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Cutting Edge: IL-6 Is a Marker of Inflammation with No Direct Role in Inflammasome-Mediated Mouse Models

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IL-6 is a known downstream target of IL-1β and is consistently increased in serum from patients with NLRP3 inflammasome-mediated conditions. Therefore, IL-6 could be a therapeutic target in the treatment of IL-1β–provoked inflammation. IL-6 was increased in serum with accompanying neutrophilia in tissues of an inducible mouse model of Muckle–Wells syndrome. However, an IL-6–null background failed to provide phenotypic rescue and did not significantly impact inflammatory cytokine levels. In a second model of IL-1β–driven inflammation, NLRP3 activation by monosodium urate crystals similarly increased IL-6. Consistent with our Muckle–Wells syndrome model, ablation of IL-6 did not impact an acute neutrophilic response in this in vivo evaluation of gouty arthritis. Taken together, our results indicate that IL-6 is a reliable marker of inflammation, with no direct role in inflammasome-mediated disease. The Journal of Immunology, 2012, 189: 000–000.

Interleukin-6 has diverse immune functions that can stimulate or inhibit inflammatory responses, depending on cellular context. As an example, IL-6 is crucial for neutrophil recruitment to sites of infection, yet it appears to be important in resolving immune responses to allow tissue repair. IL-6 can also impact the immune context itself by attracting monocytes and lymphocytes to replace neutrophils in shifting from innate to adaptive immunity. Additionally, IL-6 is a key cytokine in the development of the Th17 T cell response (1).

Early studies using Il6–null mice (Il6−/−) implicated an essential role for IL-6 in the immune response to various infectious organisms, including Listeria monocytogenes, Escherichia coli, Candida albicans, and Mycobacterium tuberculosis. Such mice exhibited profoundly reduced neutrophil titers, higher microbial loads, and reduced survival as a result of poorly mobilized inflammatory responses (2–5). Il6−/− mice have also been used to further elucidate the proinflammatory effects of IL-6 in models of collagen and Ag-induced arthritis, experimental autoimmune encephalomyelitis, colitis, and asthma (6–10). Later studies specifically examined IL-6 trans-signaling using a fusion molecule of IL-6 fused to a soluble IL-6R or, alternatively, a transgenic mouse overexpressing soluble gp130, the signal-transducing protein that complexes with IL-6R (11, 12). Trans-signaling promotes cell migration and upregulation of adhesion markers on endothelial cells, and it is crucial to various models of arthritis, chronic colitis, and peritonitis (13–16).

Although IL-6 is generally considered a proinflammatory cytokine, various studies demonstrated a dampening effect on the immune response in certain experimental models of osteoarthritis, airway inflammation, and UBV light-driven cutaneous inflammation (17–20). In these models, Il6−/− animals showed more extensive disease, with increased cytokine-mediated inflammation, leukocyte infiltration, and tissue damage. Furthermore, IL-6 was shown to induce PGE2, which may, in turn, inhibit synthesis of the proinflammatory mediators TNF-α and IL-1 (21–23).

One of the most potent activators of IL-6 production is the proinflammatory cytokine, IL-1β. Recent studies proved a central role for IL-1β in various autoimmune syndromes, complex pathologies thought to arise from dysregulation of the innate arm of the immune response. The cryopyrin-associated periodic syndromes (CAPS) are classic IL-1β–driven diseases consisting of three clinical entities: familial cold autoinflammatory syndrome, Muckle–Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disease. CAPS are caused by missense mutations in NLRP3 (cryopyrin) protein (24–26), a cytoplasmic sensor that nucleates the inflammasome, a multi-protein complex responsible for cleavage of IL-1β to its active, secreted form (27).

Although, IL-1β is difficult to quantify in the serum of patients with CAPS, ex vivo patient cells secrete high levels of cytokine in response to weak inflammatory stimuli, such as...
cold temperature (28). In contrast, IL-6 is readily measurable in serum from patients with familial cold autoinflammatory syndrome following cold room challenge, concurrent with leukocytosis and fever (29). Increased serum IL-6 was also demonstrated in a mouse model of MWS and normalized following treatment with mouse IL-1 TRAP, a regimen that increased life expectancy ∼25% (28). In a second, independent model of MWS, researchers demonstrated a Th17-skewed T cell response that potentiated disease (30), suggesting a pathogenic role for IL-6 as an upstream signal necessary to the Th17 lineage. Although dysregulation of IL-1β production appears to be the linchpin in autoinflammatory conditions, such as CAPS, downstream mediators through which IL-1β signals have not been well defined. Therefore, we elucidated the role of IL-6 in two inflammasome and IL-1β driven models: a knockin MWS mouse model and the s.c. air pouch model of gouty arthritis.

**Materials and Methods**

**Mouse strains**

The Nlrp3<sup>A350V/+</sup> strain was generated as previously described (28). Additional strains included B6.129P2-Lyzt<sup>tm1(cre)Ifo</sup> (myeloid Cre), B6.Cg-Tg(cre/Emx1)62Lmgm/J (inducible Cre), B6.129S2-Ile1<sup>tm1(cre)Kopf</sup>/J (Il1β-<sup>-/-</sup> -null), and B6.129S1-Ilt2<sup>tm1(cre)Imj</sup>/J (Il1r-<sup>-/-</sup>) (The Jackson Laboratory, Bar Harbor, ME). University of California at San Diego Institutional Animal Care and Use Committee approved all protocols.

**Mutant NLRP3 protein expression induction**

Fifteen- to eighteen-week-old Nlrp3<sup>A350V/+ CreT</sup> and wild-type (WT) mice were injected i.p. with 50 mg/kg tamoxifen-free base (MP Biomedicals, Solon, OH) in 90% sunflower seed oil from Helianthus annuus (Sigma, St. Louis, MO)/10% ethanol daily for 4 d, followed by twice weekly injections.

**Peripheral blood analysis**

Blood was obtained by submandibular puncture (adult mice) or following decapitation (neonates). IL-6 and IL-1β were measured by ELISA (R&D Systems, Minneapolis, MN). Multiplex cytokine analysis was performed using a Luminex assay (Bio-Rad, Hercules, CA), per the manufacturer’s instructions. Concentrations of serum amyloid A (SAA) were determined by ELISA (Tridelta Development, Kildare, Ireland).

**Histologic analysis**

Histologic analysis of mouse skin was performed, as previously described (29). p-STAT3 staining was done using primary Ab to detect endogenous levels of STAT3, only when phosphorylated at tyrosine 705, at a 1:400 dilution (#9145; Cell Signaling, Beverly, MA), secondary biotinylated goat Ab to rabbit IgG at a 1:200 dilution, and peroxidase-labeled avidin detection (Vector ABC PK-6101; Vector Laboratories, Burlingame, CA). Hematoxylin was used for counterstaining.

**Flow cytometric analysis**

Spleen cells were hypotonically lysed to remove RBCs; stained with anti–CD11b–PE and/or anti–GR-1–allophycocyanin (eBioscience, San Diego, CA), per the manufacturer’s instructions; and analyzed on a BD LSRII flow cytometer using FACSDiva software.

**Subcutaneous air pouch model**

Subcutaneous air pouches were generated and challenged, as previously described (31). Mice were sacrificed 6, 12, 18, or 24 h postinjection.

**Statistical analyses**

Statistical analyses and graphing were performed in Excel and GraphPad Prism programs with the Student t test. Flow cytometry data were analyzed using FlowJo software.

**Results and Discussion**

To elucidate the disease processes involved in CAPS-mediated inflammation, we used a mouse model carrying the alanine 350-to-valine mutation in Nlrp3, analogous to the human MWS mutation A352V. This line (Nlrp3<sup>A350V/+ CreT</sup>) does not express mutant NLRP3 unless a neomycin resistance cassette, placed upstream of the mutation, is first excised by cre recombinase, allowing us to take advantage of various cell-specific and inducible cre mouse systems. Nlrp3<sup>A350V/+ CreL</sup>, a mouse line expressing mutant NLRP3 restricted to the myeloid cell lineage, was shown to be a reliable model of the unprovoked innate immune inflammation characteristic of MWS, demonstrating a strong dependence on IL-1β and the inflammasome (28). Because Nlrp3<sup>A350V/+ CreL</sup> animals die within 2 wk of birth, we bred Nlrp3<sup>A350V/+ CreL</sup> mice to an inducible line (creT), creating Nlrp3<sup>A350V/+ CreT</sup> mice, in which nuclear translocation of cre and, therefore, expression of mutant NLRP3 are induced in adult animals through administration of tamoxifen.

Tamoxifen-induced expression of A350V-mutant NLRP3 produced an inflammatory phenotype consisting of steady weight loss (25% within 30 d) (Fig. 1A). Tamoxifen-treated Nlrp3<sup>A350V/+ CreT</sup> mice, but not WT animals, also developed skin inflammation with erythema and occasional ulceration. Analysis of skin sections stained with H&E revealed neutrophilic infiltrates in the dermis (Fig. 1B). These findings were similar to those noted in animals expressing mutant NLRP3 from birth (28). Flow cytometric analysis of spleens from tamoxifen-treated Nlrp3<sup>A350V/+ CreT</sup> mice showed a neutrophilic infiltrate, with a 4-fold increase in the percentage of GR-1/CD11b double-positive cells (Fig. 1C). Mice also exhibited
peripheral blood leukocytosis as the result of increased production of neutrophils, along with a concomitant lymphopenia, mild anemia, and thrombocytosis (Fig. 1D, data not shown). Additionally, Nlrp3\textsuperscript{A350V/}\textsuperscript{CreT} animals had significantly increased serum IL-6 posttamoxifen treatment, whereas cytokine levels in WT controls did not change (Fig. 1E). Certain mice with elevated serum IL-6 and/or neutrophilia pretamoxifen treatment did not demonstrate further elevated cytokine or neutrophil levels. These animals likely had increased inflammation pretreatment due to “leaky” nuclear translocation of cre, an observation previously noted (32). IL-1\textbeta and TNF-\alpha levels in tamoxifen-treated Nlrp3\textsuperscript{A350V/}\textsuperscript{CreT} mice were highly variable or were not measurable (data not shown). Given the dependence of IL-6 on IL-1\textbeta in our mice (28), we considered IL-6 an excellent marker for the onset of pathology in our inducible Nlrp3\textsuperscript{A350V/}\textsuperscript{CreT} mice, as well as a potentially important downstream therapeutic target in CAPS disease progression.

To test whether IL-6 is indeed important in pathogenesis, we bred Nlrp3\textsuperscript{A350V/}\textsuperscript{neoR/+} mice to Il6\textsuperscript{-/-} mice and then bred the resulting offspring to Cre. Il6\textsuperscript{-/-} mice. Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} pups developed skin abscesses within 1 d postbirth, giving way to scaling erythema, lack of pigmentation, alopecia, and growth delay by day 4 (Fig. 2A, 2B). All pups died within 2 wk (Fig. 2B). H&E staining analysis revealed neutrophilic infiltrates in the skin of Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} (Fig. 2C), as well as other organs, including lung, pancreas, liver, and spleen (data not shown). There was an observable progression of neutrophilic inflammation in tissues over time. Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} (knockout) animals were visually and pathologically indistinguishable from Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} littermates (heterozygotes) and Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice (Fig. 2A, 2C, Supplemental Fig. 1A, 1B, data not shown). Immunohistochemical staining for p-STAT3, a downstream signaling target of IL-6, revealed nuclear staining in multiple tissues and cell types, including neutrophils, demonstrating widespread cytokine-mediated inflammation equivalent in affected mice on both sufficient and deficient IL-6 backgrounds (Supplemental Fig. 1C).

Multiple cytokines and chemokines were upregulated in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} serum, including IL-1\textbeta and IL-6 (28). Most of these mediators were similarly elevated in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} animals (Fig. 3), whereas IL-6 was not measured, as expected. Interestingly, keratinocyte chemoattractant was significantly increased in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice compared with Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} mice (Fig. 3), suggesting that IL-6 could have a dampening effect on production of this mediator. In addition, a trend toward an increase in IL-18 was noted (Fig. 3). Lack of IL-6 signaling did not abrogate production of the inflammasome-mediated cytokine IL-1\textbeta. Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice also did not display significant differences in TNF-\alpha or additional downstream cytokines and chemokines (G-CSF, GM-CSF, MCP-1, IL-4, IL-9, IL-10, and IL-13) shown to be elevated in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} mice in our previously published report (Fig. 3, data not shown) (28). Similar to human CAPS patients, SAA levels were markedly increased in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} animals (33). Levels of this systemic inflammatory marker were significantly reduced in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice, consistent with the dependence of SAA levels on IL-6 signaling.

The lack of phenotypic rescue on the Il6\textsuperscript{-/-} background was surprising, given the prominent position of IL-6 in the IL-1\textbeta-signaling cascade and its increased presence in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} serum and skin (28). Therefore, it is possible that this cytokine is a marker for disease but is not actually pathogenic in CAPS. To extrapolate beyond CAPS to other inflammasome-mediated conditions, we investigated the

**FIGURE 2.** IL-6 is not required for disease in mice expressing A350V-mutated NLRP3. (A) At 6 d postbirth, Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} and Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice exhibit runting, skin inflammation, and alopecia compared with Nlrp3\textsuperscript{A350V/}\textsuperscript{neoR/+} littermate controls with no expression of mutated NLRP3. (B) Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} and Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice have similar survival curves, with no mice surviving >14 d (n > 10 for all groups). Error bars shown for mean weights on growth curves are SD. (C) H&E stains of Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} mice and Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice show similar neutrophilic infiltrates in the dermis (original magnification X20). The photomicrograph is a representative of skin sections taken from three mice.

**FIGURE 3.** Effect of IL-6 ablation on serum mediators and inflammatory markers. With the exception of keratinocyte chemoattractant and IL-6, no significant difference in any of the mediators examined was observed in serum from Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} mice and Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice. SAA levels were significantly elevated in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL} serum and markedly reduced in Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} mice. No significant difference in any mediator or SAA was observed between WT mice and Nlrp3\textsuperscript{A350V/}\textsuperscript{CreL Il6\textsuperscript{-/-}} littermate controls, with no expression of mutated NLRP3. Each dot represents an individual mouse.
role of IL-6 in an air pouch model, an artificially induced environment used to simulate the synovium in gouty arthritis (31). Injection of monosodium urate crystals, previously shown to activate the NLRP3 inflammasome (34), resulted in comparable neutrophil recruitment to the pouch within 6 h in both Il6−/− and WT mice (Fig. 4A). Similar neutrophil-clearance rates were also observed at later time points in both groups (Supplemental Fig. 2). In contrast, neutrophil levels in Il1r−/− mice were not significantly different from saline-injected controls, as previously reported (35). Exudate fluid from WT, but not Il1r−/−, air pouches contained increased IL-6 (Fig. 4B). No IL-6 was detected in Il6−/− fluid, as expected.

The air pouch system is a simple model of NLRP3 inflammasome activation resulting from danger-associated molecular pattern sensing, whereas murine CAPS is an in vivo model of NLRP3 inflammasome dysregulation the results from an intrinsic defect. Given the direct effect of IL-1β on IL-6 secretion, as well as the known synergy between the two cytokines in promoting various processes, such as Th17 polarization, we expected IL-6 to be critically important for full elaboration of IL-1β–dependent inflammation. However, our data suggest that downstream inflammation resulting from inflammasome activation is IL-6 independent. This is supported by studies using zymosan, an activator of the NLRP3 inflammasome (36). The acute inflammation of nonimmune-mediated arthritis induced by zymosan is IL-6 independent (37). Additionally, Il6−/− animals respond similarly to controls when challenged with LPS, a well-known inflammasome activator (3, 38).

The only reported use of an IL-6–targeted therapy in a severely affected CAPS patient resulted in an incomplete clinical response, with persistent tissue inflammation and an unfavorable outcome (39), consistent with our conclusion that CAPS is IL-6 independent. Our results suggest that IL-1β and IL-6 may not be coupled in pathogenesis; instead IL-6 is simply a marker of inflammation without having a significant causative role. In contrast, IL-6–targeted therapy was shown to be effective in the treatment of resistant systemic-onset juvenile idiopathic arthritis (40), despite the clear role of IL-1β in this systemic inflammatory disorder (41). The clinical relevance of IL-6 in other disorders along the autoinflammatory spectrum remains to be determined; however, targeting IL-6 in the context of human CAPS and acute gouty arthritis is likely not an effective therapeutic option, despite elevated tissue and serum IL-6.

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Disclosures

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