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Determining distinct roles of IL-1 α through generation of an IL-1 α knockout mouse with no defect in IL-1 β expression

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Interleukin 1 α (IL-1 α) and IL-1 β are the founding members of the IL-1 cytokine family, and these innate immune inflammatory mediators are critically important in health and disease. Early studies on these molecules suggested that their expression was interdependent, with an initial genetic model of IL-1 α depletion, the IL-1 α KO mouse (*Il1a*-KO^{line1}), showing reduced IL-1 β expression. However, studies using this line in models of infection and inflammation resulted in contrasting observations. To overcome the limitations of this genetic model, we have generated and characterized a new line of IL-1 α KO mice (*Il1a*-KO^{line2}) using CRISPR-Cas9 technology. In contrast to cells from *Il1a*-KO^{line1}, where IL-1 β expression was drastically reduced, bone marrow-derived macrophages (BMDMs) from *Il1a*-KO^{line2} mice showed normal induction and activation of IL-1 β . Additionally, *Il1a*-KO^{line2} BMDMs showed normal inflammasome activation and IL-1 β expression in response to multiple innate immune triggers, including both pathogen-associated molecular patterns and pathogens. Moreover, using *Il1a*-KO^{line2} cells, we confirmed that IL-1 α , independent of IL-1 β , is critical for the expression of the neutrophil chemoattractant KC/CXCL1. Overall, we report the generation of a new line of IL-1 α KO mice and confirm functions for IL-1 α independent of IL-1 β . Future studies on the unique functions of IL-1 α and IL-1 β using these mice will be critical to identify new roles for these molecules in health and disease and develop therapeutic strategies.

KEYWORDS

PAMP, innate immunity, inflammation, inflammasome, IL-1 α , IL-1 β , CXCL1, caspase-1

Introduction

The IL-1 family of cytokines is a diverse family made up of potent inducers of inflammation. Members of this family can either prevent or promote disease, and they have been widely recognized as potential therapeutic targets (1–5). The three members of the IL-1 sub-family, IL-1 α , IL-1 β , and IL-1 receptor antagonist (IL-1RA), bind the same IL-1 receptor (IL-1R). The cytokines IL-1 α and IL-1 β act as agonistic ligands, whereas IL-1RA is a strong antagonist; together, these molecules orchestrate robust proinflammatory immune responses (6, 7).

Among the IL-1 cytokines, significant overlap has been observed in the downstream processes they activate. However, there are also key differences between their expression and release and the biological processes they drive (8). The pro-form of IL-1 β is biologically inactive and requires proteolytic processing for its activation. Inflammasome-dependent caspase-1 activation and pyroptosis are the major mechanisms responsible for IL-1 β processing and release (9–11). Unlike IL-1 β , the pro-form of IL-1 α is constitutively expressed in most cells from healthy hosts (12, 13); it is also biologically active and can be present directly on the plasma membrane for signaling or released following membrane damage during various forms of cell death, making it a classic danger signal (5, 14, 15).

As signaling molecules, a wide range of pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) that activate innate immune signaling induce the expression and activation of both IL-1 α and IL-1 β (5, 16). IL-1 family receptors carry the cytoplasmic TIR domain, a shared feature with pathogen sensing toll-like receptors (TLRs), making them excellent amplifiers of inflammatory signaling (17). Indeed, nanomolar doses of IL-1 α and IL-1 β can trigger lethal inflammatory responses in mice and humans (18–20). Consistently, IL-1 α and IL-1 β were shown to act as self-amplifying factors and upregulate each other *via* IL-1R signaling (7, 21–24). However, studies of IL-1 α and IL-1 β have produced conflicting results with regard to how these cytokines regulate each other. Studies focused on TLR triggers reported that these self-amplifying positive feedback mechanisms are redundant or not important to amplify the production of IL-1 α and IL-1 β further (25–29). These observations differed from studies using a genetic *Il1a* knockout mice (hereafter referred to as *Il1a*-KO^{line1}), which showed substantial reduction in IL-1 β production when *Il1a* was deleted (30–32), suggesting that IL-1 α may regulate IL-1 β expression even during TLR activation. These conclusions remained debated and poorly understood for many years.

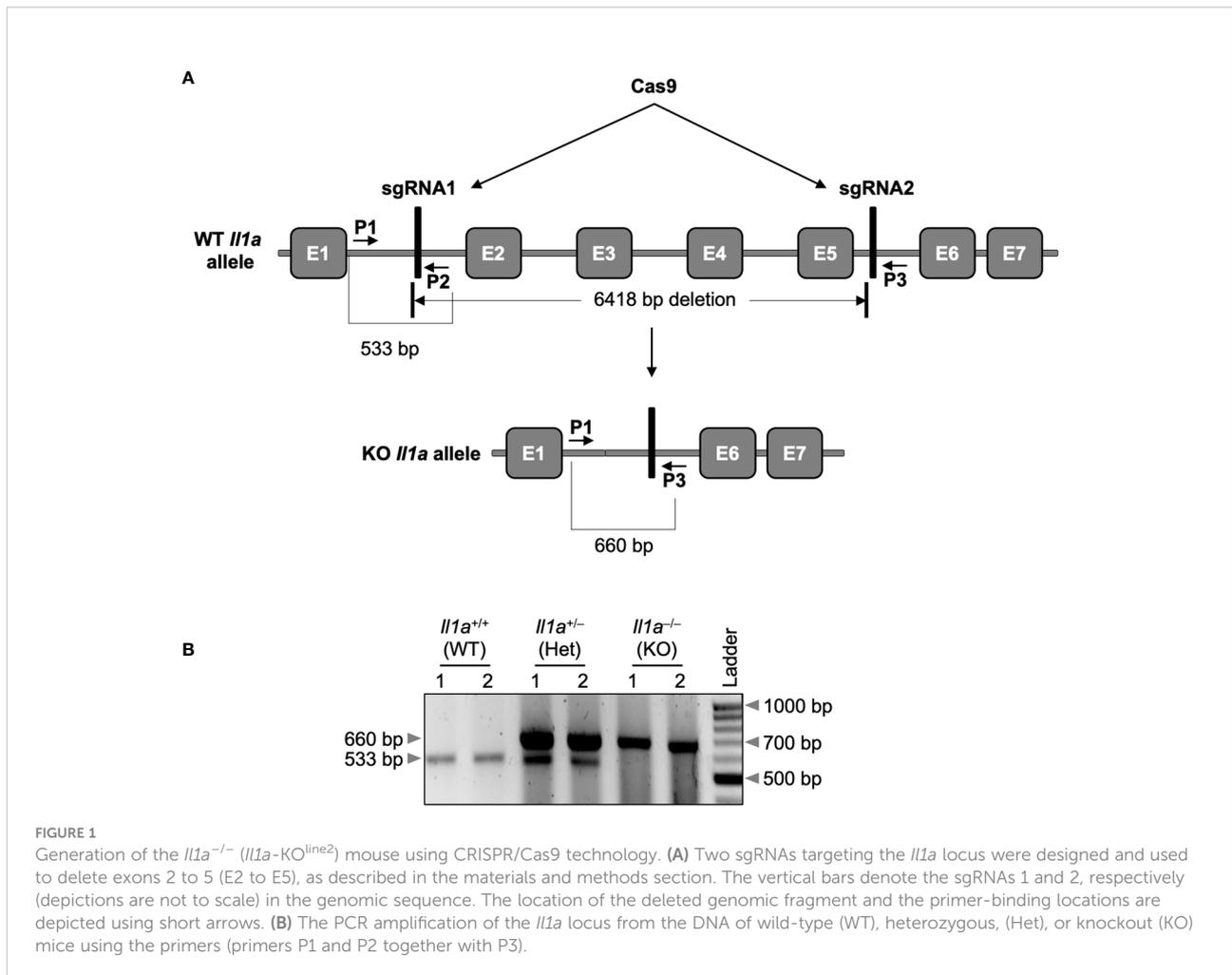
Therefore, we sought to generate a new line of *Il1a* knockout mice (hereafter referred to as *Il1a*-KO^{line2}) using CRISPR-Cas9 technology. The newly generated *Il1a*-KO^{line2} mice showed normal development, with comparable levels of basal immune cells in the blood compared with wild-type (WT) mice. Bone

marrow-derived macrophages (BMDMs) prepared from the *Il1a*-KO^{line2} mice showed no defect in expression or activation of inflammasome components in response to PAMPs and live pathogen triggers. Additionally, while the cells from *Il1a*-KO^{line1} showed reduced expression of both IL-1 α and IL-1 β , *Il1a*-KO^{line2} macrophages had no expression of IL-1 α but near-normal expression of IL-1 β . Moreover, the *Il1a*-KO^{line2} BMDMs showed a specific requirement of IL-1 α for the expression of neutrophil chemoattractant KC/CXCL1, further confirming the functional accuracy of the KO. In summary, we generated and characterized a new line of IL-1 α KO mice that improves upon the previous version and has normal IL-1 β expression. These mice can be broadly used for future studies on the unique functions of IL-1 α and IL-1 β to establish their relevance in health and disease and identify new treatment strategies.

Results

Generation of the IL-1 α KO (*Il1a*-KO^{line2}) mouse using CRISPR/Cas9 technology

Although IL-1 α has long been recognized as a critical regulator of inflammation and immune responses (8), its specific functions in physiologic and pathologic inflammatory outcomes in health and disease remain unclear. IL-1 α is subjected to complex regulation, and early genetic studies using different knockout mice produced conflicting observations (27, 30–32). To clarify the previously observed contradictory roles of IL-1 α in IL-1 β expression in *Il1a*-KO^{line1} mice, we generated a new line of IL-1 α knockout (KO) mice using CRISPR-Cas9 technology, referred to here as *Il1a*-KO^{line2} (Figure 1). Exons 2–5 of the *Il1a* gene were deleted by using simultaneous injection of two individual gRNAs with human codon optimized Cas9 mRNA (Figure 1A). We opted to use pronuclear-staged C57BL/6J zygotes for the injections to minimize the background-related genetic issues. Successful generation of the *Il1a*-deficient mice was assessed by targeted deep sequencing and further confirmed by PCR amplification of genomic DNA from the WT and mutant alleles (Figure 1B), and western blot analysis to confirm the loss of IL-1 α protein production (Figure 2A). Additionally, because IL-1 α is a multifaceted cytokine that we postulated may have a role in regulating immune cell phenotypes at basal levels, we evaluated the immune cellularity in the blood from the newly generated CRISPR *Il1a*^{-/-} mice (*Il1a*-KO^{line2}). We found that these mice did not show any gross abnormalities in the immune cellularity (Supplementary Figures 1A, B). In sum, we generated a new line of IL-1 α knockout mice, *Il1a*-KO^{line2}, and confirmed the loss of IL-1 α expression with no defects in overall blood immune cellularity.



CRISPR-based genetic deletion of *Il1a* does not affect IL-1 β expression or activation

Both IL-1 α and IL-1 β are known to be highly induced in response to pathogenic insults. Therefore, we next sought to characterize the cytokine expression in cells from the newly generated *Il1a*-KO^{line2} mice in response to PAMPs and pathogens. Treatment of BMDMs with lipopolysaccharide (LPS, a toll-like receptor 4 (TLR4) agonist from Gram-negative bacteria) induced robust and time-dependent expression of IL-1 α protein in WT cells but not in *Il1a*-KO^{line2} cells (Figure 2A). In addition, the induction of IL-1 α protein expression was not affected by *Il1b* genetic deletion, and the expression of IL-1 β in response to LPS was similar in the WT and *Il1a*-KO^{line2} cells (Figure 2A). In contrast, we observed a delay and reduction in the production of IL-1 β in the previously generated *Il1a*-KO^{line1} cells in response to LPS (Supplementary Figure 2A).

We next sought to further understand the potential roles for IL-1 α and IL-1 β in NLRP3 inflammasome priming, which is known to produce mature IL-1 β . We found that IL-1 α was not required for

upregulation of NLRP3 or IL-1 β expression in response to the innate immune triggers LPS, LPS plus ATP, Pam3CSK4 (Pam3) plus ATP, or Gram-negative bacteria *Escherichia coli* or *Citrobacter rodentium* (Figures 2A–C). Moreover, the activation of canonical and non-canonical inflammasomes, as measured by cleavage of caspase-1 and gasdermin D (GSDMD), was not reduced by deletion of *Il1a* (Figures 2B, C). Consistently, IL-1 β release was similar in WT and *Il1a*-KO^{line2} BMDMs (Figures 2D, E). In contrast, using similar experimental approaches, we observed defects in IL-1 β expression in macrophages from the earlier *Il1a*-KO^{line1} line, with pronounced reductions in IL-1 β expression at early time points in response to LPS, while the induction improved at later timepoints (Supplementary Figure 2A). We also observed reductions in IL-1 β expression in response to NLRP3 inflammasome triggers LPS plus ATP and Pam3 plus ATP (Supplementary Figure 2B). We did not observe defects in NLRP3 production or caspase-1 and GSDMD activation in *Il1a*-KO^{line1} cells (Supplementary Figures 2A, B). Together, these results show that while the previously generated *Il1a*-KO^{line1} mice had defects in IL-1 β production, *Il1a*-KO^{line2} mice did not share these defects.

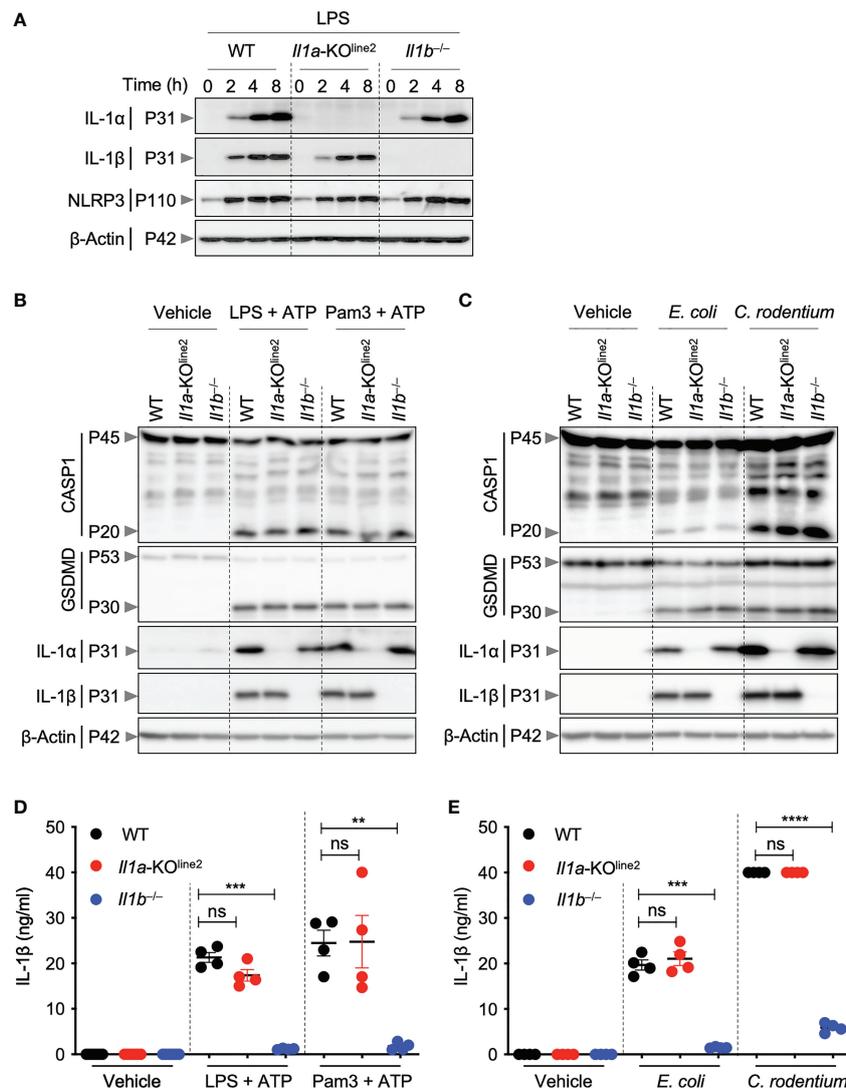


FIGURE 2

CRISPR-based genetic deletion of *Il1a* (*Il1a*-KO^{line2}) does not affect IL-1 β expression or activation. (A) Western blot analysis of pro-IL-1 α (P31), pro-IL-1 β (P31), NLRP3 (P110), and β -Actin (P42) in bone marrow-derived macrophages (BMDMs) treated with lipopolysaccharide (LPS) for indicated times. (B, C) Western blot analysis of pro- (P45) and activated (P20) caspase-1 (CASP1), pro- (P53) and activated (P30) gasdermin D (GSDMD), pro-IL-1 α (P31), pro-IL-1 β (P31), and β -Actin (P42) in BMDMs treated with LPS + ATP or Pam3CSK4 (Pam3) + ATP for 4 h (B), or BMDMs infected with *E. coli* or *C. rodentium* for 24 h (C). (D, E) Measurement of IL-1 β release in the cellular supernatants collected from BMDMs treated as detailed in panels (B) and (C), respectively, for (D) and (E). Western blot of β -actin was used as loading control. Data are representative of at least two independent experiments (A–E). Data are presented as the mean \pm SEM (D, E). Analyses of the *P* values were performed using the *t* test (D, E). ns, non-significant; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

CRISPR-based genetic deletion of *Il1a* confirms its critical role in the expression of the chemokine KC (CXCL1)

IL-1 α is a pleiotropic cytokine and critical amplifier of inflammation in response to both infection and sterile cellular insults (8). IL-1 α also plays key roles in regulating neutrophil-chemotactic factors such as the chemokine KC (CXCL1) in mice (33). Therefore, to further confirm the IL-1 α deletion in the

newly generated *Il1a*-KO^{line2} mice and assess its functional effects, we evaluated expression of TNF and KC in response to innate immune triggers. We specifically used LPS plus ATP as the stimulation to mimic the inflammasome activation conditions used above to determine differences in IL-1 β production, as this stimulus has been previously shown to be suitable for measuring inflammatory markers (34). We found that IL-1 α specifically was required to produce KC, but not TNF, in response to both PAMP- and pathogen-induced signaling in

macrophages; loss of IL-1 α resulted in significant decreases in KC release, while loss of IL-1 β did not decrease KC release (Figures 3A–D). Instead, we observed significantly increased levels of KC production in *Il1b*^{-/-} cells in response to LPS plus ATP and Pam3 plus ATP treatments (Figure 3A), suggesting a competition between IL-1 α and IL-1 β for IL-1R binding in this context, where the increased availability of IL-1R molecules for binding by IL-1 α may promote hyper-expression of select inflammatory factors in the absence of IL-1 β . Together, these findings confirm the specific role of IL-1 α for the release of KC, further supporting the functional relevance of the newly created *Il1a*-KO^{line2} mice for the evaluation of IL-1 α -mediated signaling and disease phenotypes.

Discussion

Members of the IL-1 family of cytokines play important roles as inflammatory mediators in host defense but have also been implicated in disease pathogenesis. Therefore, understanding the distinct functions of IL-1 family members is fundamental to our

understanding of the molecular basis of disease. Previous genetic models of IL-1 α deletion have displayed defects in IL-1 β production, making it difficult to determine the distinct roles of these molecules in immune responses. To overcome this obstacle, we report the generation of a genetic deletion of *Il1a* in mice using CRISPR technology that did not affect IL-1 β induction in response to microbial PAMPs and pathogens. Our findings suggest that expression of IL-1 β in response to TLR activation is not affected by loss of IL-1 α .

Growing evidence supports that IL-1 α and IL-1 β have distinct functions (35–37). Our findings further confirm that IL-1 α is a non-redundant positive regulator of the expression of the chemokine KC in macrophages, which is consistent with earlier studies reporting the specific role of IL-1 α in promoting production and recruitment of neutrophils in chronic inflammatory conditions (33, 38–40). The mechanism behind the differences between IL-1 α and IL-1 β in regulating KC requires further study but may be driven by the distinct localization of these cytokines. IL-1 α is unique in its ability to localize to the nucleus and directly regulate transcription (7),

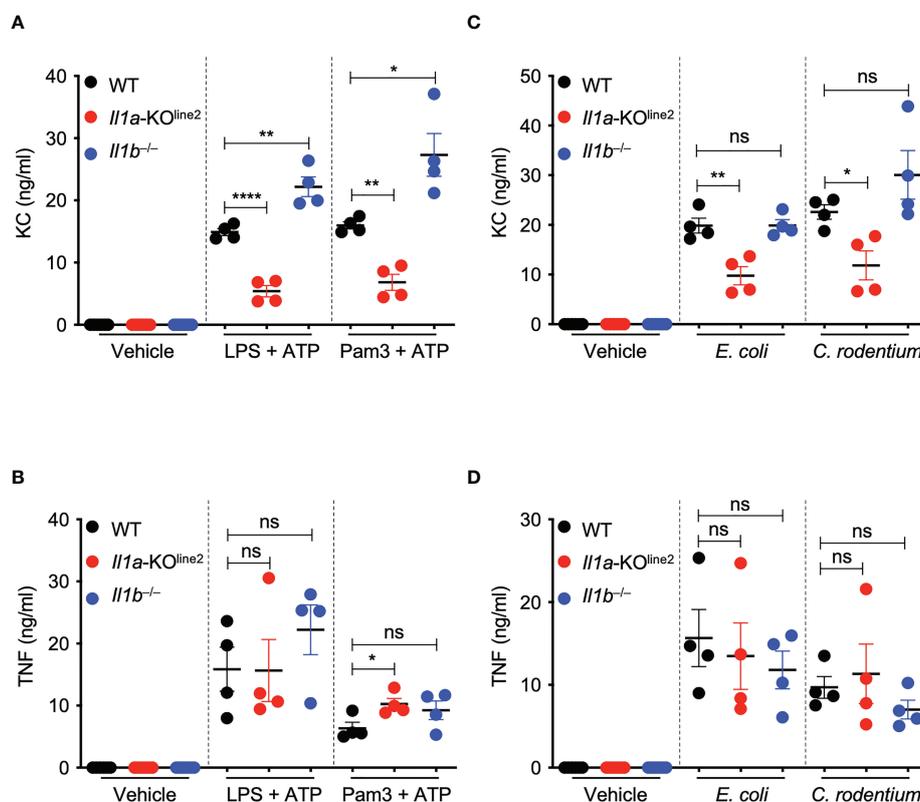


FIGURE 3

CRISPR-based genetic deletion of *Il1a* (*Il1a*-KO^{line2}) confirms its critical role in the expression of the chemokine KC (CXCL1). (A–D) Measurement of secreted cytokines KC and TNF in bone marrow-derived macrophages (BMDMs) treated with lipopolysaccharide (LPS) + ATP or Pam3CSK4 (Pam3) + ATP for 4 h (A, B) or infected with *E. coli* or *C. rodentium* for 24 h (C, D). Data are representative of at least two independent experiments (A–D). Data are presented as the mean \pm SEM (A–D). Analyses of the *P* values were performed using the *t* test (A–D). ns, non-significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.0001.

and it may selectively modulate the transcription of the *Cxcl1* gene. However, IL-1 β has also been shown to be important for the induction of neutrophil growth- and chemotactic-factors (41–45). Therefore, it is plausible that IL-1 β might also contribute to neutrophil-mediated inflammatory conditions as a result of the activation of cell death modalities that drive IL-1 β maturation *via* the activation of caspase-1 or other proteases (46), though this requires further study.

Additionally, previous studies using the earlier *Il1a*-KO^{line1} mice distinguished unique functions of IL-1 α and IL-1 β in the development of chronic autoinflammatory diseases (40, 42, 43, 47, 48). Our results suggest that the ability to use the *Il1a*-KO^{line1} mice to successfully identify this differential phenotype is due to the chronic nature of the disease. We found that the reduction of IL-1 β expression in *Il1a*-KO^{line1} cells was pronounced only at early time points following stimulation, and that prolonged stimulation resulted in similar levels of IL-1 β in WT and *Il1a*-KO^{line1} cells, in response to both PAMPs and pathogens. This suggests that WT and *Il1a*-KO^{line1} mice would have similar levels of IL-1 β during chronic disease, allowing differential phenotypes between *Il1b*^{-/-} and *Il1a*^{-/-} mice to be observed.

Given the critical roles of IL-1 family cytokines in inflammation and pathology, these cytokines have been targeted in several therapeutic strategies which have further highlighted unique functions for IL-1 α and IL-1 β . For example, the recent SAVE-MORE trial showed that anakinra, which blocks both IL-1 α and IL-1 β , reduced the risk of clinical progression in patients with COVID-19, when co-administered with dexamethasone (49). Accordingly, anakinra was authorized for the treatment of COVID-19 in Europe by the EMA. In contrast, the CAN-COVID trial, which was designed to evaluate the efficacy of canakinumab (a specific IL-1 β blocking antibody) failed to improve the survival of patients with COVID-19 (50). These studies further expand the concept that IL-1 α plays a dominant and potentially specific role in driving IL-1 β -independent inflammatory immune responses and pathology in some contexts.

Together, these observations show that caution should be used when interpreting previous studies and highlight the need to authenticate genetic resources for future work. The development of the *Il1a*-KO^{line2} mouse line, which does not seem to display acute or chronic defects in IL-1 β production, may help address many of the critical, long-standing questions in the field of immunobiology regarding the shared and unique functions and context-dependent interdependencies of IL-1 α and IL-1 β cytokines to improve understanding of the molecular basis of disease and inform therapeutic strategies.

Materials and methods

Mice

Il1b^{-/-} (51) and *Il1a*^{-/-} (*Il1a*-KO^{line1}) (52) mice were both previously described. *Il1a*^{-/-} (*Il1a*-KO^{line2}) mice were generated

in the current study and are described below. All mice were generated on or extensively backcrossed to the C57BL/6 background. All mice were bred at the Animal Resources Center at St. Jude Children's Research Hospital and maintained under specific pathogen-free conditions. Mice were maintained with a 12 h light/dark cycle and were fed standard chow. Animal studies were conducted under protocols approved by the St. Jude Children's Research Hospital committee on the Use and Care of Animals.

Generation of the new IL-1 α KO (*Il1a*-KO^{line2}) mouse strain

The new *Il1a*-KO^{line2} mouse was generated using CRISPR/Cas9 technology in collaboration with the St. Jude Transgenic/Gene Knockout Shared Resource facility. Pronuclear-staged C57BL/6J zygotes were injected with human codon-optimized *Cas9* mRNA transcripts (50 ng/ μ l) combined with two guide RNAs (120 ng/ μ l each; sgRNA1 for the 5' of exon 2: AAAAGCTTCTGACGTACCACagg, and sgRNA2 for the 3' of exon 5: AAGTAACAGCGGAGCGCTTTtgg (pam sequences are underlined)) to generate a long deletion encompassing exons (E) 2–5 of the *Il1a* gene (Figure 1A). Zygotes were surgically transplanted into the oviducts of pseudo-pregnant CD1 females, and newborn mice carrying the desired deletion in the *Il1a* allele were identified by PCR agarose gel-electrophoresis (Figure 1B) and Sanger sequencing. The WT allele was PCR amplified by using the primers IL1a_F1 (5'-GGGCACACGAATTCACACTCACA-3'; primer P1) and IL1a_R1 (5'-GGAGAACTTGGTTCCTGTTAGGGTGA-3'; primer P2), and the KO allele was amplified by using IL1a_F1 and IL1a_R2 (5'-TGATTAGCTTCCTTTGGGCTTTGA-3'; primer P3) primer pairs. The details of the generation of the CRISPR reagents were described previously (53). The uniqueness of sgRNAs and the off-target sites with fewer than three mismatches were found using the Cas-OFFinder algorithm (54).

Macrophage differentiation and stimulation

BMDMs were prepared as described previously (55). In short, bone marrow cells were cultured in IMDM supplemented with 30% L929 cell-conditioned medium, 10% FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin for 6 days to differentiate into macrophages. On day 6, BMDMs were counted and seeded at 10⁶ cells per well in 12-well culture plates in DMEM containing 10% FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin. iBMDMs (immortalized BMDMs from *Il1a*^{-/-} (*Il1a*-KO^{line1}) mice) were maintained in DMEM supplemented with 5% L929 cell-conditioned medium, 10% FBS, 1% nonessential amino acid, and 1% penicillin-streptomycin. Stimulations were performed

with LPS alone (100 ng/ml) for the indicated times, LPS (100 ng/ml) or Pam3 (1 µg/ml) for 3.5 h followed by the addition of ATP (5 mM final concentration) for 30 min, or *E. coli* (MOI, 20) or *C. rodentium* (MOI, 20) for 24 h.

Flow cytometry and analysis of cellularity

The cellular phenotypes of immune cells in the blood were analyzed either by flow cytometry (for T cell subsets and B cells) or by using an automated hematology analyzer machine (for % lymphocytes, % neutrophils, % monocytes, red blood cell (RBC) counts, hemoglobin (HB) quantification, and platelet (PLT) quantification). The following antibodies were used for cell staining: anti-CD19 (APC, clone ID3), anti-CD45.2 (FITC, clone 104), and anti-TCRβ (PECy7, clone H57-597) from Biolegend, and anti-CD8a (eFluor450, clone 53-6.7) from eBiosciences. Samples were assessed and data were acquired on LSR II Flow Cytometer from BD Biosciences and analyzed using the FlowJo software (Tree Star), version 10.2 (FlowJo LLC).

Western blotting

Samples for immunoblotting of caspase-1 were prepared by mixing the cell lysates with culture supernatants (lysis buffer: 5% NP-40 solution in water supplemented with 10 mM DTT and protease inhibitor solution at 1× final concentration); samples for all other protein immunoblotting were prepared without the supernatants in RIPA lysis buffer. Samples were mixed and denatured in loading buffer containing SDS and 100 mM DTT and boiled for 12 min. SDS-PAGE-separated proteins were transferred to PVDF membranes and immunoblotted with primary antibodies against IL-1α (503207, Biolegend), IL-1β (12426, Cell Signaling Technology), caspase-1 (AG-20B-0042, Adipogen), NLRP3 (AG-20B-0014, Adipogen), GSDMD (ab209845, Abcam), and β-Actin (sc-47778 HRP, Santa Cruz). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-Armenian hamster [127-035-099], anti-mouse [315-035-047], and anti-rabbit [111-035-047], Jackson ImmunoResearch Laboratories) were used as described previously (56). Immunoblot images were acquired on an Amersham Imager using Immobilon[®] Forte Western HRP Substrate (WBLUF0500, Millipore).

Cytokine analysis

Cytokines and chemokines were measured by multiplex ELISA (Millipore), as per the manufacturer's instructions.

Statistical analysis

GraphPad Prism 9.0 software was used for data analysis. Data are presented as mean ± SEM. Statistical significance was determined by *t* tests (two-tailed) for two groups.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the St. Jude Children's Research Hospital committee on the Use and Care of Animals.

Author contributions

RKSM and T-DK designed the study. RKSM, RRB, BK, and BS performed experiments. AB and SP performed the CRISPR-based knockout generation and initial breeding. RKSM, RRB, and T-DK analyzed the data. RKSM and RRB wrote the manuscript with input from all authors. T-DK oversaw the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

T-DK is a consultant for Pfizer.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1068230/full#supplementary-material>

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