Nlpr12 mutation causes C57BL/6J strain-specific defect in neutrophil recruitment

Tyler K. Ulland1,2,*, Nidhi Jain1,3,*, Emma E. Hornick1,4, Eric I. Elliott1,2,3,5, Gwendolyn M. Clay2,5, Jeffrey J. Sadler1, Kathleen A.M. Mills1, Ann M. Janowski1,4, A. Paige Davis Volk1,6, Kai Wang7, Kevin L. Legge4,5,8, Lokesh Gakhar9,10, Mohammed Bourdi11, Polly J. Ferguson6, Mary E. Wilson2,4,5,12,13,14, Suzanne L. Cassel1,3,4,12,*,** & Fayyaz S. Sutterwala1,2,3,4,5,12,13,*,**

The inbred mouse strain C57BL/6J is widely used in models of immunological and infectious diseases. Here we show that C57BL/6J mice have a defect in neutrophil recruitment to a range of inflammatory stimuli compared with the related C57BL/6N strain. This immune perturbation is associated with a missense mutation in Nlpr12 in C57BL/6J mice. Both C57BL/6J and NLRP12-deficient mice have increased susceptibility to bacterial infection that correlates with defective neutrophil migration. C57BL/6J and NLRP12-deficient macrophages have impaired CXCL1 production and the neutrophil defect observed in C57BL/6J and NLRP12-deficient mice is rescued by restoration of macrophage NLRP12. These results demonstrate that C57BL/6J mice have a functional defect in NLRP12 and that macrophages require NLRP12 expression for effective recruitment of neutrophils to inflammatory sites.

1 Inflammation Program, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 2 Interdisciplinary Program in Molecular and Cellular Biology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 3 Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, California 90048, USA. 4 Interdisciplinary Program in Immunology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 5 Medical Scientist Training Program, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 6 Department of Pediatrics, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 7 Department of Biostatistics, University of Iowa College of Public Health, Iowa City, Iowa 52242, USA. 8 Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 9 Department of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 10 Protein Crystallography Facility, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 11 Molecular and Cellular Toxicology Section, Laboratory of Molecular Immunology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA. 12 Department of Internal Medicine, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. 13 Veterans Affairs Medical Center, Iowa City, Iowa 52246, USA. 14 Department of Microbiology, University of Iowa Carver College of Medicine, Iowa City, Iowa 52242, USA. * These authors contributed equally to this work. ** These authors jointly supervised this work. Correspondence and requests for materials should be addressed to F.S.S. (email: fayyaz.sutterwala@cshs.org).
The genetic background of a transgenic or knockout mouse has an important, but often overlooked, role in its phenotype. The founder C57BL/6J substrain originated in the 1920s and has been maintained at the Jackson Laboratories since 1948. A colony was established at the National Institutes of Health in 1951 and designated as the C57BL/6N substrain. Over the intervening 65 years there has been substantial genetic drift. Comparison of the genome between these two substrains revealed 40 single nucleotide polymorphisms (SNPs) and 30 indels. This genetic difference is relevant because large projects have utilized different substrains for their work. The International Mouse Phenotyping Consortium, for example, uses C57BL/6N embryonic stem cells for the generation of knockout mice. By contrast, the Mouse Genome Sequencing Consortium has relied on the C57BL/6J substrain. In addition, many commercially available knockout mice are on a mixed background with varying genetic contributions from both C57BL/6J and C57BL/6N substrains. Both substrains are widely used in biomedical research and hence it is critical to understand the phenotypic differences, their genetic causes, and in particular how these differences affect the outcomes of immunological studies using these mice. The nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family of proteins is a group of intracellular receptors that are important for sensing both pathogen-associated and danger-associated molecular patterns. NLRP2 was initially identified as a member of the NLR family reported to attenuate inflammatory responses. NLRP2 has also been shown to inhibit colonic inflammation and tumorigenesis in mouse models through negative regulation of nuclear factor kappa-light-chain (NF-κB). The role for NLRP2 in controlling bacterial infections is less clear. Vladimer et al. suggest that NLRP2 serves a pro-inflammatory role during infection with a modified Yersinia pestis strain through its ability to form a caspase-1 activating inflammasome that regulates interleukin (IL)-1β and IL-18 secretion. In marked contrast, Zaki et al. found an anti-inflammatory role for NLRP2 showing that NLRP2-deficient mice were resistant to infection with Salmonella typhimurium due to increased NF-κB and extracellular signal-regulated kinases activation. In yet a separate study, Allen et al. identified no role for NLRP2 in the in vivo host response to Klebsiella pneumoniae and Mycobacterium tuberculosis. Given these markedly discrepant findings and postulated mechanisms of action of NLRP2 in bacterial infection, a closer examination is warranted.

In this study, we demonstrate that C57BL/6J mice have a defect in recruitment of neutrophils to inflammatory sites in vivo. This immune perturbation in C57BL/6J mice is associated with a missense mutation within the leucine-rich repeat domain of Nlrp2 resulting in an arginine to lysine change (R1034K). The failure of neutrophil migration in C57BL/6J mice was phenocopied in Nlrp2<sup>−/−</sup> mice. By comparing C57BL/6N, C57BL/6J and Nlrp2<sup>−/−</sup> mice we further demonstrate that the role of NLRP2 in the recruitment of neutrophils to the site of inflammation is critical for host defense against bacterial pathogens. Expression of C57BL/6N NLRP2 in macrophages is required to mediate appropriate neutrophil recruitment. Furthermore, there is a defect in macrophages from C57BL/6J and Nlrp2<sup>−/−</sup> mice to produce the neutrophil chemotactic chemokine CXCL1 in response to inflammatory stimuli. Altogether, our findings demonstrate that NLRP2 controls neutrophil migration to inflammatory sites and that a missense mutation in Nlrp2 in C57BL/6J mice renders these mice with a functional NLRP2 deficit.

**Results**

**Reduced neutrophil recruitment in C57BL/6J mice.** Although differences in response to acetaminophen-induced hepatotoxicity and endotoxic shock have been noted in C57BL/6 substrains the genetic cause has not been determined. We evaluated the ability of C57BL/6N and C57BL/6J mice to recruit neutrophils in response to inflammatory stimuli in vivo. C57BL/6N and C57BL/6J mice were challenged intranasally (i.n.) with lipopolysaccharide (LPS) or *F. tularensis* live vaccine strain (LVS) and the number of neutrophils in the bronchoalveolar lavage (BAL) quantified by flow cytometry. The total number of neutrophils present in the BAL of C57BL/6J mice was diminished compared with that from C57BL/6N mice for both stimuli (Fig. 1a,b, Supplementary Fig. 1a–i). This C57BL/6J substrain difference was specific to the neutrophilic response, as numbers of CD45.2<sup>+</sup>Gr1<sup>−</sup> cells were not significantly different between C57BL/6N and C57BL/6J mice challenged i.n. with LPS (Supplementary Fig. 1g,h).

C57BL/6J mice also demonstrated increased mortality in response to i.n. challenge with *F. tularensis* LVS compared with C57BL/6N mice (Fig. 1c). Taken together, these data demonstrate that C57BL/6J mice have diminished inflammatory responses compared with C57BL/6N mice and this translates to increased susceptibility to bacterial infection in vivo.

C57BL/6J Nlrp12 mutation causes defective neutrophil recruitment. Commensal intestinal bacteria can have profound effects on the host immune system. Ivanov et al. demonstrated that differences in segmented filamentous bacterium (SFB) colonization between C57BL/6 mice from Jackson Laboratories and Taconic Farms influenced Th17 cell differentiation. To assess the possibility that the differences in neutrophilic responses observed between C57BL/6N and C57BL/6J mice was due to differences in intestinal microbiota, C57BL/6N and C57BL/6J mice were co-housed for 4 weeks before i.n. LPS challenge. A significant decrease in neutrophil recruitment in the BAL was still observed in C57BL/6J mice that had been co-housed with C57BL/6N mice (Fig. 1d) suggesting a predominant genetic rather than environmental cause for this phenotypic difference. We did observe that C57BL/6N mice that had been co-housed with C57BL/6J mice had decreased neutrophilic responses compared with separately housed C57BL/6N mice (Fig. 1d) implying that there may be an additional role for microbiota in neutrophil recruitment.

To identify the genetic cause of the neutrophil defect observed in C57BL/6J mice we intercrossed C57BL/6N and C57BL/6J mice (Supplementary Fig. 2a). We found F<sub>1</sub> offspring had neutrophil recruitment similar to C57BL/6J mice in response to i.n. LPS challenge. We did observe that C57BL/6N mice that had been co-housed with C57BL/6J mice had decreased neutrophilic responses compared with separately housed C57BL/6N mice and this translates to increased susceptibility to bacterial infection in vivo.

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Haematopoietic NLRP12 controls neutrophil recruitment. To specifically determine if NLRP12 was required for effective neutrophil recruitment to inflammatory stimuli, we utilized mice in which *Nlrp12* had been genetically ablated. Similar to C57BL/6J mice, *Nlrp12*−/− mice challenged i.n. with LPS displayed significantly diminished neutrophilic influx into the BAL fluid from lungs, as compared with wild-type (WT) C57BL/6J mice (Fig. 2a). *Nlrp12*−/− mice backcrossed onto a BALB/cJ background also had a neutrophil recruitment defect in response to intranasal LPS challenge (Fig. 2b). Taken together, these data demonstrate a defect in neutrophil migration in response to LPS in *Nlrp12*−/− mice.

In addition to neutrophilic infiltration, vascular leakage has an important role in the pathology of LPS-induced acute lung injury. Therefore, we examined the role of NLRP12 in LPS-induced increase in microvascular permeability. In WT (C57BL/6N) mice, LPS inhalation caused an increase in Evans blue leakage into the lungs (Fig. 2c). In contrast, *Nlrp12*−/− mice were protected from...
Evans blue leakage into the lungs compared with WT (C57BL/6N) mice, suggesting diminished vascular leakage in response to inflammatory stimuli (Fig. 2c).

We next sought to determine whether the defect in neutrophil migration in Nlrp12−/− mice was due to the loss of NLRP12 in the haematopoietic compartment. We generated bone marrow chimeric mice in which NLRP12 deficiency was restricted to the haematopoietic or non-haematopoietic compartment. WT (C57BL/6N) mice that received Nlrp12−/− bone marrow had defective neutrophil recruitment to the lungs in response to an i.n. LPS challenge and recapitulated the phenotype observed in NLRP12-deficient mice (Fig. 2d). Conversely, Nlrp12−/− mice that received WT (C57BL/6N) bone marrow had intact neutrophil recruitment to the lungs in response to an i.n. LPS challenge in comparison to WT (C57BL/6N) mice that received WT (C57BL/6N) bone marrow (Fig. 2d). These results suggest that defective neutrophil recruitment in Nlrp12−/− mice was due to a deficiency of NLRP12 within the haematopoietic compartment.

**Neutrophil extrinsic NLRP12 regulates neutrophil migration.**

To determine if neutrophil intrinsic Nlrp12 expression was required for appropriate neutrophil recruitment, we utilized an adoptive transfer model. In all, 5 × 10⁶ CD45.1+ WT (confirmed to be Nlrp12B6N/B6N) bone marrow cells, as a source of neutrophils, were transferred intravenously into CD45.2+ WT (C57BL/6N) or CD45.2+ Nlrp12−/− mice that were then challenged i.n. with LPS. Six hours later the number of CD45.1+ neutrophils in the BAL fluid was quantified by flow cytometry (Fig. 3a). As expected, the total number of neutrophils recovered from the BAL of Nlrp12−/− mice was diminished compared with that from WT (C57BL/6N) recipient mice (Fig. 3b). In addition, the number of CD45.1+ WT (Nlrp12B6N/B6N) donor neutrophils in the BAL of Nlrp12−/− recipient mice was also significantly diminished (Fig. 3c), indicating that normal CD45.1+ WT (Nlrp12B6N/B6N) donor neutrophils exhibited defective recruitment in an NLRP12-deficient environment. To confirm that the donor CD45.1+ neutrophils had not been specifically eliminated in the Nlrp12−/− recipient mouse, we compared the frequency of CD45.1+ donor neutrophils in the peripheral blood of WT (C57BL/6N) and Nlrp12−/− recipient mice. A similar frequency of CD45.1+ neutrophils were measured in the blood of both WT (C57BL/6N) and Nlrp12−/− recipient mice (Fig. 3d).

To confirm these findings, we performed the complementary experiment wherein 5 × 10⁶ CD45.2+ WT (C57BL/6N) or Nlrp12−/− mice were transferred intravenously into CD45.1+ WT (Nlrp12B6N/B6N) mice. Mice were challenged i.n. with LPS and 6 h later the number of donor CD45.2+ neutrophils in the BAL fluid was quantified by flow cytometry (Fig. 3e). The total number of neutrophils recovered with either PBS or LPS (0.0125 mg kg⁻¹ of body weight). Six hours post challenge BAL were performed and the number of Gr1+ cells quantified by flow cytometry (n = 2, PBS-treated group; n = 12, LPS-treated group). (b) WT (BALB/c) or Nlrp12−/− mice on a BALB/c background were challenged i.n. with LPS (0.0125 mg kg⁻¹ of body weight). Six hours post challenge BAL were performed and the number of Gr1+ cells quantified by flow cytometry (n = 17 per group). (c) WT (B6N) or Nlrp12−/− mice were challenged i.n. with either PBS or LPS (0.5 mg kg⁻¹ of body weight). In all, 5.5 h post challenge mice were injected i.v. with Evans blue dye (20 mg kg⁻¹). At 6 h post LPS challenge, the amount of extravasated Evans blue dye into the lung was calculated (n = 2, PBS-treated group; n ≥ 6 LPS-treated group). (d) Bone marrow chimeras (donor→recipient) were challenged i.n. with LPS (0.5 mg kg⁻¹ of body weight). Six hours post challenge, the number of Gr1+ cells in the BAL was determined by flow cytometry (n = 8 per group). *P < 0.05, **P < 0.01, ***P < 0.005, NS, not significant by Mann–Whitney U-test (a–c) or Kruskal–Wallis test with Dunn’s multiple comparisons test (d).
Figure 3 | The requirement for NLRP12 in neutrophil recruitment is neutrophil extrinsic. (a–d) A schematic illustrating the experimental approach is shown (a). WT CD45.1+ bone marrow was adoptively transferred into CD45.2+ WT or CD45.2+ Nlrp12−/− mice. Mice were challenged i.n. with LPS (0.5 mg kg−1 of body weight); 6 h post challenge the total number of Gr1+ cells (b) and the number of CD45.1+ Gr1+ cells (c) in the BAL was determined by flow cytometry. The percentage of CD45.1+ Gr1+ cells in the blood was also measured by flow cytometry (d). (e–h) A schematic illustrating the experimental approach is shown e. CD45.2+ WT or CD45.2+ Nlrp12−/− bone marrow was adoptively transferred into CD45.1+ WT mice. Mice were challenged i.n. with LPS (0.5 mg kg−1 of body weight); 6 h post challenge the total number of Gr1+ cells (f) and the number of CD45.2+ Gr1+ cells (g) in the BAL was determined by flow cytometry. The percentage of CD45.2+ Gr1+ cells in the blood was also measured by flow cytometry (h). Data are representative of two independent experiments. *P<0.05, NS not significant by Mann–Whitney U-test.
from the BAL of CD45.1+ WT (Nlrp12B6N/B6N) mice that had received bone marrow cells from either WT (C57BL/6N) or Nlrp12−/− mice was similar (Fig. 4b). Moreover, we saw no difference in the number of donor CD45.2+ WT (C57BL/6N) or Nlrp12−/− neutrophils in the BAL of CD45.1+ WT (Nlrp12B6N/B6N) recipient mice or in the frequency of donor CD45.2+ WT (C57BL/6N) and Nlrp12−/− neutrophils in the peripheral blood of CD45.1+ WT (Nlrp12B6N/B6N) recipient mice (Fig. 3g,h). These data suggest that Nlrp12 expression is required in a haematopoietic cell extrinsic to the neutrophil to appropriately direct neutrophil migration to inflammatory stimuli.

Macrophage NLRP12 is critical for neutrophil recruitment. Based on our finding that NLRP12 extrinsic to neutrophils modulated neutrophilic recruitment to inflammatory sites, we hypothesized that macrophage expression of NLRP12 might instruct neutrophil migration indirectly. To examine this hypothesis, we transferred WT (C57BL/6N) or NLRP12-deficient bone marrow-derived macrophages (BMDM) i.n. into WT (C57BL/6N) or Nlrp12−/− mice. Sixteen hours later mice were challenged i.n. with LPS and then the neutrophilic influx was determined in the BAL fluid (Fig. 4a,b). Transfer of WT (C57BL/6N), but not NLRP12-deficient, BMDM into Nlrp12−/− mice rescued the neutrophil migration defect in vivo in response to LPS challenge (Fig. 4b). These data suggest that NLRP12 expression in macrophages is required to direct appropriate neutrophil migration in vivo.

Next, we applied the findings from the NLRP12-deficient mice to C57BL/6J mice that carry a missense polymorphism in Nlrp12. To determine if the mechanism for the diminished neutrophil recruitment in C57BL/6J mice is also through defective macrophage function we transferred WT (C57BL/6N) or Nlrp12−/− BMDM i.n. into C57BL/6J mice. Sixteen hours later mice were challenged i.n. with LPS and neutrophil influx determined in the BAL fluid. Transfer of WT (C57BL/6N) BMDM into C57BL/6J recipient mice resulted in the rescue of neutrophil recruitment in response to i.n. LPS as compared with WT (C57BL/6N) recipient mice (Fig. 4c).

Importantly, transfer of NLRP12-deficient BMDM into C57BL/6J mice failed to rescue the neutrophil migration defect in vivo in response to LPS challenge (Fig. 4c), suggesting that defective NLRP12 function in C57BL/6J macrophages is responsible for the diminished neutrophil recruitment to inflammatory insults in vivo in these mice.

Nlrp12−/− mice are more susceptible to bacterial infections. To determine if the defective neutrophil recruitment observed in Nlrp12−/− mice affected their ability to control bacterial infections in vivo, analogous to the C57BL/6J mice with the missense polymorphism in Nlrp12, we utilized a pulmonary F. tularensis LVS infection model. ASC-, NLRP3-, NLRP6- and NLRP12-deficient mice were challenged i.n. with a sub-lethal inoculum of F. tularensis LVS and survival monitored. As previously reported, mice deficient in ASC (a critical component of several inflammasomes including the AIM2 inflammasome) were highly susceptible to infection with F. tularensis LVS with all ASC-deficient mice succumbing to infection by day 13 post infection (Fig. 5a; (refs 17,18). However, NLRP3- or NLRP6-deficiency did not compromise the ability of mice to survive an i.n. infection with F. tularensis LVS (Fig. 5a). In contrast, but consistent with the results for C57BL/6J mice (Fig. 1c), NLRP12-deficient mice were highly susceptible to i.n. infection with F. tularensis LVS with >75% of NLRP12-deficient mice succumbing to infection by day 12 post infection (Fig. 5a).

The increased susceptibility of Nlrp12−/− mice to infection with F. tularensis LVS compared with WT (C57BL/6N) mice was also reflected by increased bacterial burdens in infected organs 6 and 9 days post infection (Fig. 5b and Supplementary Fig. 3). NLRP12-deficient mice were also more susceptible to an intra-peritoneal route of infection with F. tularensis LVS compared with WT mice, suggesting that the role of NLRP12 in host defense is not restricted to the lung (Fig. 5c).

In order to determine if NLRP12 had a more global role in host defense against pathogens, we infected NLRP12-deficient mice
i.n. with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Similar to the findings with *F. tularensis* LVS, *Nlrp12*−/− mice had significantly higher bacterial burdens of *S. aureus* and *P. aeruginosa* in their lungs compared with WT (C57BL/6N) mice (Fig. 5d,e). Taken together, these data suggest that NLRP12 has an important role in controlling not only intracellular Gram-negative bacteria but also extracellular Gram-negative and Gram-positive pathogens.

**Reduced neutrophil recruitment to *F. tularensis* in *Nlrp12*−/− mice.** Neutrophils are rapidly recruited to inflammatory sites
through a number of well-defined steps\textsuperscript{19}. Neutrophils are initially mobilized from the bone marrow into the peripheral circulation. After receiving the appropriate signals neutrophils interact with the endothelium and undergo tethering, rolling, adhesion, crawling and transendothelial migration. In some instances, neutrophils can additionally exit the tissue parenchyma via transepithelial migration. To define at what point neutrophil migration was disrupted in $Nlrp12^{-/-}$ mice, we evaluated the in vivo mobilization of inflammatory cells in response to infection with $F.\, tularensis$ LVS. Circulating neutrophils were markedly increased at day 3 post infection in both WT (C57BL/6N) and $Nlrp12^{-/-}$ mice (Fig. 6a). This suggested two things: first that the absence of NLRP12 did not impact the development of neutrophils or second impact their release from the bone marrow into peripheral circulation. In contrast, significantly fewer neutrophils were recovered from the BAL fluid following infection with $F.\, tularensis$ LVS in $Nlrp12^{-/-}$ mice compared with WT (C57BL/6N) (Fig. 6b and Supplementary Fig. 4).

Movement from the circulation into the alveolar space requires the neutrophils to cross first an endothelial and then an epithelial barrier. To differentiate if neutrophil migration was impaired at the level of transendothelial or transepithelial migration, we assessed the number of neutrophils in the lung parenchyma by flow cytometry. Analogous to the loss of neutrophils in the BAL fluid, we also observed a similar defect in the number of neutrophils recruited across the endothelial barrier into the lung parenchyma of $Nlrp12^{-/-}$ mice 6 days post infection with $F.\, tularensis$ LVS (Fig. 6c). The impact of NLRP12 deficiency was limited to the neutrophilic response, as the number of parenchymal macrophages and dendritic cells were not significantly different between WT (C57BL/6N) and $Nlrp12^{-/-}$ mice (Fig. 6c). Taken together, these data suggest

**Figure 6** NLRP12 is required for neutrophil recruitment following $F.\, tularensis$ infection. (a, b) WT (B6N) or $Nlrp12^{-/-}$ mice were infected i.n. with $5 \times 10^5$ colony-forming unit (CFU) of $F.\, tularensis$ LVS. At the indicated time post infection blood was collected and BAL performed. Cell count and differential on blood (a) and BAL (b) was performed. (c) Three days post i.n. infection with $5 \times 10^3$ CFU of $F.\, tularensis$ LVS lungs were harvested, prepared into single cell suspensions, stained and analysed by flow cytometry for dendritic cells (CD11b$^+$, CD11c$^{high}$, Gr1$^+$), macrophages/monocytes (CD11b$^+$, CD11c$^{low}$, Gr1$^-$) and neutrophils (CD11b$^+$, CD11c$^-$, Gr1$^+$). Pooled data from 2-3 independent experiments is shown (a, b) or is representative of two independent experiments (c). Data are expressed as the mean $\pm$ s.e.m. (a, b). *$P<0.05$, **$P<0.01$, ***$P<0.005$, NS, not significant by Mann–Whitney $U$-test.
that the defect in neutrophil migration observed in Nlrp12−/− mice infected with F. tularensis LVS lies at the level of transendothelial migration out of the vasculature and into the lung parenchyma.

NLRP12-deficient macrophages have defective CXCL1 production. To further characterize the role of NLRP12 in the response to F. tularensis LVS infection, we next asked what the mechanism is by which NLRP12 acts in macrophages to direct neutrophil migration. We examined the induction of cytokines and chemokines in BAL fluid from WT (C57BL/6N) and NLRP12-deficient mice 3 days post infection. Whereas IL-6, tumor-necrosis factor (TNFα), IL-17A, macrophage inflammatory protein (MIP)-1α, IL-18 and IL-1β levels were not different in Nlrp12−/− mice compared with WT (C57BL/6N) mice, the neutrophil chemotactic chemokine CXCL1 levels were significantly diminished in the BAL fluid from Nlrp12−/− mice compared with WT (B6N) (Fig. 7a and Supplementary Fig. 5a).

Consistent with our in vivo findings of diminished CXCL1 in BAL from Nlrp12−/− mice following infection with F. tularensis LVS, we observed that Nlrp12−/− BMDM secreted significantly less CXCL1 in response to F. tularensis LVS, S. aureus and P. aeruginosa (Fig. 7b). We observed a similar defect in CXCL1 production in the absence of NLRP12 in bone marrow-derived dendritic cells (BMDC) challenged with F. tularensis LVS, S. aureus or P. aeruginosa (Supplementary Fig. 5b). However, the production of IL-6, TNFα and IL-10 in response to infection with F. tularensis LVS, S. aureus or P. aeruginosa was

![Figure 7](image-url)
similar between WT (C57BL/6N) and Nlrp12$^{-/-}$ BMDM (Fig. 7b).

To determine if the defect in CXCL1 production was restricted to infection with viable bacteria, we challenged BMDM from WT (C57BL/6N) and Nlrp12$^{-/-}$ mice with a variety of Toll-like receptor (TLR) agonists. Whereas NLRP12-deficient BMDM produced amounts of IL-6 and TNFα similar to those of WT (C57BL/6N) BMDM, NLRP12-deficient cells released less CXCL1 in response to all TLR agonists tested (Fig. 7c and Supplementary Fig. 5c). In support of the enzyme-linked immunosorbent assay (ELISA) data, intracellular cytokine staining also demonstrated less CXCL1 production in NLRP12-deficient BMDM compared with that in WT (C57BL/6N) challenged with LPS (Fig. 7d).

Examination of Cxcl1 expression by quantitative reverse transcription–PCR also found diminished Cxcl1 expression in NLRP12-deficient BMDM compared with WT (C57BL/6N) BMDM stimulated with LPS (Fig. 7e). Evaluation of mitogen-activated protein kinases (MAPK), canonical NF-κB and non-canonical NF-κB pathways in response to LPS stimulation did not reveal any substantial differences between BMDM from WT (C57BL/6N) and Nlrp12$^{-/-}$ mice (Supplementary Fig. 5d–g). Taken together, these data suggest that NLRP12 was specifically required for CXCL1 production and that this regulation occurred at the level of Cxcl1 transcript abundance.

Vladimer et al. have shown that Y. pestis can activate macrophage caspase-1 in an NLRP12-dependent manner and result in the generation of IL-1β and IL-18. To determine if NLRP12 was required to trigger inflammasome activation in response to F. tularensis LVS, S. aureus or P. aeruginosa, we measured IL-1β secretion and caspase-1 activation from BMDM from WT (C57BL/6N) and Nlrp12$^{-/-}$ mice challenged with F. tularensis LVS, S. aureus or P. aeruginosa. The production of IL-1β and caspase-1 activation between WT (C57BL/6N) and NLRP12-deficient BMDM following challenge with F. tularensis LVS, S. aureus or P. aeruginosa were similar (Supplementary Fig. 6,a,b). Similarly, no defect in the production of IL-1β in the absence of NLRP12 was observed in BMDC challenged with F. tularensis LVS, S. aureus or P. aeruginosa (Supplementary Fig. 6c). These data demonstrate that NLRP12 did not participate in the assembly or activation of an inflammasome in response to F. tularensis LVS, S. aureus or P. aeruginosa, and that the increased susceptibility of Nlrp12$^{-/-}$ mice to infection with these organisms was independent of caspase-1 activation.

Discussion

Our work demonstrates differences in inflammatory responses between C57BL/6N and C57BL/6J mouse substrains with C57BL/6J mice having a failure of neutrophil recruitment to a wide variety of inflammatory stimuli compared with the related C57BL/6N substrain. C57BL/6J mice carry a missense polymorphism in chromosome 7 at position 3,222,537 (G to A) related to the change of an arginine for lysine in the C-terminal leucine-rich repeat domain of NLRP12 (ref. 1). Our findings show that the Nlrp12 SNP is associated with the difference in neutrophil recruitment found between C57BL/6N and C57BL/6J substrains and that NLRP12 is required in macrophages to direct neutrophil recruitment. This failure by macrophages, both the NLRP12-deficient and the NLRP12 missense mutant, to recruit neutrophils is associated with defective production of the neutrophil chemoattractant chemokine CXCL1. These data suggest a single seemingly conservative amino acid change within NLRP12 can render it dysfunctional and profoundly impact a wide range of immune responses both in vitro and in vivo. Our data suggest that C57BL/6J mice carrying the Nlrp12 missense polymorphism may be inappropriate controls to study NLRP12 function. Furthermore, the use of C57BL/6J mice, as well as knockout mice backcrossed onto the C57BL/6N substrain, in studies that depend upon intact neutrophilic responses or that assess control of infectious pathogens may significantly confound their interpretation due to the presence of a dysfunctional NLRP12.

The literature surrounding NLRP12 is complex as several lines of investigation suggest contradictory roles for NLRP12 in response to infection with bacterial pathogens. NLRP12 has been shown to be important for host defense against Y. pestis through the production of caspase-1-dependent IL-18 (ref. 10). In contrast, NLRP12 has a detrimental role in infection with S. typhimurium through negative regulation of NF-κB and MAPK11. In the current study, we demonstrate a novel role for NLRP12 in host defense against infection with F. tularensis LVS, S. aureus and P. aeruginosa. In contrast to the findings with Y. pestis10, we did not observe a defect in caspase-1 mediated IL-1β production in Nlrp12$^{-/-}$ mice in response to F. tularensis LVS, S. aureus and P. aeruginosa, suggesting that NLRP12 did not regulate inflammasome activation in response to these pathogens. However, Nlrp12$^{-/-}$ mice failed to control replication of F. tularensis LVS, S. aureus and P. aeruginosa in vivo, a failure that correlated with a defective recruitment of neutrophils to the site of infection. Mobilization of neutrophils from the bone marrow into the peripheral circulation was intact in the absence of NLRP12 in mice infected i.n. with F. tularensis LVS. However, these newly recruited neutrophils were defective in their ability to exit from the peripheral circulation and migrate into the lung parenchyma in Nlrp12$^{-/-}$ mice, thereby suggesting a defect in transendothelial migration. Consistent with our findings a study by Cai et al. demonstrated that NLRP12-deficient mice were more susceptible to pulmonary infection with K. pneumoniae in vivo and that this was in part due to defective CXCL1 production. Interestingly, a study by Arthur et al. demonstrated...
an in vitro migration defect of NLRP12-deficient neutrophils to CXCL1. In contrast, Zamoshnikova et al.22 demonstrated an increase in neutrophil migration in vitro to the higher of two doses of CXCL1, but not in migration to Leishmania major. Although our in vivo assays did not reveal a neutrophil intrinsic role for NLRP12, the findings of Arthur et al. and Zamoshnikova et al. suggest there may be an additional neutrophil intrinsic role for NLRP12 (refs 21,22).

Perivascular macrophages in a variety of tissues are in close proximity to blood vessels and potently express CXCL1 and CXCL2 in response to challenge with infectious stimuli23. Chemokines secreted by perivascular macrophages are thought to create the precise cytokine milieu triggering both the adhesion and transmigration of neutrophils out of the blood stream23. Consistent with a role for a perivascular macrophage-like cell, local pulmonary reconstitution of Nlrp12−/− mice with WT (C57BL/6N) macrophages, but not Nlrp12−/− macrophages, corrected defective neutrophil recruitment to the lungs in response to LPS. These findings suggest that the NLRP12-dependent production of a chemotactic signal from local macrophages is a necessity for protective neutrophil recruitment in response to inflammatory stimuli in vivo. In further support of this role for macrophage-derived NLRP12, we observed a specific defect in the ability of NLRP12-deficient macrophages to secrete CXCL1 in response to a variety of pathogens and TLR agonists. These findings are consistent with a previous study in which diminished CXCL1 production was observed in NLRP12-deficient dendritic cells stimulated with the TLR2 agonist Pam3Cys4 compared with WT dendritic cells8.

In conclusion, our results demonstrate that NLRP12 is critical in the regulation of neutrophil recruitment to inflammatory triggers and that this is through control of the production of CXCL1 by local tissue macrophages. This neutrophil-extrinsic mechanism in turn is important for host defense against a variety of bacterial pathogens. Furthermore, a missense mutation in Nlrp12 in C57BL/6J mice renders them with defective NLRP12 function and hence diminished neutrophil recruitment to inflammatory stimuli in vivo. It is important to note that although the mutation in Nlrp12 in C57BL/6J mice results in defective neutrophil migration in vivo, it remains to be seen if other roles for NLRP12 are affected by this missense mutation. Further studies are also needed to define how the C57BL/6J missense mutation in NLRP12 affects its structure and binding to potential ligands and downstream signalling molecules. In addition, the relevance of these findings to human NLRP12 function will require further investigation.

Methods

Mice. The generation of NLRP12−/−, NLRP6−/−, NLRP1- and ASC-deficient mice has been described previously21,24,25. Mice were backcrossed onto the C57BL/6N genetic background for at least nine generations. Nlrp12−/− mice were backcrossed onto a BALB/c background for eight generations. C57BL/6N mice were purchased from National Cancer Institute and used as WT controls unless otherwise stated; BALB/c and C57BL/6N mice were purchased from Jackson Laboratories. CD45.1+ C57BL/6 mice (B6-129S6/SvJae; CD45.1) were purchased from National Cancer Institute. Both male and female mice between 6 and 12 weeks of age were used, however individual experiments utilized age and sex matched animals. The Institutional Animal Care and Use Committee at the University of Iowa approved all protocols used in this study. Bone marrow chimeras were generated as described26. Reconstitution was >92% in Nlrp12−/− mice reconstituted with WT bone marrow and 89% in WT mice reconstituted with Nlrp12−/− bone marrow.

Bacterial strains and growth conditions. F. tularensis ssp. holarctica LVS was obtained from American Type Culture Collection (ATCC 29684). F. tularensis LVS was grown on DiCso cysteine heart agar supplemented with 9% sheep red blood cells (SRBC) for 48 h at 37°C. For in vivo studies, bacteria were grown overnight in modified Mueller-Hinton (MMH) broth (Becton Dickinson) supplemented with 1% (wt/vol) glucose, 0.025% ferric pyrophosphate, and 2% IsoVitalex and then subcultured for 4 h before use. F. tularensis LVS expressing green fluorescent protein (GFP)27 were grown on DiCso cysteine heart agar supplemented with 9% SRBC and 25 μl/ml spectinomycin for 48 h at 37°C. Bacteria were then cultured in supplemented MMH broth with 25 μl/ml spectinomycin at 37°C overnight, and subcultured for 4 h in supplemented MMH broth. P. aeruginosa PAK strain was obtained from Barbara Kazmierczak (Yale University). P. aeruginosa was cultured in lysogeny broth overnight at 37°C and then subcultured for 4 h in supplemented MMH broth. S. aureus Newman strain was obtained from William Nauseef (University of Iowa); S. aureus was cultured overnight in tryptic soy broth at 37°C and then subcultured for 4 h before use.

In vivo infection and LPS challenge. For in vivo infections mice were anaesthetized with ketamine/xylazine and injected with the indicated number of bacteria resuspended in 50 μl of PBS. Alternatively, mice were injected intraperitoneally with 5 x 106 colony-forming unit of F. tularensis LVS resuspended in 200 μl of PBS. Mice were monitored every 12–24 h for lethality; mice found to be in a moribund state for more than 4 h were considered terminal and euthanized. Bacterial burdens in the lung, spleen and liver of infected mice were determined at the indicated times post infection by dilution plating of tissue homogenates onto DiCso cysteine heart agar supplemented with 9% SRBC for F. tularensis LVS, lysogeny broth for P. aeruginosa or TSA for S. aureus. For in vivo infection challenge mice were anaesthetized with ketamine/xylazine and challenged i.n. with the indicated amount of LPS (E. coli serotype 0111:B4; Invivogen) in 50 μl of PBS. BAL was performed on a subset of mice at the indicated time by cannulation of the trachea and lavage with either 3 ml for cell assay or 1 ml for cytokine determination (PBS28). Red blood cells were lysed and total nucleated cell counts were obtained using a haemocytometer. Cytospin slides were prepared by Wright-Giemsa staining with HEMA 3 (Fisher) and numbers of neutrophils, lymphocytes and dendritic cells were counted and cell differentials were determined by flow cytometry. At the indicated time, post infection blood was collected and blood count/differential was performed for the Clinical Pathology Laboratory at Iowa State University.

Microvascular permeability. We assessed microvascular permeability through extravasation of Evans blue dye. Thirty minutes before euthanasia, mice were injected intravenously with Evans blue dye (20 mg kg−1). Lungs were perfused with PBS to remove intravascular Evans blue dye. Lungs were harvested and the Evans blue dye extracted as previously described29. Briefly, lungs were harvested, homogenized and incubated overnight with 16.7% formamide in PBS at 37°C. Lung homogenates were filtered through a 70 μm mesh and plated into 96-well flat bottom plates. Absorbance at 620 and 740 nm were measured and used to correct for the presence of haeme pigments using the following equation: A620 (corrected) = A620 − (1.426 x A740 + 0.03) (refs 29,30). The amount of extravasated Evans blue dye was then calculated against a standard curve.

Adaptive cell transfer. For adoptive transfer mice were injected intravenously in the tail vein with 5 x 106 bone marrow cells from WT or Nlrp12−/− mice in PBS into congenically mismatched recipients before intranasal LPS challenge. Adoptive transfer of BMDM was accomplished by intranasal delivery of 4 x 106 BMDM derived from either donor WT or Nlrp12−/− mice into the corresponding recipient; 16–18 h later mice were i.n. challenged with LPS.

Flow cytometry. Single cell suspensions of lungs prepared by pressing tissues through a wire mesh screen or BAL were used and live cells enumerated using trypan blue exclusion. In all, 0.1–1 x 106 cells per well were stained in a 96-well plate and blocked with 2% rat serum or Fc Block (anti-mouse CD16/CD32, clone 2.4G2; 1:500; BioLegend). Anti-FITC, anti-CD54 (PerCP-Cy5.5; or PE-Cy7; clone 104; 1:100; BioLegend), anti-CD54 (FITC or PE; clone a26; 1:100; BioLegend), anti-CD11b (FITC, PE or PE-Cy7; clone M170; 1:100; eBioscience), anti-CD11c (PerCP-Cy5.5; clone N418; 1:200; eBioscience), anti-Gr1 (APC or FITC; clone RB6-8C5 or 1A8; 1:200; eBioscience) and anti-F4/80 (PE; clone BM8; 1:100; eBioscience) as described. Cells were subsequently fixed with FACS lysing buffer (BD Biosciences) per manufacturer’s instructions and resuspended in PBS. For CXCL1 intracellular staining BMDMs were left unstimulated or stimulated for 4 h with LPS (50 ng ml−1) in the presence of brefeldin A (5 μg ml−1) and monensin (2 μg ml−1) in the presence of brefeldin A (5 μg ml−1) and monensin (2 μg ml−1) in the presence of brefeldin A (5 μg ml−1) and monensin (2 μg ml−1) in the presence of brefeldin A (5 μg ml−1) and monensin (2 μg ml−1). Secondary staining was accomplished with streptavidin PE (eBioscience). Data were acquired on a BD LSR II and analysed using FlowJo Software (Treestar, Inc.).

In vitro stimulation of macrophages and dendritic cells. BMDM and BMDC31,32 were either left unprimed or primed with 50 ml ml−1 LPS for 4 h and then infected with F. tularensis LVS (MOI 50:1), P. aeruginosa PAK strain (MOI 1:1), or S. aureus Newman strain (MOI 1:1) for 8 h and supernatants collected and analysed for cytokine production by ELISA. For C57BL/6N mice leukocytes were lysed in lysis buffer (50 μM TRIS - HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and a protease inhibitor mixture (Roche) and stored at – 80°C until
analysed. Proteins were separated on a NuPAGE gel (Invitrogen) and transferred for the IL-1B and Nlrp12B6N proteins on a 1% agarose gel (Sigma–Aldrich). Proteins were separated on a NuPAGE gel (Invitrogen) and transferred to ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13180 | www.nature.com/naturecommunications for the IL-1B from the corresponding author upon request.

Nlrp12B6N University of Iowa. HEK-TLR4 cells were transfected with 2 μg plasmids (HEK-TLR4) (ref. 34) were a generous gift from Dr Jerrold Weiss at the University of Pennsylvania. Nlrp12B6N

Cytokine ELISA. Culture supernatants and BAL samples were assayed for IL-1β, MIP-1α, TNF-α, IL-6, IL-10, IL-18, IL-17A and CXCL1 by ELISA. Antibody pairs for the IL-1β (MAB401 and BAFL01; clone 30311 and polyclonal; R&D Systems; 8 μg/ml) and Nlrp12B6N

Transfection. To prepare vectors with B6N and B6J Nlrp12B6N

Real-time PCR and sequencing. Complementary DNA was generated at the indicated time point stimulation with LPS and real-time PCR performed using PerfeCTa SYBR Green Fast Mix (Quanta) and RT2 quantitative PCR primers (Quans) for Cxcl1. For quantification, standards were generated by cloning the PCR product into the pcR 2.1 vector using TOPO TA cloning kit (Life Technologies). To identify genetic differences between C57BL/6N and C57BL/6J mice the SNP

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
Conceptualization: T.K.U., N.J., S.L.C. and F.S.S.; methodology: T.K.U., N.J., S.L.C. and F.S.S.; investigation: T.K.U., N.J., G.C.C., E.E.H., E.I.E., J.J.S., K.A.M.M., and A.M.J.; formal analysis: K.W. and L.G.; resources: K.L.L.; writing original draft: T.K.U., S.L.C. and F.S.S.; writing review and editing: T.K.U., N.J., E.E.H., A.P.D.V., K.L.L., M.B., P.J.F., M.E.W., S.L.C. and F.S.S.; funding acquisition: S.L.C. and F.S.S.; supervision: S.L.C. and F.S.S.

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