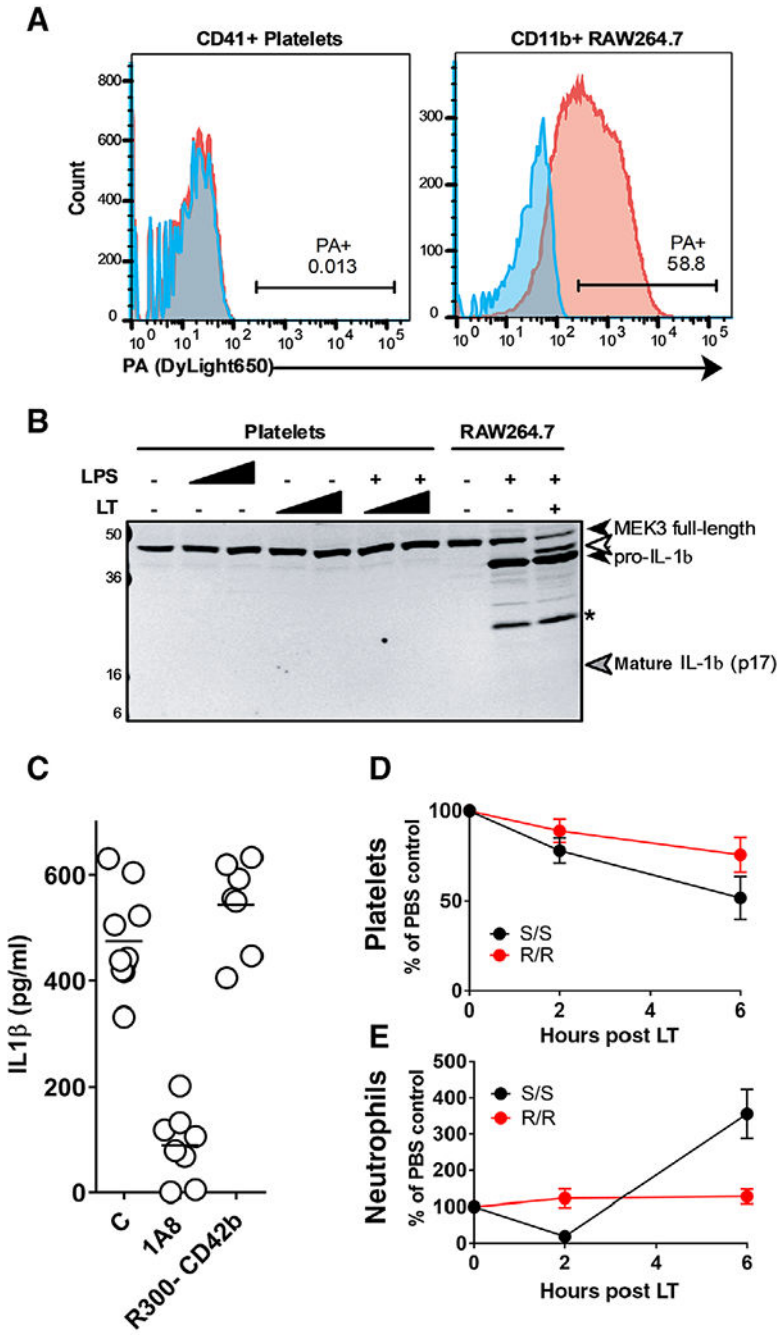


**FIGURE 5. Platelets cannot take up LF and their depletion does not inhibit toxin-mediated IL-1*β* release.**

(**A**) RAW264.7 cells or platelet-rich plasma (PRP) isolated from peripheral whole blood of BALB/cJ (NLRP1b$^{S}$) mice were treated with or without DyLight-650-labeled PA (4 μg/ml) for 30 min and analyzed for PA binding by flow cytometry. Blue fill represents untreated controls, and red fill represents cells treated with PA. (**B**) PRP was treated with LPS (1 μg/ml) for 2 or 6 h, LT (1 μg/ml) for 2 or 6 h, LPS for 2 h followed by LT for 2 or 6 h, or vehicle. RAW264.7 macrophages were treated with or without LPS for 6 h, followed by

LT for 75 min. MEK3 and IL-1$\beta$ levels and cleavage were assessed by WB. The white-filled arrowhead indicates the MEK3 cleavage product resulting from LF treatment. The asterisk indicates a breakdown product of pro-IL-1$\beta$ commonly seen in this cell line after induction of the cytokine, that is not the cleavage product. The approximate location of cleaved IL-1$\beta$, which has already been secreted from the cells before lysate preparation is also shown. (**C**) $C57^{Nlrp1b\ S/S}$ ($n = 3$) or BALB/cJ ($NLRP1b^{S}$) mice ($n = 4$) were platelet depleted using anti-CD42b or neutrophil depleted using anti-Ly6G A8 clone prior to toxin challenge (LT, 100 μg i.p.) and terminal bleeds at 4 h for cytokine assessment. **C** (control) is either LTF2 control antibody used at same dose or PBS. Each circle represents a single animal. The control group and platelet depleted group are not significantly different ($P = 0.14$) but each is significantly different from the 1A8 group ($P < 0.0001$). (**D** and **E**) CBC analyses of EDTA bleeds from congenic $C57^{Nlrp1b\ S/S}$ and $C57^{Nlrp1b\ R/R}$ mice at 2 and 6 h following treatment with LT (100 μg, i.p.). Graphs show platelets and neutrophils levels relative to PBS-treated control mice. The averages shown are for $n = 3$ mice, per time point, per group. PBS-treated controls were $n = 2$ mice bled a 2 and 6 h after PBS injection and arbitrarily assigned as 100% at time zero in these graphs

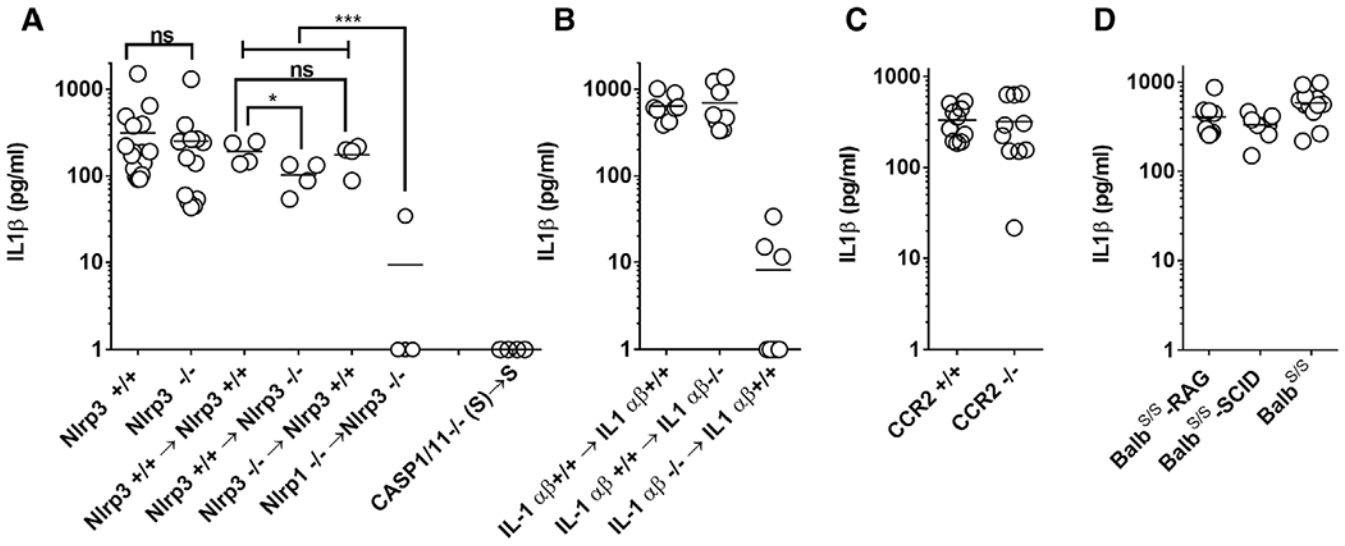


**FIGURE 6. LT-induced IL-1β originates from the hematopoietic compartment independently of NLRP3 or CCR2 mediated events.**

(**A** and **B**) Cytokine levels were assessed in irradiated mice reconstituted with donor marrow or in knockout mice after LT toxin challenge (100 μg, i.p.). In panel (**B**), the first two groups are not significantly different from each other ($P = 0.74$) but differ significantly ($P < 0.0001$) from the third group. CCR2 knockout mice and littermate WT controls (**C**) and RAG or SCID mice on the BALB/cJ background (**D**) were injected with mice in the same manner as in (**A** and **B**) for cytokine assessment. The groups in panel **C** and **D** are not significantly different in their response ($P > 0.8$ for all comparisons). All bleeds were performed at 5–6 h post-toxin challenge. All mice are homozygous for *Nlrp1b*$^{S}$ alleles. Each circle represents a single animal. Arrows indicate the direction of bone marrow transfer (donor → recipient)

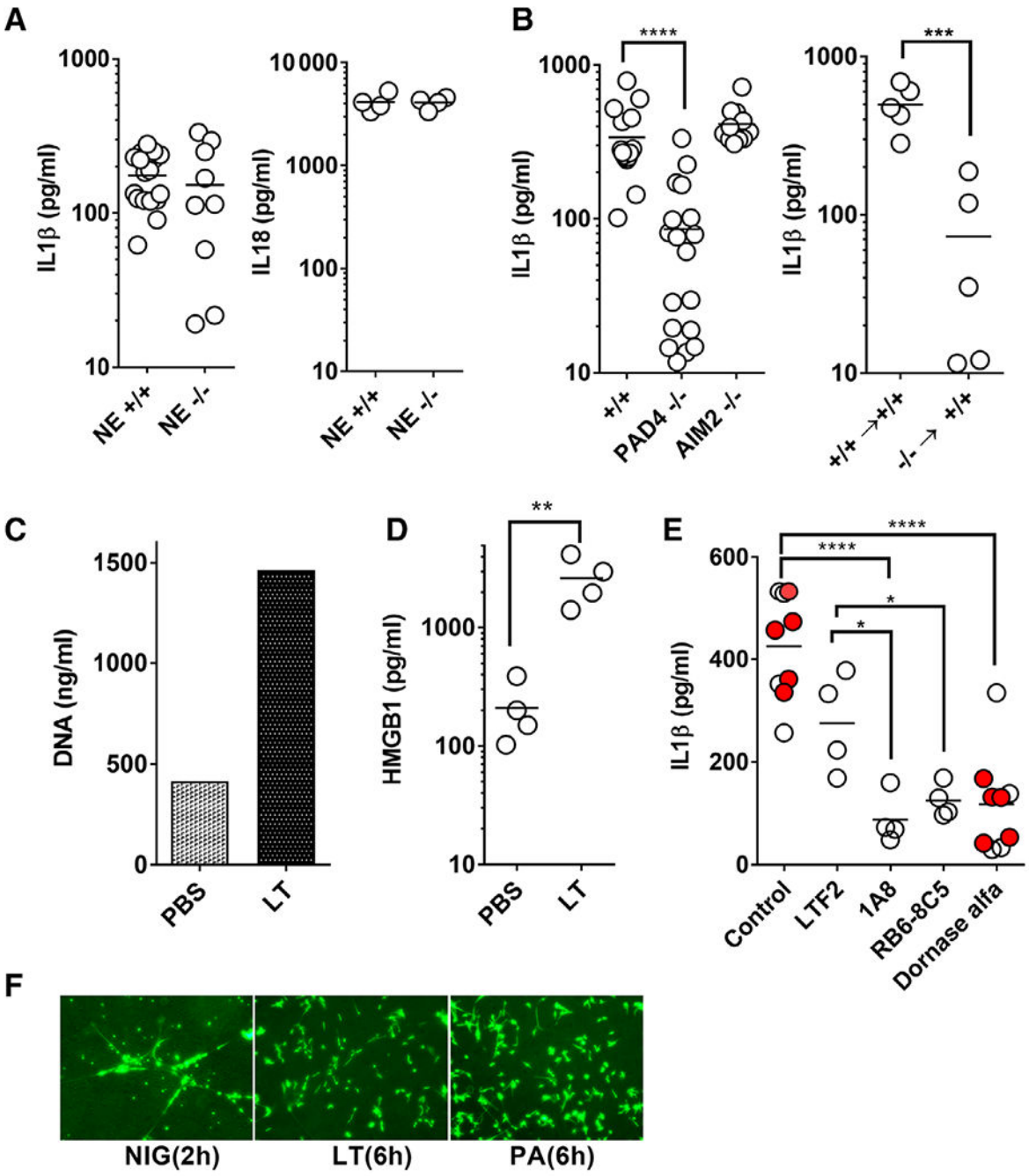


**FIGURE 7. LT-induced IL-1β production does not require neutrophil elastase (NE) but is reduced in PAD4-deficient mice and following DNase treatment.**

(**A**) Mice were injected with LT (100 μg, i.p.) and bled at 5 h for cytokine assessment. The *n* values are $n = 17$ (NE +/+) and $n = 9$(NE −/−) for the left panel and $n = 4$ for the IL-18 measurements. *P* value comparing the two IL-1β groups by unpaired *t*-test is 0.545 and the two IL-18 groups is 0.916. All mice are on the C57BL/6J background, homozygous for *Nlrp1b*$^{S}$ alleles. (**B**) Left panel, mice were injected with 50 μg LT-OS, i.p. and bled at 5 h post-injection. Right panel, IL-1β was assessed after LT challenge of irradiated WT

mice, which were reconstituted with either PAD4 knockout or WT donor marrow. Mice were challenged with LT as in the left panel, at 10 weeks after reconstitution. All mice are homozygous for *Nlrp1b*$^{S}$ alleles. (**C**) Plasma (400 μl) from three BALB/cJ mice treated with LT (100 μg, i.p.) and three mice treated with PBS were pooled and processed through a DNA binding column. DNA level was calculated from absorbance at 260 nm (A260) readings, adjusted for 1.2 ml of plasma. (**B**) HMGB1 levels in sera from BALB/cJ mice treated with LT (100 μg, i.p.) were assessed. *P* value comparing two groups is 0.0076. (**E**) BALB/cJ mice were pretreated with PBS or Dornase Alfa DNAse (400 μg, i.p. at −18 and −2 h), antibodies to deplete neutrophils (1A8), anti-Gr1 (Ly6C/Ly6G) (RBG-8C5) or a control antibody, LTF2 (all used at 50–200 μg, i.p., injected at −18 h) followed by toxin challenge with LT-OS (50 μg, i.p.) at time zero. Bleeds were performed 4–5 h post-toxin injection. Each circle represents a single mouse, with colors indicating separate experiments. (**F**) BMNs were treated with nigericin (15 μM, 2 h), LT (1 μg/ml, 6 h) or PA (1 μg/ml, 6 h) followed by staining for NET formation and membrane permeability using NucGreen Dead stain. Panels are representative of the *highest* level of individual staining observed in 12 experiments with varying levels of background neutrophil death at 6 h (range = 10-30%) with no difference noted between PA or LT-treated cells