IL-1 INHIBITION

EDITED BY: Francesca Oliviero, Paolo Sfriso, Leonardo Punzi and Jean-Michel Dayer

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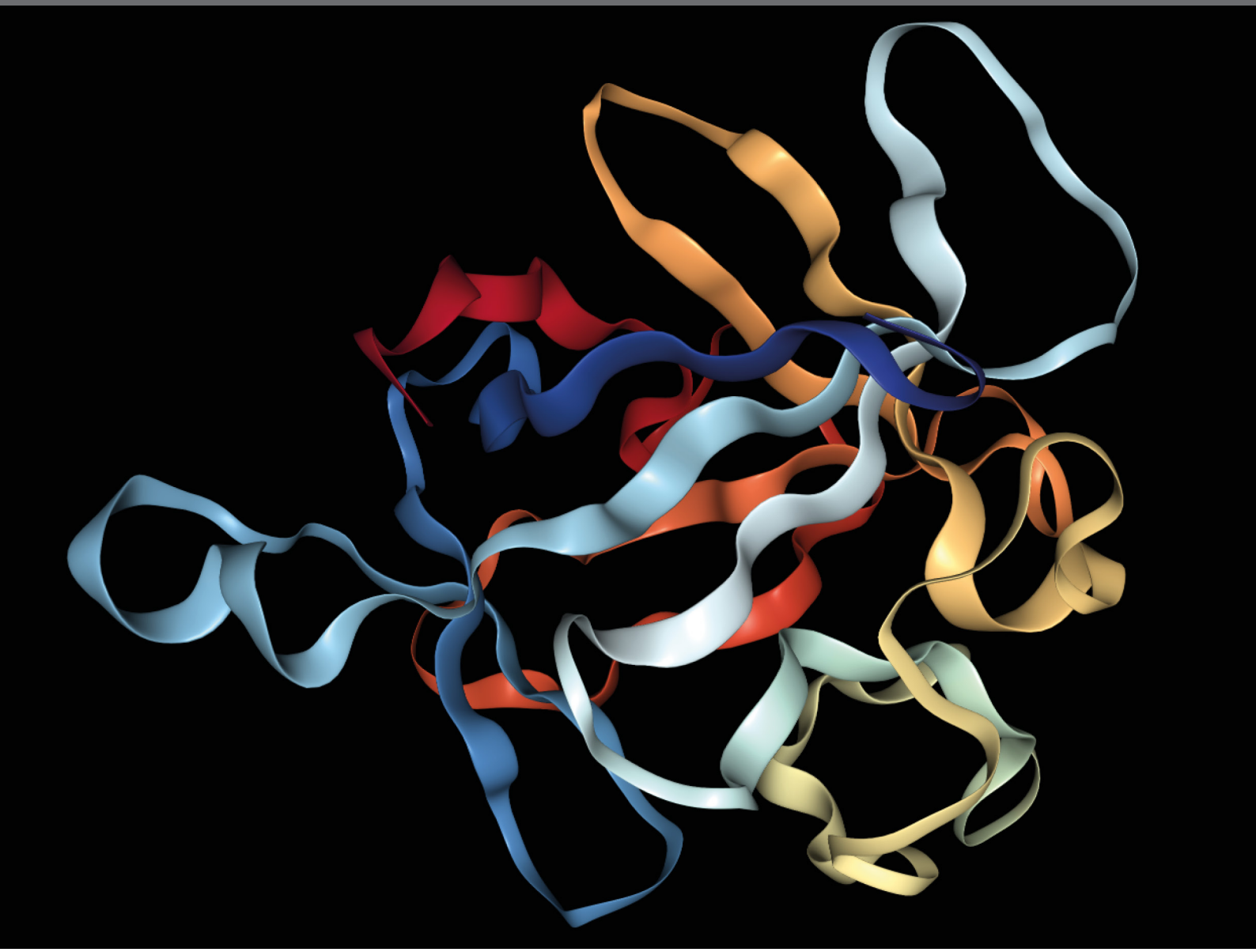

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IL-1 INHIBITION

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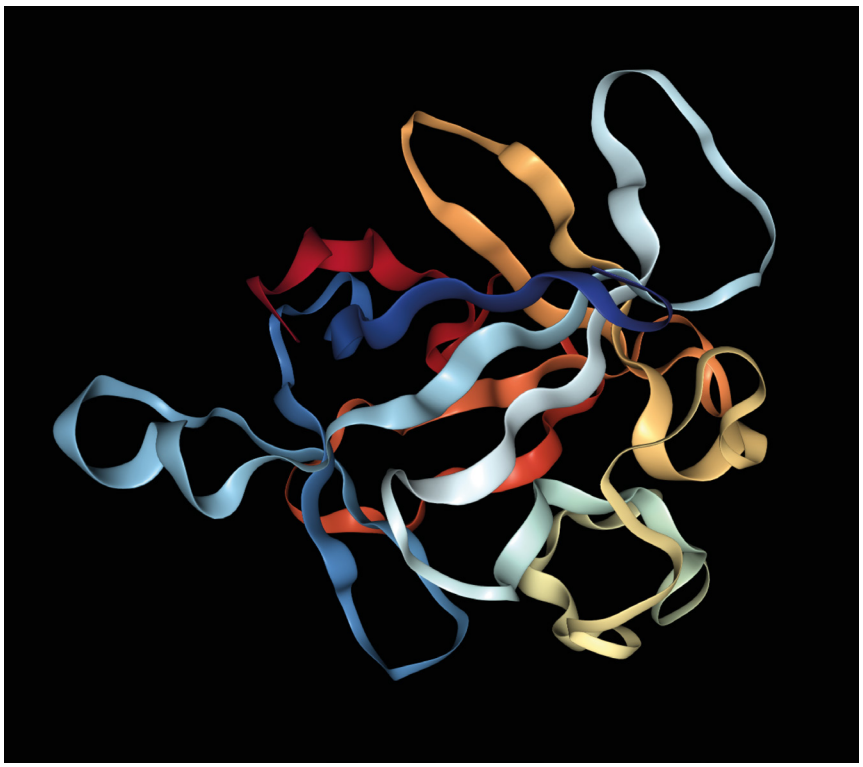

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This special article collection of *Frontiers in Pharmacology* includes reviews and original articles on different aspects of IL-1 inhibition. Since the time IL-1 and its natural antagonist IL-1Ra have been discovered, specific IL-1 targeted therapies have been developed to cure an increasing number of diseases.

The purpose of this Research Topic is to provide an overview of the different clinical uses of IL-1 blockade and new insights in basic research issues.

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NOVEL INSIGHT ON IL-1 PRODUCTION, RELEASE AND REGULATION

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EDITORIAL
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Editorial: IL-1 Inhibition

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Keywords: IL-1 (interleukin-1), IL-1 blockade, anakinra, canakinumab, rheumatic diseases, inflammatory chronic diseases, autoinflammatory diseases

Editorial on the Research Topic

IL-1 Inhibition

Interleukin 1 (IL-1) is a crucial mediator of the inflammatory response, playing an important part in the body's natural responses and the development of acute pathological conditions leading to chronic inflammation and tissue destruction. In the last decades, specific IL-1-targeting therapies have produced beneficial effects in a wide spectrum of IL-1 driven diseases, the most dramatic one being autoinflammatory disorders. Indeed, IL-1 continues to be a key attractive therapeutic target for many inflammatory, metabolic, skin, and heart disease. The main purpose of this Research Topic is to discuss and evaluate some current knowledge on IL-1 biology and the rational approach toward its blockade in different conditions. The topic covers both basic scientific as well as clinical aspects and includes 8 reviews, 8 original articles, 1 perspective, and 1 opinion. The topic focusing mainly on IL-1ß and its antagonism does not preclude for the importance of other members of the IL-1 family.

Back to history, Dayer et al. reminds the discovery of the monocytic origin of IL-1 in Rheumatology, the seminal description of the mechanism of IL-1 receptor antagonist (IL-1Ra) acting as ligand binding to its IL-1 receptor and its change levels in serum of adult-onset Still's disease.

The inflammasome, the expansion of the IL-1 family, as well as the role on the IL-1 and IL-1Ra in crystal induced arthropathies, osteoarthritis and in cartilage and bone biology, are discussed.

Following a useful update on IL-1, Cavalli and Dinarello review the importance of IL-1 blockade by anakinra, the recombinant form of the naturally occurring IL-1Ra. The Authors focus on anakinra treatment of a broad spectrum of acute as well as chronic inflammatory diseases, ranging from rare autoinflammatory diseases to common conditions such as gout and rheumatoid arthritis, type 2 diabetes, atherosclerosis, and acute myocardial infarction.

The efficacy of IL-1 blockade in autoinflammatory-associated skin diseases is extensively described in the article by Fenini et al. The Authors review the different IL-1ß antagonists available in the market, inflammasome inhibitors and IL-1α and IL-18 blockers under development and investigation in both monogenic and polygenic autoinflammatory diseases (Note of Editors: The biotherapies in adult-onset Still's disease and other rare inflammatory disorders, including blockade of IL-18 has been more recently discussed in the editorial of Guilpain et al., 2018).

The use of IL-1 inhibitors in the management of systemic juvenile idiopathic arthritis is discussed in the article of Giancane et al. As not all patients respond to anti-IL-1 therapy in that disease, the Authors argue about the possible causes of the different clinical responses focusing on the heterogeneous nature of systemic juvenile idiopathic arthritis.

Dusser and Koné-Paut discuss the key role of IL-1 inhibition in Kawasaki disease which shares phenotypical and immunological similarities with systemic juvenile idiopathic arthritis. Few clinical trials on IL-1 blockade in young patients affected with Kawasaki disease have been conducted and the Authors discuss the importance of this therapeutic approach to treat refractory forms.

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The article of Colafrancesco et al. reports clinical results obtained from a large Italian multicentric retrospective observational study conducted in patients with adult-onset Still's disease. In this study, the efficacy and safety of two IL-1 inhibitors are evaluated in patient's refractory to other therapies. A good response was noted at 3 months after therapy onset in both groups.

Peiró et al. summarize the main experimental and clinical findings obtained with pharmacological IL-1β inhibitors in patients with diabetes mellitus and cardiovascular complications and discuss the perspectives of IL-1β inhibitors as novel therapeutic tools for treating these patients. (Note of Editors: In fact, just recently a large clinical study "CANTOS" shows promising results both in biological parameters and clinical outcomes (Ridker et al., 2017). At the 2018 Annual Meeting of the American College of Rheumatology, IL-1β inhibition with Canakinumab (Schieker et al., 2018) was shown to be associates with reduced rates of total hip and knee replacement and osteoarthritis).

Vitale et al. describe a nationwide Italian observational study on the on-label and off-label use of IL-1 inhibitors in children and adults. The Authors report demographic, clinical, and therapeutic data highlighting the wide use of these drugs as off-label indications in Italy. Their data confirm the good safety profile of IL-1 inhibitors.

The safety of anakinra combined with immunosuppressive drugs is discussed in the article of Mulders-Manders et al. They report three case series of patients undergoing renal transplantation treated with IL-1Ra in addition to common therapy during the pre- and post-operative period. These anecdotal reports illustrate a possible protective effect of anti-IL-1 therapy after solid organ transplantation. Peri- and postoperative use of anakinra is safe and effective in patients undergoing renal transplantation.

In the article of Assier et al. anti-cytokine vaccination approaches in autoimmune models are discussed with regards to IL-1 target. The Authors review the advantages and the limits of more specific targeting of IL-1β obtained with vaccination pointing out the promising results achieved with a peptide-based vaccine.

A second group of papers of this Research Topic focuses on basic scientific aspects. Novel insights in IL-1 production and release pathways, as well as its regulation in some rheumatic diseases are discussed.

Giuliani et al. review the role of P2X7 receptor (P2X7R), one of the molecules involved in IL-1β maturation. This ATP-gated ion channel acts through the recruitment of the NACHT-LRRPYD-containing protein-3 (NLRP) 3 inflammasome -caspase-1 complex. The authors summarize evidence supporting the role of the P2X7R in IL-1ß production, with special emphasis on the mechanism of release, a process that is still a matter of controversy. They discuss four different mechanisms of release: exocytosis via secretory lysosomes, microvesicles shedding from plasma membrane, release of exosomes, and passive efflux across a leaky plasma membrane during pyroptotic cell death.

In their article, Madej et al. investigate a novel mechanism of IL-1ß release and propose that innate memory appeared to limit the amount of active IL-1ß produced by macrophages in response to a bacterial challenge, while enhancing the responsiveness of monocytes.

The paper by Nasi et al. revisit the role of IL-1 in osteoarthritis. Through the meniscectomy model of murine osteoarthritis, the Authors do not support the role of IL-1α and ß as key mediators in that disease.

Armbruster et al. describe the protective effects of foamy viral vectors expressing IL-1Ra in an *in vitro* model of chondrogenesis. The Authors show that these vectors are capable of efficient gene transfer to mesenchymal stem cells which, in turn, efficiently block the effects of IL-1β. This gene transfer tool for mesenchymal stem cells could be a base for therapies for cartilage repair.

Oliviero and Scanu discuss various endogenous and exogenous factors involved in the self-limiting course of crystal-induced inflammation. The authors pay attention to novel mechanisms that intervene in the resolution of this process through the inhibition of IL-1ß production. Resolution of the inflammation remaining an important issue.

The importance of the regulation of IL-1ß production in crystal-induced inflammation is discussed in the paper of Khameneh et al. In their original research carried out in a murine model of urate crystal-induced peritonitis, the Authors identify the complement protein C5a as a key determinant of IL-1-mediated inflammatory cell recruitment. C5a generated upon MSU-induced complement activation increases neutrophil recruitment *in vivo* by promoting IL-1 production via the generation of reactive oxygen species, which activate the NLRP3inflammasome.

Finally, two articles investigate the beneficial effects of plant-derived natural compounds in *in vitro* and animal models of inflammation.

Liang et al. demonstrate that piperine can reduce adenosine triphosphate -induced pyroptosis in macrophages and to decrease systemic IL-1ß levels in a murine bacterial sepsis model.

The effect of curcumin is discussed in the original research by Kong et al. In this paper the authors show how this natural polyphenol effectively suppressed the expression of NLRP3 inflammasome in macrophages involving toll-like receptor (TLR)4/myeloid differentiation primary response 88 (MyD88)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and P2X7R pathways.

Overall, the various contributions provide an overview on therapeutic potential of IL-1 inhibition and discuss some original approaches on IL-1 production, regulation and action.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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A Brief History of IL-1 and IL-1 Ra in Rheumatology

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The history of what, in 1979, was called interleukin-1 (IL-1), orchestrator of leukocyte inter-communication, began many years before then, initially by the observation of fever induction via the endogenous pyrogen (EP) (1974) and then in rheumatology on the role in tissue destruction in rheumatoid diseases via the induction of collagenase and PGE_2 in human synovial cells by a mononuclear cell factor (MCF) (1977). Since then, the family has exploded to presently 11 members as well as many membrane-bound and soluble receptor forms. The discovery of a natural Interleukin-1 receptor antagonist (IL-1Ra) in human biological fluids has highlighted the importance of IL-1 and IL-1Ra in human diseases. Evidence delineating its role in autoinflammatory syndromes and the elucidation of the macromolecular complex referred to as "inflammasome" have been instrumental to our understanding of the link with IL-1. At present, the IL-1blockade as therapeutic approach is crucial for many hereditary autoinflammatory diseases, as well as for adult-onset Still's disease, crystal-induced arthropathies, certain skin diseases including neutrophil-triggered skin diseases, Behçet's disease and deficiency of IL-1Ra and other rare fever syndromes. Its role is only marginally important in rheumatoid arthritis and is still under debate with regard to osteoarthritis, type 2 diabetes mellitus, cardiovascular diseases and cancer. This brief historical review focuses on some aspects of IL-1, mainly IL-1β and IL-Ra, in rheumatology. There are many excellent reviews focusing on the IL-1 family in general or with regard to specific diseases or biological discoveries.

Keywords: interleulin-1, interleukin-1 antagonist, rheumatoid arthritis, inflammasome, autoinflammatory diseases

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IL-1 AND ITS ROOTS

Historically in rheumatology, the seminal observation of the production of human interstitial collagenase (later called MMP-1) by human synovial cells in patients with rheumatoid arthritis (RA) led to the unraveling of the link between matrix degradation and the biological function of inflammatory molecules produced by immune cells. It was found that the main cellular source of both interstitial collagenase (matrix metalloproteases, MMPs) and prostaglandin E_2 (PGE_2) in synovial tissue were adherent stellate fibroblast-like cells (ASC), also called type B fibroblastic synovial lining cells (Dayer et al., 1976). Presently, the fibroblast subpopulation in inflamed synovial tissue has proved to be far more complex (Croft et al., 2016).

The question was which are the driving forces for stimulating the synovial cells to produce MMP and PGE_2. Seminal studies at the Arthritis Unit, Massachusetts General Hospital, Harvard Medical School) have shown that partial purification of the conditioned medium revealed a protein of approximately 15 kDa, and the factor responsible for this biological activity was

called mononuclear cell factor (MCF) (Dayer et al., 1977a,b, 1981) (**Figure 1**). Interestingly, interactions between T cells and monocyte-macrophages were found to play an important role in the production of IL-1 by monocyte-macrophages, giving rise to the first descriptions of the pathways going from lymphocytes to monocyte-macrophages and synovial fibroblast cells (Dayer et al., 1979a,b).

At this early stage of the discovery, before the cloning, different investigators analyzed the soluble factors derived from leucocyte culture supernatants in their respective functional bioassays in order to identify the putative functional specificity. Purified 'MCF' proved to have similar properties to the lymphocyte-activating factor (LAF), a factor which had the same molecular weight of approximately 15 kDa and shared chromatographic as well as other biochemical properties (Mizel et al., 1981). It was not until 1979 that the nomenclature and the terminology of interleukin-1 (IL-1), orchestrator of leukocyte communication, was coined at a meeting (Ermatingen, Switzerland) and it referred to products previously identified by several investigators using different bioassays, including lymphocyte activating factor (LAF), MCF, B lymphocyte activating factor (BAFF) and of course endogenous pyrogen (EP) that induced fever. At that time, cytokines were defined only by biochemistry (Aarden et al., 1979). It was defined for the biochemistry properties by a m.w. between 12,000–18,000 with an isoelectric point of 4.5–5.5 in mouse and 6.5–7.5 in human, pH 2 insensitive, does not contain Ia and for the biologic properties as produced by macrophages, with H-2 unrestricted activity, unrestricted species activity, distinguishing bioactivity and does not possess the ability to promote and maintain *in vitro* long-term cultures of T cells in contrast to IL-2.

When human IL-1 was cloned using an antibody to EP and based on its EP bioactivity, the protein was expressed and the recombinant IL-1 found to have the same biological functions as MCF (Dayer et al., 1986). However, it must be emphasized that in addition to IL-1, natural tumor necrosis factor-cachectin (TNFα) was also demonstrated early on to induce MMP and PGE2 production in synovial cells (Dayer et al., 1985).

As far as LAF is concerned, despite the initial description of its role in the early activation of murine T cells from thymocytes, diseases characterized by an adaptive immune response to specific autoantigens are usually not part of the spectrum of IL-1β-driven disorders.

With regard to the field of rheumatology, other partially purified molecules like catabolin – inducing cartilage degradation – and osteoclast-activating factor (OAF) inducing calcium[45] release from bone, to name but a few, were also found to have functions similar to those of IL-1 (Dayer, 2004).

The discovery of the other aspects of the IL-1 and IL-1 receptor families, related to the seminal features of EP and to several other important functions, has been extensively reviewed (Dinarello, 2010, 2015; Dinarello and van der Meer, 2013; Garlanda et al., 2013; Fantuzzi, 2016) as well as the biology of IL-1 alpha (Di Paolo and Shayakhmetov, 2016). The role of IL-1 in rheumatology and its specific interaction with MMPs have also been recently reviewed (Schett et al., 2016; Dayer et al., 2017).

THE STORY BEHIND IL-1Ra

As early as 1984, before IL-1 was cloned, the existence of a potential natural inhibitor to IL-1 was suspected. In the bioassay for stimulating collagenase and prostaglandin production, no IL-1 biological activities were detected in the serum or urine of febrile patients (Balavoine et al., 1984). In the light of that finding, Dayer and colleagues (University of Geneva, Switzerland) reported in 1984 that, after biochemical purification,

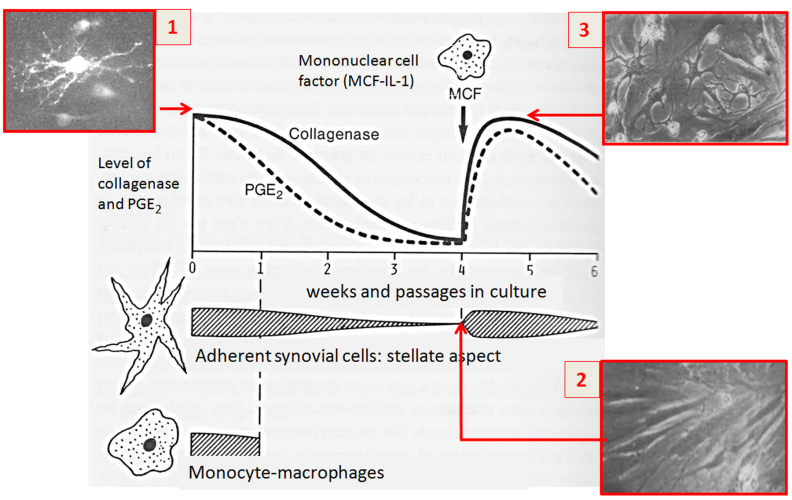

FIGURE 1 | Seminal observation about the link between matrix degradation and the biological function of inflammatory molecules produced by immune cells (1976–1977). (1) Cultures of synovial explants from patients with rheumatoid arthritis release large quantities of collagenase and prostaglandins (PGE2) into the media. Secretion of PGE2 declines more rapidly with respect to collagenase whose levels decline after different passages in culture; (2,3) The addition of monocyte-macrophages or their culture supernatants restored collagenase and PGE2 levels. The soluble factor responsible of this effect was later identified in mononuclear cell factor (MCF).

IL-1 was masked by a factor of approximately 17 kDa in the urine of febrile patients with monocytic leukemia and juvenile rheumatoid arthritis (JRA). This seminal observation was presented in 1984 at the Fourth International Lymphokine Workshop (Balavoine et al., 1984). The factor specifically blocked the biological activities of IL-1 (Dayer, 1985; Balavoine et al., 1986).

The first description of the concept underlying the interleukin-1 receptor antagonist (IL-1Ra) was that this natural antagonist could block the binding of a cytokine from the same family to its receptor without affecting those of TNFα (Seckinger et al., 1987a,b). In the field of cytokine, it was the first observation of an inhibitor factor that blocks ligand binding of cytokine and its activities. The discovery of the competitive binding assay of the IL-1 inhibitor (apparent m.w. around 17 kd) to IL-1 at the IL-1 receptor level was crucial for researchers at Synergen (Boulder, CO, United States) who purified and cloned IL-1Ra in 1990. Before IL-1Ra was cloned, the first clinical description of natural IL-Ra was made by following the time course of this specific IL inhibitor in the serum and urine of children with systemic juvenile chronic arthritis, a typical inflammatory syndrome (Prieur et al., 1987). Arend et al. (1985) reported the presence of an inhibitor of IL-1 activity under *in vitro* conditions, but neither its molecular weight nor the concept of binding to the IL-1 receptor was identified.

IL-1 AND ITS EXTENDED FAMILY

First described as a secreted product of monocytes and neutrophils ∼35 years ago, interleukin (IL)-1 refers to IL-1α and IL-1β. At present, the IL-1 family comprises a total of 11 members, consisting of the two activating cytokines IL-1α and IL-1β, the IL-1Ra, as well as IL-18, IL-33, four isoforms of IL-36 [IL-36α, IL-36β, IL-36γ and IL-36 receptor antagonist (IL-36Ra)], IL-37 and IL-1 family member 10 (also known as IL-38). IL-1 is processed and activated by a caspase-1-dependent mechanism as well as by caspase-1-independent processes that involve neutrophil proteases (see history in Fantuzzi, 2016).

Interleukin-1 receptor antagonist, IL-36Ra and IL-37 are predominantly anti-inflammatory cytokines. IL-36Ra shares homology with IL-1Ra but is unable to bind to the IL-1R1 (Netea et al., 2015). It has an important role in regulating skin inflammation and its deficiency (linked to mutations in IL-36RN) is associated to a rare form of pustular psoriasis also called deficiency of IL-36-receptor antagonist or DITRA (Marrakchi et al., 2011). Skin lesions, along with multifocal osteomyelitis and periostitis are associated also to IL-1Ra deficiency, termed DIRA, an autosomal recessive autoinflammatory disease caused by mutations affecting IL1RN (Aksentijevich et al., 2009).

INFLAMMASOME, THE MACROMOLECULAR COMPLEX

The discovery of the macromolecular complex inflammasome resulted from observations made by investigators working with rare diseases (autoinflammatory syndromes), experts in apoptosis, and biochemists analyzing how cells sense the presence of danger. The first to identify the mechanism was a French consortium of scientists in 1997, which was due to the mutation causing familial Mediterranean fever (FMF). The mutated gene encoding an unusual structure of unknown function – named at that time marenostrin (from *mare nostrum,* referring to the Mediterranean Sea) – a pyrin involved in periodic fevers (French FMF Consortium, 1997). The protease which processes interleukin-1β [IL-1β-converting enzyme (ICE) = caspase-1] was identified in 1989 (Black et al., 1989) and cloned in 1992 (Cerretti et al., 1992; Thornberry et al., 1992). The identification of CARDIAK, a death domain associated with caspase-1, was identified in 1998 (Thome et al., 1998). The mutation in the gene (currently called NLRP3-inflammasome) in recurrent and chronic inflammation was initially detected in a group of rare autoinflammatory conditions, termed cryopyrin-associated periodic syndromes (CAPS) (Hoffman et al., 2001); this protein also belongs to the pyrin family. The link between pyrin, cryopyrin, NLRP3, death domain (CARD) and caspase-1 was established and this macromolecule complex was termed "inflammasome" (Martinon et al., 2002; Srinivasula et al., 2002) (**Figure 2**).

IL-1 RECEPTORS

The search for IL-1 receptors began in 1985 when a high-affinity plasma membrane receptor for human interleukin was identified (Dower et al., 1985); between 1985 and 1988, numerous investigators were working in this field of research (Bird and Saklatvala, 1986; Kilian et al., 1986; Kroggel et al., 1988).

The gene sequence for IL-1β and IL-1Ra receptors was described by Sims et al. (1988). Stylianou et al. (1992) was well established that the type I and not type II receptor led to signal transduction.

Eastgate et al. (1990) demonstrated the existence of an IL-1β binding protein, followed by the findings of Francesco Colotta and Alberto Mantovani in 1993 that the shed molecule of type II receptor was a decoy receptor and probably similar to the IL-1β-binding protein described by Eastgate and Duff.

In a recent book entitled "Body Messages: the Quest for the Proteins of Cellular Communication. Evolution of Message Discovery: The IL Family," Fantuzzi (2016) elegantly describes the different facets, controversies and surprises characterizing the discovery of the IL-1, IL-1Ra and interleukin receptors families.

INFLAMMASOME, IL-1 AND IL-Ra IN CRYSTAL-INDUCED ARTHROPATHIES

The first observation that IL-1 could contribute to the pathogenesis of crystal-induced arthritis Duff et al. (1983) demonstrated that monosodium urate (MSU) crystals were able to stimulate mononuclear phagocytes *in vitro* to produce EP. The authors failed to extend these results to other pathogenic crystals such as hydroxyapatite and calcium pyrophosphate

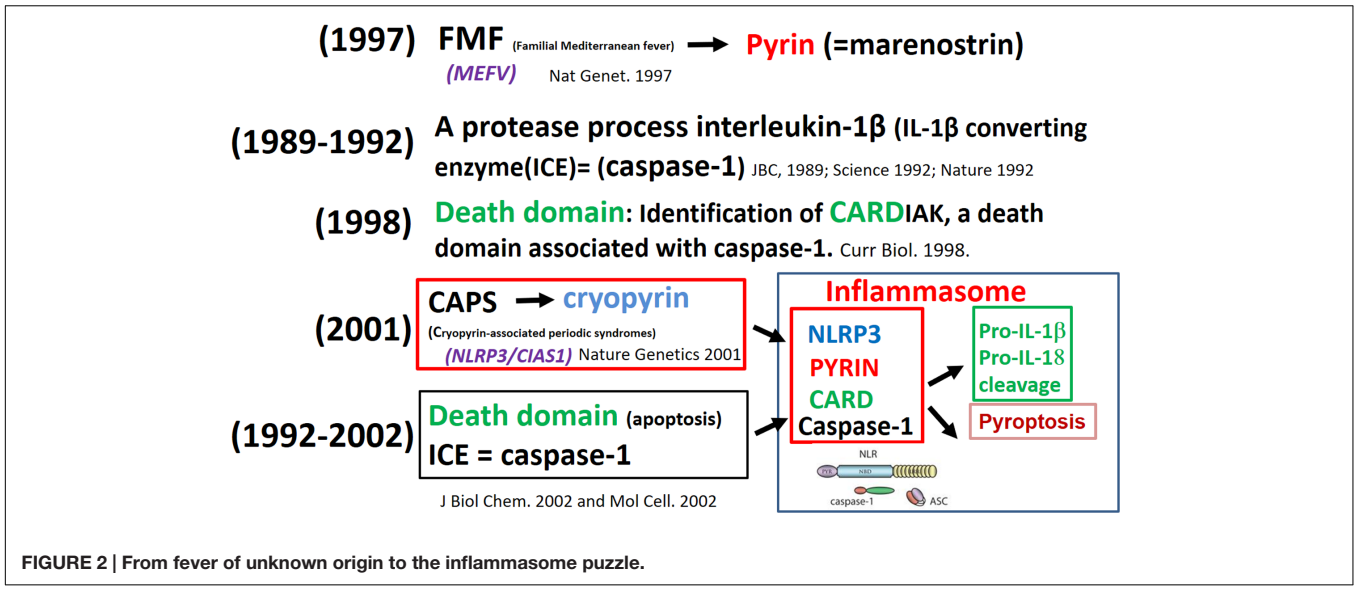

FIGURE 2 | From fever of unknown origin to the inflammasome puzzle.

(CPP), hypothesizing that the joint had to be, in some way, "conditioned" to propagate an inflammatory stimulus and that the source of IL-1 could lie outside the joint (Malawista et al., 1985). Subsequently, it became clear that purified crystals alone could not induce IL-1but that an additional stimulus was needed *in vitro* to generate IL-1 (Giamarellos-Bourboulis et al., 2009). Further *ex vivo* models identified free fatty acids (Joosten et al., 2010) and synovial fluid (SF) proteins (Scanu et al., 2016) as important triggers of IL-1 release.

An imbalance between the production of IL-1 and IL-1Ra induced by crystals in activated cells was pointed out in 1994 when it became clear that MSU and CPP crystals did not affect IL-1Ra production in neutrophils (Roberge et al., 1994).

Martinon et al. (2006) demonstrated for the first time the involvement of the inflammasome NLRP3 in caspase-1 activation and IL-1 release induced by crystals. IL-1R was also found to play a critical role in amplifying the inflammatory response through a MyD88-dependent mechanism (Chen et al., 2006). These observations, along with the discovery that uric acid was capable to stimulate the innate immune system (Shi et al., 2003), led to the addition of gout to the spectrum of autoinflammatory diseases (Punzi et al., 2012).

The key role that IL-1β plays in gout and pseudo-gout was further confirmed by the effectiveness of blocking agents to IL-1 in that it reduced acute attacks, initially observed after the administration of IL-1Ra anakinra, and then to the anti-IL-1β monoclonal antibody canakinumab (So et al., 2007; Schlesinger et al., 2012; Punzi and So, 2013). Accordingly, the recent EULAR recommendations for the management of gout advocate considering the administration of IL-1 blockers in those patients with frequent flares and contraindications to colchicine, NSAIDs and corticosteroids (Richette et al., 2017). In contrast, Rilonacept, a soluble receptor fusion protein binding both IL-1α and IL-1β, provided no benefit over indomethacin in an RCT (Terkeltaub et al., 2013). Other promising molecules, such as the recombinant human alpha-1-anti-trypsin (AAT)-IgG1 Fc fusion protein, have been shown to mainly target IL-1 in gout (Joosten et al., 2016).

EFFECT OF IL-1 ON CARTILAGE AND BONE IN OA

The presence of a low molecular weight peptide released from synovial tissue and able to affect cartilage homeostasis was demonstrated at the end of the 1970s (Dingle et al., 1979). This messenger called "catabolin" was able to stimulate chondrocytes to degrade both cartilage proteoglycans and collagen (Saklatvala and Dingle, 1980), and soon its similarity to IL-1 was established (Saklatvala et al., 1984).

Gowen et al. (1983) demonstrated for the first time that IL-1-like factor altered the breakdown of bone by modulating both bone resorption in RA and bone formation in OA. As far as bone resorption is concerned, IL-1Ra was later shown to block the resorptive effect induced by IL-1 *in vitro* (Seckinger et al., 1990).

Despite the fact that the altered expression of proinflammatory cytokines and chemokines in OA cartilage and synovium is well documented (Kapoor et al., 2011; Raghu et al., 2016), the role of IL-1β has not been fully clarified. Very low levels of IL-1β have been determined in the synovial membrane and SF of patients with early and end-stage OA (Scanzello et al., 2009) although levels were more elevated in the SF cells of OA than in those of controls (Sauerschnig et al., 2014). However, IL-1β levels are significantly lower in OA than in RA (Farahat et al., 1993). *In vitro* studies have revealed that IL-1β plays a critical role in driving the production of proteolytic enzymes such as MMPs and ADAMTS-4 in OA (Bondeson et al., 2006). But results obtained from different OA animal models showed discrepancies when anti-IL-1β therapy (Fernandes et al., 1999; Zhang et al., 2004) or mice deficient in IL-1 (Clements et al., 2003) were utilized, thus emphasizing that other, IL-1-independent, pathways may be involved in OA pathology (Bondeson et al., 2010).

As far as the association between the IL-1 gene polymorphisms and OA is concerned, the haplotypes IL1A-IL1B-IL1RN and IL1B-IL1RN have, respectively, been shown to confer a higher and a decreased risk of OA (Smith et al., 2004). A study investigating the contribution of gene polymorphisms to dysregulated cytokine expression in OA cartilage revealed significant associations between the TNFα (high) chondrocyte phenotype and IL-1Ra allele 2, and between the TNFα (low) phenotype and IL-1β allele 2 (Moos et al., 2000).

An association between severe hand OA and single nucleotide polymorphisms in the IL-1R1 gene has been observed in family based and case-control analyses (Nakki et al., 2010). Several studies assessing the polymorphism in OA are ongoing, and some have been reviewed in a meta-analysis (Kerkhof et al., 2011).

TOWARD CLINICAL IMPLICATIONS

Although IL-1 itself has been used as a therapeutic agent in oncology patients, alone it has little antitumor activity in melanoma, renal cell carcinoma, ovarian carcinoma or other diseases.

Considering that IL-1 seems to protect and restore the bone marrow from lesions induced by radiation or chemotherapy, it has been proposed to use it for treatment, but its modest hematopoietic effects probably do not counterbalance the toxicity necessary to achieve said effects (Veltri and Smith, 1996). IL-1α and IL-1β have also been proposed as vaccine adjuvants (Staats and Ennis, 1999).

The initial attempts to block IL-1 in infectious diseases have been unsuccessful and were accompanied by an increase in infections and side effects (Freeman and Buchman, 2001). Trials monitoring effects of IL-1Ra in patients with RA, which were begun in 1991, have produced interesting results although long-term results have not proved impressive and have demonstrated that this therapy is less effective than anti-TNF therapies. The initial trial targeting IL-1 in RA has been reviewed in detail (Dayer and Bresnihan, 2002).

At present, therapeutic blockade of IL-1 is crucial in many hereditary autoinflammatory diseases, as well as in patients with Still's disease, crystal-induced arthropathies, certain skin diseases including neutrophil-triggered skin diseases, Behçet's disease and deficiency of IL-1Ra, and other rare fever syndromes. Its effects have been marginal in RA patients and are still under debate in OA, type 2 diabetes mellitus, cardiovascular diseases and cancer (Larsen et al., 2009; Cantarini et al., 2015; Cavalli and Dinarello, 2015; Sfriso et al., 2016).

Relevant developments are still be expected in future with regard to the IL-1 family and the macromolecular complex which is inflammasome. The complexity of the various forms of the IL-1 receptor family, not discussed here in the history, is far from being fully understood and may lead to new avenues for pharmacological interventions.

NOTE

We regret that given the brevity of the review with particular focus on rheumatological aspects, it was impossible to acknowledge all the important researchers involved in this field of study and their discoveries and contributions.

AUTHOR CONTRIBUTIONS

J-MD conceived and drafted the manuscript. FO and LP contributed to the drafting and the revision of the work. All authors approved it for publication.

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REVIEW
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Anakinra Therapy for Non-cancer Inflammatory Diseases

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Interleukin-1 (IL-1) is the prototypical inflammatory cytokine: two distinct ligands (IL-1α and IL-1β) bind the IL-1 type 1 receptor (IL-1R1) and induce a myriad of secondary inflammatory mediators, including prostaglandins, cytokines, and chemokines. IL-1α is constitutively present in endothelial and epithelial cells, whereas IL-1β is inducible in myeloid cells and released following cleavage by caspase-1. Over the past 30 years, IL-1-mediated inflammation has been established in a broad spectrum of diseases, ranging from rare autoinflammatory diseases to common conditions such as gout and rheumatoid arthritis (RA), type 2 diabetes, atherosclerosis, and acute myocardial infarction. Blocking IL-1 entered the clinical arena with anakinra, the recombinant form of the naturally occurring IL-1 receptor antagonist (IL-1Ra); IL-1Ra prevents the binding of IL-1α as well as IL-1β to IL-1R1. Quenching IL-1-mediated inflammation prevents the detrimental consequences of tissue damage and organ dysfunction. Although anakinra is presently approved for the treatment of RA and cryopyrin-associated periodic syndromes, off-label use of anakinra far exceeds its approved indications. Dosing of 100 mg of anakinra subcutaneously provides clinically evident benefits within days and for some diseases, anakinra has been used daily for over 12 years. Compared to other biologics, anakinra has an unparalleled record of safety: opportunistic infections, particularly *Mycobacterium tuberculosis*, are rare even in populations at risk for reactivation of latent infections. Because of this excellent safety profile and relative short duration of action, anakinra can also be used as a diagnostic tool for undefined diseases mediated by IL-1. Although anakinra is presently in clinical trials to treat cancer, this review focuses on anakinra treatment of acute as well as chronic inflammatory diseases.

Keywords: interleukin 1, IL-1β, IL-1α, rheumatology, inflammation

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INTRODUCTION

Historical Background of IL-1 and IL-1Ra

The history of interleukin 1 (IL-1) dates back to the purification of the endogenous fever-producing protein called leukocytic pyrogen, as reviewed in Dinarello (2015). During the purification of the leukocytic pyrogen, two fever-inducing proteins were observed with different molecular weights and distinct isoelectric points. Specifically, human blood monocytes produced both a high (35 kDa) as well as a low (15 kDa) molecular weight leukocytic pyrogen (Dinarello et al., 1974),

with two distinct isoelectric points at 5.1 and 6.8, respectively (Dinarello et al., 1974). Murphy et al. (1981) also reported two leukocytic pyrogens, with isoelectric focusing points of 5.1 and 7.0 from rabbit cells. The specific biologic activity of purified human leukocytic pyrogen was first reported in 1977 as the induction of fever in rabbits at 10 ng/kg (Dinarello et al., 1977). Thus, the *in vivo* potency of IL-1 was established in 1977 and later confirmed in animals and humans with recombinant IL-1β. In 1979, based on the ability of purified human leukocytic pyrogen to enhance T-cell proliferation in response to antigen recognition, the name "leukocytic pyrogen," or "lymphocyte activation factor" was replaced with the current nomenclature "IL-1" (Rosenwasser et al., 1979). The 1984 cDNA cloning of IL-1β in humans (Auron et al., 1984) and IL-1α in mice (Lomedico et al., 1984) univocally established that there were in fact two distinct genes coding for IL-1. Looking back today, the higher molecular weight fever-producing molecule was likely the IL-1α precursor, which unlike the IL-1β precursor is biologically active without processing. In contrast, the IL-1β precursor requires processing and proteolytic cleavage in order to generate the lower molecular weight and biologically active IL-1β.

Interleukin-1β exerts clinically marked pro-inflammatory effects at very low concentrations and correlations of circulating levels of IL-1β with disease severity is often not possible due to the limited sensitivity of immunoassays. Instead, human plasma has been assayed for IL-1 bioactivity by enhancement of PHA-induced proliferation of mouse thymocytes *in vitro*. This assay was reliable in that indirect readouts of IL-1 activity were found in plasma samples of subjects with endotoxemia and in women during the menstrual cycle (Cannon and Dinarello, 1985). The bioassay for IL-1 required chromatographic separation of each plasma sample in order to remove inhibitory proteins present in the plasma. Specifically, plasma from healthy human subjects was obtained before and after intravenous inoculum of a low dose of endotoxin. Before the administration of the endotoxin, the plasma fractions had no effect on thymocyte proliferation; however, 4 h after endotoxin administration, at the peak of the fever, fractions suppressed thymocyte proliferation. Thus, there was an endotoxin-inducible suppressor "factor" specifically inhibiting IL-1-mediated thymocyte proliferation in the circulation (Dinarello et al., 1981).

Subsequent to this observation, others reported the presence of a "specific" inhibitor of IL-1 bioactivity in supernatants of human monocytes (Arend et al., 1985) and in the serum and urine of children with systemic juvenile arthritis (Prieur et al., 1987). In 1987, this "IL-1 inhibitor" isolated from the urine was shown to prevent binding of IL-1 to cells (Seckinger et al., 1987), thus providing evidence for its mechanism of action. The IL-1 inhibitor was purified in 1990 (Hannum et al., 1990); the cDNA sequence first reported that same year (Eisenberg et al., 1990) and the term IL-1 receptor antagonist (IL-1Ra) was used for the first time in that report. Following the cDNA cloning of IL-1Ra, a radioimmunoassay for IL-1Ra was developed and used to assay the plasma samples from subjects during experimental endotoxemia. Endogenous IL-1Ra is found at very low levels in the circulation of healthy subjects (less than 200 rpg/mL), but levels steeply rise to a mean level of 1435 pg/mL 2 h after the infusion of endotoxin, and to top levels of 6400 pg/mL at the peak of the fever 4 h after infusion of endotoxin (Granowitz et al., 1991). Of note, IL-1β levels reached peak levels of only 20 pg/mL in the same samples. In that study, the kinetics of IL-1Ra matched the induction of the specific suppressor of IL-1-mediated thymocyte proliferation reported during experimental endotoxemia in 1981. In addition, the study also revealed that there was a molar excess of at least 100-fold IL-1Ra over IL-1β. It was not until the reports of subjects with a genetic deletion of IL-1Ra that the critical function of this endogenous inhibitor, which naturally suppresses IL-1 mediated inflammation, was fully revealed (Aksentijevich et al., 2009).

As stated in our review (Cavalli and Dinarello, 2018), in 1981 we described a circulating suppressor factor from humans during experimental endotoxemia as assayed for specific inhibition of IL-1 activity *in vitro* (Dinarello et al., 1981). We believe this circulating suppressor factor was the first description of IL-1Ra, and we confirmed our findings in a report published in *Lancet* in 1991 using a specific radioimmunoassay for IL-1Ra (Granowitz et al., 1991). However, in 1984, there was documentation from the group of Jean-Michel Dayer describing a specific inhibitor of IL-1 activity isolated from the urine of patients with monoblastic leukemia (Balavoine et al., 1984). This was an essential contribution to the history of the discovery of the antagonist. In 1985, there was another report from the Dayer laboratory "Collagenase- and PGE2-Stimulating Activity (Interleukin-1-Like) and Inhibitor in Urine from a Patient with Monocytic Leukemia," as published in *Progress in Leukocyte Biology*, vol. 2 (New York, NY: Alan R. Liss, 1985 p. 429). These reports were followed by another publication in the *Journal of Clinical Investigation* (Balavoine et al., 1986). As stated in our Review, "the IL-1 inhibitor" isolated from the urine was shown to prevent binding of IL-1 to cells (Seckinger et al., 1987), thus providing for the first time evidence for its mechanism of action. Because of the widespread and beneficial use of anakinra (the recombinant form of the nature IL-1Ra) to treat human diseases, the contributions of Jean-Michel Dayer as well as those of William Arend are paramount.

Synthesis and Release of IL-1β

Interleukin-1β is not produced or detectable with standard immunoassays in healthy tissues; rather, IL-1β is mainly produced by inflammatory cells of the myeloid compartment: blood monocytes, tissue macrophages, and dendritic cells. **Figure 1** summarizes the mechanisms of IL-1 activation and signaling.

Production is stimulated by exogenous Toll-like receptor (TLR) agonists or by endogenous cytokines such as TNFα (Dinarello et al., 1987). IL-1α and IL-1β induce themselves. This self-sustained induction of IL-1 is a key mechanism of autoinflammation. In order to prevent unwanted release and runaway inflammation, IL-1β is synthesized as an inactive precursor, whose activation is contingent on proteolytic cleavage by caspase-1, an intracellular cysteine protease. In turn, activation of caspase-1 requires the oligomerization and assembly of the

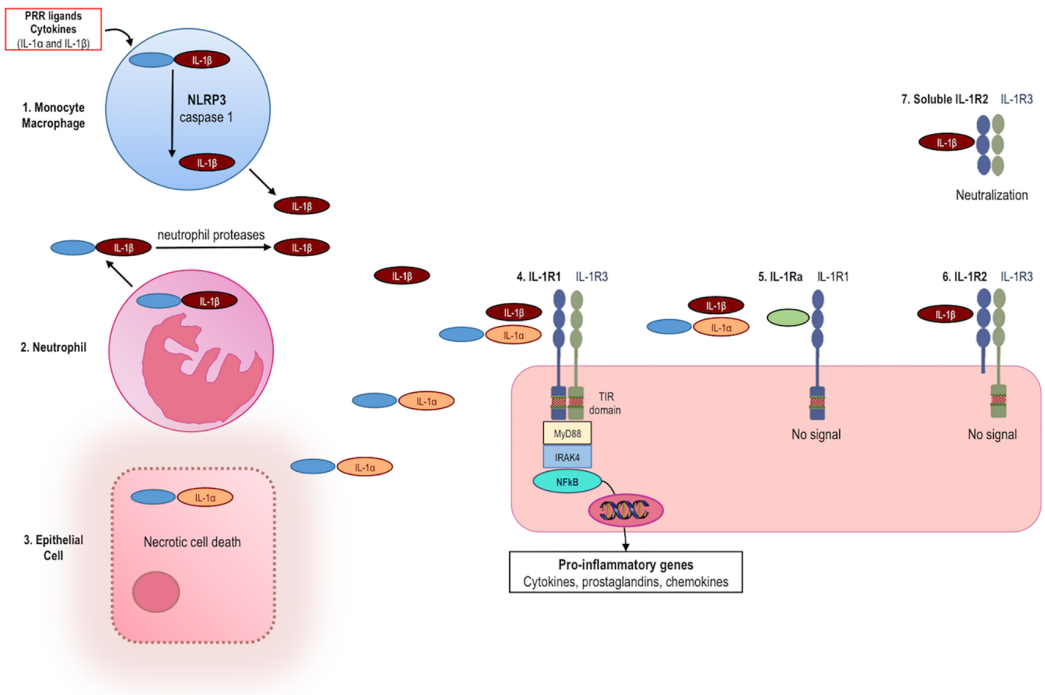

FIGURE 1 | Production and release of IL-1, signaling and inhibition of IL-1 activities. (1) The IL-1β precursor is induced in monocytes/macrophages following engagement of pattern recognition receptors (PRR) or by pro-inflammatory cytokines, including IL-1α and IL-1β. IL-1β is synthesized as an inactive precursor (pro-IL-1β). Release of biologically active IL-1β takes place by enzymatic cleavage of the precursor protein by caspase-1. Activation of caspase-1 requires induction of the NLRP3 inflammasome. (2) Neutrophils release the IL-1β precursor into the extracellular space where it is cleaved to active IL-1β by neutrophil-derived proteases. (3) The IL-1α precursor is constitutively present in most epithelial cells and is fully active. Upon cell necrosis, the intracellular IL-1α precursor is released and acts as an alarmin. (4) Both IL-1α and IL-1β bind to IL-1 receptor type 1 (IL-1R1), which is followed by recruitment of the co-receptor IL-1R3 (formerly termed IL-1 receptor accessory protein, IL-1RAcP). The heterotrimer results in the approximation of the intracellular TIR domains of IL-1R1 and IL-1R3. MyD88, IL-1 receptor-associated kinase 4 (IRAK4), and NFκB are phosphorylated. NFκB induces transcription of pro-inflammatory genes. Mechanisms physiologically counteracting the activity of IL-1α and IL-1β include: (5) The IL-1 receptor antagonist (IL-1Ra, green) binds IL-1R1 and prevents binding of IL-1α and IL-1β, thereby resulting in no signal. (6) The IL-1 receptor type 2 (IL-1R2) preferentially binds IL-1SS, but lacking a cytoplasmic domain, acts as a decoy receptor and there is no signal. (7) Soluble IL-1R2 (extracellular domain only) binds IL-1β and forms a complex with soluble IL-1R3, resulting in neutralization of IL-1β.

"inflammasome," a complex of intracellular proteins (Martinon et al., 2009). Once activated, caspase-1 cleaves the N-terminal amino acid of the inactive IL-1β precursor, thus enabling the release of the processed, biologically active form of this cytokine. Assembly and activation of the inflammasome thereby represents a critical safety mechanism preventing deregulated release of IL-1β. Unrestricted activation of caspase-1 and secretion of IL-1β lead to systemic and multi-organ sterile inflammation, which characterizes autoinflammatory diseases (Hoffman and Wanderer, 2011).

Anakinra Reveals the Nature of Autoinflammatory Disorders

The term IL-1 is often used without distinguishing between the two gene products, IL-1α and IL-1β. This is because both cytokines bind to the same signaling receptor, the IL-1 receptor 1 (IL-1R1), and hence there is no significant difference between the biological activities of either cytokine. IL-1Ra, also an endogenous member of the IL-1 family, binds to the IL-1R1 and therefore blocks the activity of both IL-1α and IL-1β. A recombinant form of IL-1Ra (anakinra) is used to treat a broad variety of diseases, ranging from common conditions such as rheumatoid arthritis (RA), gout, and idiopathic pericarditis, to rare hereditary diseases. Specific mutations in diseases such as familial Mediterranean fever (FMF) and cryopyrin-associated periodic syndrome (CAPS) result in deregulated release of active IL-1β, which is clinically manifested as periodic fevers with systemic and local inflammation. These diseases do not involve T-lymphocytes, which characteristically represent the effector cells of every autoimmune disease, nor autoantibodies. Therefore, these diseases are not considered autoimmune diseases, but rather termed "autoinflammatory" syndromes. In autoinflammatory diseases, the effector cell is a myeloid cell, characteristically a monocyte or macrophage (Dinarello et al., 2012). The central role of IL-1 in the pathogenesis of autoinflammation is well established. Monocytes from patients with autoinflammatory diseases release more IL-1β, but not TNFα, compared to healthy persons (Pascual et al., 2005; Goldbach-Mansky et al., 2006; Gattorno et al., 2007).

Autoinflammatory diseases can be regarded as a "natural experiment", which reveals the clinical and pathologic consequences of deregulated IL-1-mediated inflammation

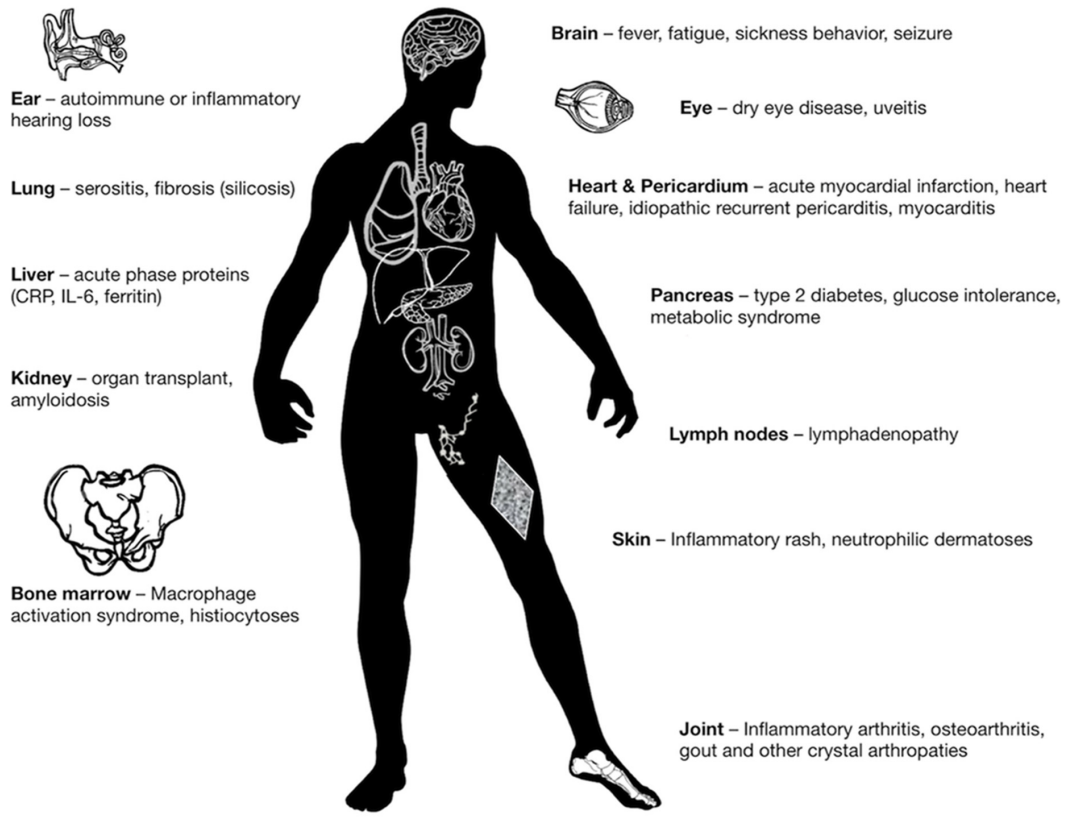

FIGURE 2 | Clinical manifestations of IL-1-mediated inflammation, which are reversible upon treatment with anakinra.

in humans. Lessons from autoinflammatory diseases extend and apply beyond this group of rare conditions: deregulated activation of the myeloid compartment and IL-1 also mediate several common diseases, which can also be classified as autoinflammatory disorders (i.e., gout, pericarditis), or at least include autoinflammation as part of disease pathogenesis (i.e., heart failure, diabetes, myocarditis; Cavalli et al., 2016a; Hayashi et al., 2016; Netea et al., 2017). Because of the safety and rapid onset of action, IL-1 inhibition with anakinra can be used as a diagnostic as well as a treatment tool for patients with undefined signs or symptoms of autoinflammation (Harrison et al., 2016). **Figure 2** illustrates the broad variety of organ manifestations of IL-1-mediated inflammation, which can be treated with anakinra.

AUTOINFLAMMATORY DISEASES

Autoinflammatory diseases are a spectrum of hereditary or multifactorial conditions variably manifested with clinical and hematologic features of IL-1-mediated inflammation: these include fever, fatigue, myalgia, arthralgia, arthritis, serositis, gastrointestinal involvement, skin rashes, and multi-organ involvement, often accompanied by neutrophilia and elevated inflammatory markers. Most autoinflammatory diseases occur in recurrent flares. In some autoinflammatory diseases, causative mutations resulting in deregulated release of active IL-1 have been identified. However, in other autoinflammatory diseases, a specific mutation to account for excessive IL-1 activity has not yet been determined; in some cases, defects in regulatory molecules counteracting the biologic activity of IL-1 can be determined, or postulated (Aksentijevich et al., 2009; Cavalli et al., 2016b; Cavalli et al., 2017c,d; Ballak et al., 2018; Cavalli and Dinarello, 2018). Regardless of the underlying mechanisms, disease manifestations are characteristically controlled by IL-1 blockade with anakinra; as different pharmacokinetics result in more prolonged duration of action, neutralizing antibodies directed against IL-1β (canakinumab) represent an alternative in patients enduring frequent disease flares. The efficacy of anakinra in the treatment of these conditions is discussed below; more detailed lists of hereditary as well as non-hereditary inflammatory diseases responsive to anakinra treatment are provided in **Tables 1**, **2**.

Cryopyrin-Associated Periodic Syndromes (CAPS)

The term "CAPS" encompasses a spectrum of three hereditary diseases: familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal onset multi-inflammatory diseases (NOMID). The clinical phenotypes of FCAS, MWS, and NOMID are characterized

by escalating severity, ranging from self-limited episodes of fever, myalgia, and skin rash (FCAS), to chronic systemic and organ-specific inflammation with major complications (NOMID). The pathologic hallmark of CAPS is the presence of activating mutations in the NLRP3 inflammasome, which result in induction of caspase 1 and deregulated release of IL-1β (Agostini et al., 2004; Goldbach-Mansky et al., 2006; Gattorno et al., 2007). Anakinra, as well as other IL-1-blocking agents, is dramatically effective in the treatment of CAPS (reviewed in Cavalli and Dinarello, 2015).

Familial Mediterranean Fever (FMF) and Amyloidosis

Familial Mediterranean fever is a prototypical autoinflammatory disease characterized by recurrent bouts of fever, rash, and serositis, which are typically self-limited in 3–5 days. The disease is autosomal recessive, and determined by mutations in the MEFV gene, encoding for pyrin, a protein involved in inflammasome formation, activation of caspase 1, and release of active IL-1β (Chae et al., 2006). Colchicine is the cornerstone of treatment for FMF, but is ineffective in some patients. Patients refractory to conventional treatment are successfully treated with anakinra (Meinzer et al., 2011; Ozen et al., 2011).

Interleukin-1 induces serum amyloid A (SAA), which is thereby commonly elevated in several chronic inflammatory diseases. Progressive deposition of amyloid fibrils in tissues results in amyloidosis, a condition leading to severe organ dysfunction, including lethal kidney or heart failure, which are major causes of death in untreated FMF and CAPS. However, effective dampening of chronic inflammation with IL-1 blockers prevents progression to amyloidosis and organ failure (Moser et al., 2009; Ait-Abdesselam et al., 2011; Ozen et al., 2011; Stankovic Stojanovic et al., 2012). Intriguingly, amyloid deposition leading to organ dysfunction is also a feature of other, multifactorial conditions in which IL-1 has emerged as a pivotal pathologic mediator: amyloidosis of insulin-producing islets and brain characterizes T2D and Alzheimer disease, respectively (Tan et al., 2007; Masters et al., 2010). In both conditions, IL-1 blockade may represent a suitable therapeutic strategy to hinder disease progression.

TNF-Receptor Associated Periodic Syndrome (TRAPS)

TNF-receptor associated periodic syndrome (TRAPS) is an autosomal dominant disease caused by mutations in TNF-receptor type 1 (McDermott et al., 1999), and clinically manifested as recurrent flares of fever, rash, and serositis. A deficiency of soluble TNFα receptors, which neutralizes circulating TNFα, was postulated. Nevertheless, clinical response to TNFα inhibition is only partial, and even absent in many patients. Current understanding rather postulates a deficit in membrane translocation of TNF-receptor type 1, which leads to an unfolded protein response, cellular stress, and consequent release of IL-1β (Cantarini et al., 2012b). This view is also substantiated by clinical efficacy of anakinra, even in refractory cases (Simon et al., 2004; Gattorno et al., 2008a).

TABLE 1 | Anakinra for hereditary systemic inflammatory diseases.

Familial Mediterranean fever (FMF; Meinzer et al., 2011; Ozen et al., 2011)
CAPS (Hawkins et al., 2003; Goldbach-Mansky et al., 2006; Kullenberg et al., 2016)
TRAPS (Simon et al., 2004; Gattorno et al., 2008a)
PAPA (Dierselhuis et al., 2005; Brenner et al., 2009; Braun-Falco et al., 2011; Schellevis et al., 2011)
PASH (Braun-Falco et al., 2011; Marzano et al., 2013)
DIRA (Aksentijevich et al., 2009; Reddy et al., 2009; Sakran et al., 2013)
Blau syndrome/granulomatous arthritis (Arostegui et al., 2007; Punzi et al., 2011)
Mevalonate kinase deficiency/hyper-IgD syndrome (Ruiz Gomez et al., 2012)
Majeed syndrome (Herlin et al., 2013)
NLRP12 autoinflammatory syndrome (Jeru, 2011)

TABLE 2 | Anakinra for systemic and local inflammatory diseases.

Schnitzler syndrome (Ryan et al., 2008)
Behçet disease (Cantarini et al., 2015b)
Secondary amyloidosis (Moser et al., 2009; Ait-Abdesselam et al., 2011; Stankovic Stojanovic et al., 2012)
Henoch–Schonlein purpura (Boyer et al., 2011)
Idiopathic recurrent pericarditis (Picco et al., 2009; Brucato et al., 2016)
Systemic-onset juvenile idiopathic arthritis (Gattorno et al., 2008b; Vastert et al., 2014)
Adult-onset still disease (Fitzgerald et al., 2005; Cavalli et al., 2015b; Colafrancesco et al., 2017)
Macrophage activation syndrome (Gattorno et al., 2008b; Miettunen et al., 2012; Rajasekaran et al., 2014; Vastert et al., 2014; Sonmez et al., 2018)
Sweet's syndrome/neutrophilic dermatoses (Delluc et al., 2008; Kluger et al., 2011; Pazyar et al., 2012; Belani et al., 2013)
Neutrophilic panniculitis (Behrens et al., 2006; Aronson and Worobec, 2010; Lipsker et al., 2010)
Erdheim–Chester/histiocytoses (Aouba et al., 2010; Diamond et al., 2016; Tomelleri et al., 2018)
SAPHO (Colina et al., 2010; Eleftheriou et al., 2011; Wendling et al., 2012)
PFAPA (Stojanov et al., 2011; Cantarini et al., 2012a)
Multicentric Castleman disease (Galeotti et al., 2008)
Jessner–Kanof disease (Sparsa et al., 2012)
Primary Sjögren syndrome fatigue (Norheim et al., 2012)
Kawasaki disease (Cohen et al., 2012)
Colitis in chronic granulomatous disease (van de Veerdonk et al., 2011)
Hidradenitis suppurativa (Tzanetakou et al., 2016)
Autoimmune inner ear disease (Vambutas et al., 2014)

Hyper-IgD Syndrome (HIDS)

Also known as mevalonate kinase deficiency, Hyper-IgD syndrome (HIDS) is an autosomal recessive autoinflammatory disorder characterized by recurrent fever, myalgia, skin rash, and lymphadenopathy. Episodes usually last 4–6 days and can be triggered by infections. Multiple intracellular pathways link mevalonate kinase deficiency with deregulated release of IL-1 production (Stoffels and Simon, 2011). Consistently, IL-1

inhibitors effectively reduce the frequency and severity of the attacks of HIDS (Bodar et al., 2011).

Adult Onset Still's Disease (AOSD) and Systemic Onset Juvenile Idiopathic Arthritis (SOJIA)

Adult onset Still's disease (AOSD) is a rare, systemic inflammatory syndrome characterized by arthritis, fever, rash, multi-organ inflammation, and strikingly elevated serum inflammatory indexes, particularly ferritin. Consistent with observations that the NLRP3 inflammasome is highly expressed and activated in AOSD (Hsieh et al., 2017), IL-1β blockade, even with anakinra as monotherapy, represents the mainstay of biologic treatment and effectively controls disease manifestations (Fitzgerald et al., 2005; Naumann et al., 2010; Cavalli et al., 2015b; Colafrancesco et al., 2017; Junge et al., 2017).

Rather than being distinct clinical entities, SOJIA and AOSD are considered different manifestations of the same disease, occurring in infancy and adulthood, respectively. The efficacy of anakinra in SOJIA is thereby not unexpected, even in patients refractory to treatment with steroids, methotrexate, or TNFα blockers (Quartier et al., 2011). Both in AOSD and SOJIA, two distinct clinical phenotypes can be identified. One is characterized by rampant systemic inflammation with neutrophilia and elevated acute phase proteins, and by a lower number of inflamed joints: this form is dramatically and characteristically responsive to IL-1 blockade (Gattorno et al., 2008b). On the other hand, a clinical phenotype characterized by more severe arthritis and limited systemic inflammation may not respond as brilliantly to IL-1 inhibition.

Of note, treatment of SOJIA poses challenges beyond the mere achievement of disease control, related to the problematic use of immunosuppressive therapies in a pediatric population. For instance, steroid treatment is associated with growth retardation. In this scenario, IL-1 inhibition may be particularly advantageous, as treatment with anakinra or canakinumab results in reduced glucocorticoid dosing and catch-up growth (Ruperto et al., 2012). A prospective study evaluated anakinra (2 mg/kg) as a first-line drug in 20 children with new-onset SOJIA, and documented a near complete clinical response within 3 months of treatment initiation, which was sustained at 32 months of follow-up and allowed most patients to discontinue treatment (Vastert et al., 2014).

Schnitzler Syndrome (SchS)

Schnitzler syndrome (SchS) is characterized by chronic urticarial, fever, and development of hematopoietic malignancies, particularly Waldenström macroglobulinemia. The SchS International Registry reports nearly 100% efficacy with anakinra treatment, which leads to clinical improvement within hours and remission within days (Ryan et al., 2008). Remission upon treatment is durable, but disease flares may occur at discontinuation (Mertens and Singh, 2009a). The efficacy of anakinra is SchS is so distinctive that diagnosis should be reconsidered in the event of treatment failure. Canakinumab is also highly effective in SchS (de Koning et al., 2013). Of note, a recent study reported that some patients with SchS have myeloid-lineage restricted somatic mosaicism for mutations in NLRP3, which is associated with increased IL-1 activity in monocytes. This phenomenon likely explains the late onset of disease in some patients (de Koning et al., 2015).

TABLE 3 | Anakinra for the heart.

↓ Inflammation in acute myocardial infarction
↑ Exercise performance in heart failure
↑ Oxygen consumption in heart failure
↓ Systemic inflammation in heart failure
↓ Hospitalizations for recurrent acute heart failure
↓ Pain and inflammation in recurrent idiopathic pericarditis
↑ Function in acute myocarditis and heart failure
↑ Exercise tolerance in heart failure associated with rheumatoid arthritis

ANAKINRA FOR THE HEART

The central role of IL-1-mediated inflammation is established in the pathogenesis of atherosclerosis, ischemia-reperfusion injury, cardiac remodeling, and myocardial infarction (Pomerantz et al., 2001; Kamari et al., 2007; Duewell et al., 2010; Ridker et al., 2017). The beneficial effects of IL-1 blockade with anakinra in heart disease are discussed hereby, and summarized in **Table 3**.

Atherosclerosis

Chronic inflammation is central to the pathogenesis of atherosclerosis (Libby et al., 2002), and IL-1 specifically promotes the formation, growth, and rupture of vascular atherosclerotic plaques, which account for ischemic cardiovascular complications (Peiro et al., 2017; Buckley and Abbate, 2018). Both IL-1β and IL-1α are highly expressed in atherosclerotic lesions, and promote recruitment of leukocytes by inducing endothelial cells to express adhesion molecules; in addition, IL-1 impairs vasodilation while inducing oxidative stress and pro-coagulant mediators (Peiro et al., 2017). Experimental pre-clinical evidence shows that many of these effects can be reversed by IL-1β inhibition, thus pointing at selective pharmacological blockade as a suitable treatment strategy to dampen progression of atherosclerotic lesions (Peiro et al., 2017). Although the ability of anakinra or other IL-1β blocking drugs to prevent progression of atherosclerosis in humans has not been specifically studied, IL-1 inhibition with anakinra and canakinumab proved beneficial in the treatment of major clinical complications of atherosclerosis, such as acute myocardial infarction and ischemic cardiovascular disease, as detailed in the following sections of this review.

Acute Myocardial Infarction

The first studies of anakinra in acute ischemic heart disease involved patients who had suffered a ST-elevated myocardial infarction (STEMI; Abbate et al., 2010, 2013). In these studies, anakinra 100 mg daily was administered subcutaneously for 2 weeks, following stent placement and in addition to optimal standard of care. Seventy-two hours after the acute event, despite optimal standard of care, inflammation develops due to

myocardial ischemia; CRP reaches peak levels, which correlate with the size of the infarcted area. Anakinra treatment resulted in a significant reduction in CRP levels (Abbate et al., 2013), thus reducing the progressive inflammatory response and myocardial damage. Infiltration of neutrophils and monocytes into the area surrounding the ischemic tissue contributes to further damage, which is significantly reduced by anakinra treatment in animal studies (Toldo et al., 2013). Twelve weeks following myocardial infarction, heart function of patients was evaluated as residual left ventricular ejection fraction. Compared to the placebo-treated group, anakinra-treated patients exhibited improved functional status, but did not reach statistical significance (Abbate et al., 2013). A second trial was performed with 30 patients (Abbate et al., 2013). Again, anakinra significantly reduced CRP levels 72 h after myocardial infarction; after 10–14 weeks, this reduction in CRP correlated with a reduction in left ventricular end-systolic volume (Abbate et al., 2013). Patients treated with anakinra exhibited an overall reduction in the development of heart failure (New York Heart Association Grade III and IV) compared to placebo-treated patients (Abbate et al., 2013).

Subsequent studies confirmed that anakinra treatment effectively dampens inflammation associated with myocardial infarction. Anakinra treatment was started after standard of care for the acute event and protracted for 14 days in 182 patients with non-STEMI myocardial infarction (Morton et al., 2015). For 7 days following the acute event, a significant reduction in CPR was observed in patients receiving anakinra compared to placebo (21 compared to 43 mg day/L); levels rose again 16 days after cessation of anakinra (Morton et al., 2015).

In the massive Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), 10,061 patients with prior myocardial infarction and evidence of systemic inflammation as determined by elevated serum CRP were randomized to receive either placebo or canakinumab (50, 150, or 300 mg every 3 months). At 48 months, patients treated with canakinumab 150 mg exhibited a 15% reduction in the primary endpoint of recurrent non-fatal myocardial infarction and non-fatal stroke, or cardiovascular death, as well as reduced need for coronary revascularization, compared to placebo (Ridker et al., 2017). These definitive results indicate that IL-1β blockade with canakinumab in patients with atherosclerotic cardiovascular disease can prevent recurrent cardiovascular events. Of note, this benefit was closely related to suppression of inflammation, as patients exhibiting the greatest reduction in CRP had improved survival upon treatment (Ridker et al., 2018).

Heart Failure

Several years ago, *ex vivo* studies with human atrial heart strips revealed that IL-1β suppresses cardiac contractility, even at picomolar concentrations (Cain et al., 1999). In recent years, various studies examined the effects of anakinra on heart failure with poor exercise tolerance and signs of systemic inflammation. For example, mice treated with a single dose of recombinant human IL-1β have a 76% reduction in response to isoproterenol and a 32% reduction in left ventricular function. In a clinical trial, seven patients with heart failure and markers of systemic inflammation despite standard of care treatment received 100 mg of anakinra daily for 14 days. Compared to baseline, treatment with anakinra was associated with a statistically significant improvement in oxygen consumption, a marker of exercise capability (Van Tassell et al., 2012). This study first established a role for anakinra treatment in patients with refractory heart failure.

Besides impaired left ventricular contractility, heart failure with preserved ejection fraction can also occur and be associated with reduced exercise tolerance. When patients with this condition were treated with anakinra 100 mg daily for 14 days in a double-blind, randomized, placebo controlled study, patients receiving treatment exhibited a significant increase in oxygen consumption of 1.2 mL/kg/min and a concomitant 74% reduction in CRP.

Patients with acute, decompensated heart failure often exhibit signs of systemic inflammation. Thirty patients with acute decompensated heart failure, ejection fraction less than 40%, and elevated CRP were randomized to receive either anakinra or placebo (Van Tassell et al., 2016). Upon entering the trial, patients received either 100 mg anakinra or placebo twice daily for 3 days followed by 11 days of once daily dosing. Three days into the trial, CRP decreased by 61% in the anakinra group compared to the placebo-treated group (Van Tassell et al., 2016). Although the study was not powered to determine a clinical benefit, it showed that IL-1β inhibition with IL-1 receptor blockade reduced the systemic inflammation associated with acute heart failure.

In all these trials on heart failure, patients received anakinra for only 14 days. Although clinical and objective data indicate a functional improvement as well as reduced inflammation already with short-term treatment, it is likely that a prolonged course of anakinra would result in a more marked benefit. For example, patients hospitalized with an episode of acute decompensated heart failure are at high risk for repeated hospitalizations due to recurrent episodes. Therefore, a trial was conducted comparing two different treatment durations (2 versus 12 weeks) of anakinra 100 mg daily in patients discharged from the hospital following an episode of acute decompensated heart failure. In this study, patients treated with 12 weeks of anakinra had reduced hospital readmission rates and improved aerobic capacity, oxygen consumption, and quality of life compared to patients receiving either placebo or 2 weeks of anakinra (Van Tassell et al., 2017). Of note, patients receiving anakinra for RA exhibited improved cardiac contractility, even within 3 h of a single administration (Ikonomidis et al., 2008).

Idiopathic Recurrent Pericarditis

Pericarditis is an autoinflammatory manifestation, often encountered as part of the clinical spectrum of inherited autoinflammatory disorders such as TRAPS, FMF, and CAPS, and successfully treated with anakinra (Cantarini et al., 2010; Kuemmerle-Deschner et al., 2011b). Patients with AOSD also can have bouts of pericarditis (Gerfaud-Valentin et al., 2014) and respond to anakinra (Cavalli et al., 2015b; Colafrancesco et al., 2017).

However, inflammation of pericardium can occur as an isolated, recurrent manifestation with no clear genetic predisposition (idiopathic recurrent pericarditis), which often

develops after a viral illness. Anakinra is highly effective in treating these patients and provides a rapid and sustained reduction in pain, particularly in patients refractory to conventional treatment with colchicine (Scott et al., 2011; Imazio, 2014; Brucato et al., 2016). Treating pericarditis with etanercept or infliximab has not been successful (Ambrose and O'Connell, 2007; Devasahayam et al., 2012).

Myocarditis and Dilated Cardiomyopathy

Clinical observations indicate a central role of IL-1 in the pathogenesis of cardiac inflammation. For example, myocardial involvement is part of the clinical spectrum of inflammatory (Cavalli et al., 2013b, 2014; Campochiaro et al., 2015) or autoinflammatory diseases (Lopetuso et al., 2013; van de Veerdonk and Netea, 2013), such as AOSD or SOJIA, which are characteristically mediated by IL-1. IL-1 blockade is highly effective in these conditions (Cavalli and Dinarello, 2015), as exemplified by several published cases of myocarditis associated with SoJIA and AOSD and promptly controlled by anakinra (Raffeiner et al., 2011; Movva et al., 2013; Choi et al., 2014; Cavalli et al., 2015b). However, there is emerging evidence that anakinra can be effective in the treatment of fulminant myocarditis irrespective of the initiating trigger or underlying condition (Raffeiner et al., 2011; Cavalli et al., 2016c, 2017b; De Luca et al., 2018b). In patients with myocarditis-associated acute heart failure, beneficial effects of anakinra on myocardial contractile function are particularly striking, and generally consistent with the observed benefit in patients with heart failure. It remains to be determined whether anakinra would increase myocardial function in non-acute myocarditis as it does in the acute condition. Blocking TNFα in myocarditis is contraindicated. There are case reports of anakinra treatment for myocarditis in patients non-responsive to anti-IL-6 (Waghmare et al., 2015).

A recent report described the efficacy of anakinra in a patient with dilated cardiomyopathy, a severe, irreversible heart disease characterized by left ventricular systolic dysfunction and dilation, which are not explained by abnormal loading or coronary artery disease. It is likely that several etiologic types of myocardial damage confluence in this common end-stage condition, which is histologically characterized by loss of contractile tissue, remodeling, and fibrosis. In the patient described in this study, histologic analyses of heart specimens had revealed subtle inflammation: treatment with IL-1 inhibition was thereby started, and led to a prompt clinical improvement in contractile function and arrhythmic burden (De Luca et al., 2018a). These results point at a possible role of low-grade chronic inflammation in the pathogenesis of dilated cardiomyopathy.

DIABETES AND METABOLIC SYNDROME

Interleukin-1-mediated inflammation plays a critical role in the progressive loss of β cells, which characterizes progression from insulin resistance to T2D (van Asseldonk et al., 2011). Specifically, IL-1β gene expression is dramatically elevated in β-cells of T2D patients compared to controls (Donath and Shoelson, 2011), whereas IL-1Ra levels are locally reduced and insufficient to protect β-cells from inflammation-mediated damage (Boni-Schnetzler et al., 2018).

Mechanistically, high glucose concentrations trigger β cells to produce IL-1β (Maedler et al., 2002), which in turn contributes to β-cell loss by promoting deposition of amyloid (Masters et al., 2010). These pivotal observations delineated a new concept of T2D as a chronic inflammatory disease, in which IL-1-driven inflammation results in progressive loss of β cell function (Donath and Shoelson, 2011), and provided rationale for testing anakinra in T2D. In a randomized trial, treatment with anakinra for 13 weeks led to improved insulin production and glycemic control. Reduction in IL-1-mediated inflammation was confirmed by decreased levels of CRP and IL-6 (Larsen et al., 2007), and likely particularly relevant and sustained at the islet level, as treatment responders required 66% less insulin to maintain glycemic control in the 39 weeks following discontinuation (Larsen et al., 2009). This pilot study led to subsequent, large trials of anti-IL-1β monoclonal antibodies gevokizumab, LY2189102, and – particularly – canakinumab in T2D, which all confirmed clinical benefits (Cavelti-Weder et al., 2012; Rissanen et al., 2012; Sloan-Lancaster et al., 2013).

Human fat tissue is an inflammatory environment in which infiltrating macrophages produce IL-1β (Stienstra et al., 2011). Anakinra treatment was thereby also evaluated in non-diabetic patients with metabolic syndrome, and was associated with a decrease in CRP and a corresponding increase in disposition index, thus reflecting improved β cell function (van Asseldonk et al., 2011).

Given the association between cardiovascular disease and T2D, the potential of IL-1 blocking agents to improve cardiovascular health and glucose metabolism was assessed in the large CANTOS trial, which determined that treatment with the anti-IL-1β monoclonal antibody canakinumab reduces re-occurrence of ischemic events in patients with prior cardiovascular accidents (Ridker et al., 2017). The CANTOS trial met its primary and secondary endpoints in both T2D subjects as well as in those without diabetes. Consistent with anakinra treatment in T2D, canakinumab reduced HbA1c during the first 6–9 months of treatment (Everett et al., 2018). However, after nearly 4 years of canakinumab treatment, prevention of progression to overt T2D in subjects with impaired glucose tolerance at enrollment was not observed and there was no sustained improvement in glycemic control (Everett et al., 2018). Thus, there is likely a role for IL-1α in T2D. Indeed, two studies in recent onset T1D tested anakinra versus canakinumab. Increased C-peptide was reported in subjects treated with anakinra but not canakinumab (Moran et al., 2013).

JOINT DISEASES

Rheumatoid Arthritis and Associated Comorbidities

The efficacy of anakinra treatment in RA was evaluated in several controlled studies (Mertens and Singh, 2009b). Anakinra monotherapy or in association with methotrexate significantly

reduced disease severity, joint space narrowing, radiographic joint damage, and bone erosions, while also improving quality of life (Bresnihan et al., 2004). However, other biologics, including TNFα blockers, dominate the field of biologic treatments for RA. No direct comparison is available between the efficacy of IL-1 blockade and the overwhelming number of competing biologic agents; based upon indirect comparisons, anakinra seems moderately efficacious (Singh et al., 2010). Currently, anakinra is mostly administered to those RA patients in whom other biologics proved ineffective or are contraindicated, for example, due to previous malignancy or recurrent infections. In patients refractory to anti-TNFα therapy, anakinra was shown to be effective in controlling disease activity (Genant et al., 2001; Bresnihan et al., 2004; Botsios et al., 2007). Similar to anakinra, the anti-IL-1β monoclonal antibody canakinumab has reduced disease severity in RA patients, including those unresponsive to anti-TNFα therapies (Alten et al., 2011); however, unlike anakinra, long-term preservation of joint function with canakinumab remains unstudied.

Compared to the general population, RA patients exhibit a higher incidence of T2D and cardiovascular events (Primdahl et al., 2013). In particular, infection and cardiovascular disease are the leading causes of death in RA patients, whereas T2D and metabolic syndrome are burdensome comorbidities (Kelly and Hamilton, 2007). An ideal treatment should thereby not only reduce pain and prevent articular damage, but also aim at treating associated comorbidities with minimal adverse effects. Given the IL-1-mediated nature of these comorbidities, and the documented favorable effects of IL-1 blockade on cardiovascular and metabolic diseases (discussed above in the present review; Dinarello et al., 2012), benefits of anakinra in RA may extend beyond the mere efficacy on articular inflammation and are worth further exploration.

Gout and Other Forms of Crystal-Induced Arthritis

Monosodium urate crystals activate the NLRP3 inflammasome and induce the release of active IL-1β, with a contribution of free fatty acids, which likely account for the diet-related flares of gout (Joosten et al., 2010). Given the prominent neutrophil infiltration, extracellular processing by neutrophil proteases also likely accounts for activation of IL-1β precursor in gouty joints (Joosten et al., 2009). Traditional options for managing acute flares include colchicine, non-steroidal anti-inflammatory drugs (NSAIDs), and steroids. Treatment with anakinra is dramatically effective at dampening articular inflammation (McGonagle et al., 2007), while also resulting in prolonged periods without flares. Of note, the short half-life and excellent safety profile makes anakinra an ideal therapeutic option for the treatment of acute flares of gouty arthritis, and possibly of patients with underlying chronic kidney disease. Pyrophosphate crystal arthritis, a disease highly reminiscent of gout, is also characteristically responsive to anakinra (McGonagle et al., 2008; Announ et al., 2009), as is another common crystal-induced inflammatory condition, that is, acute calcific periarthritis of the shoulder (Zufferey and So, 2013). In these conditions, short-course anakinra treatment is associated with durable reduction of pain and function impairment, and with normalization of inflammatory indexes.

Osteoarthritis

There is clinical and experimental evidence that IL-1 is involved in the pathogenesis of osteoarthritis. Thereby, previous studies evaluated the efficacy of direct instillation of anakinra into affected knee joints. Nevertheless, intra-articular injections of anakinra in patients with knee osteoarthritis yielded limited clinical benefit, which did not extend beyond one month from administration (possibly due to short-term persistence of anakinra in the joint space; Chevalier et al., 2005, 2009). Anakinra has demonstrated some efficacy against joint pain and swelling in erosive osteoarthritis of the hand (Bacconnier et al., 2009). IL-1 inhibition with antibodies to the IL-1 receptor has also been evaluated, again with only modest improvement (Cohen et al., 2011). Recent data from the worldwide CANTOS trial supports a role for IL-1β in osteoarthritis. Although this was not the intent of the study, a highly significant reduction in osteoarthritis pain and improved joint function was reported by those treated with canakinumab compared to patients receiving placebo (Ridker et al., 2017). Patients receiving 150 mg of canakinumab fives times each year reported a low incidence of osteoarthritis (1.67 per 100 person-years for placebo versus 1.12 for canakinumab, $p < 0.001$). Supporting evidence to a role for IL-1β in osteoarthritis from the CANTOS database is derived from (i) the large number of patients enrolled world-wide, (ii) the randomized, placebo controlled nature of the trial, and (iii) the specificity of IL-1β neutralization. The demographics of the CANTOS population include age, high BMI, and type 2 diabetes, each of which is characteristic of the osteoarthritis population. Not unexpectedly, there was also a significant reduction in gouty arthritis (Ridker et al., 2017). Although canakinumab treatment was effective in reducing osteoarthritis, systemic treatment with anakinra or canakinumab is an unlikely therapy for the disease.

MULTIFACTORIAL INFLAMMATORY CONDITIONS

Macrophage Activation Syndrome

Role of IL-1 and IL-18 in Macrophage Activation Syndrome

Macrophage activation syndrome (MAS), also known as hemophagocytic lympho-histiocytosis (HLH), is a rare, life-threatening condition characterized by a severe hyper-inflammatory state. It is clinically manifested with fever, elevated ferritin, liver enzymes, triglycerides, and pancytopenia due to phagocytosis of bone marrow hematopoietic precursors. Both genetic (familial HLH) and acquired forms of MAS have been described, the latter associated with infection with Epstein–Barr virus, cytomegalovirus, other herpes viruses, and intracellular bacteria, and also of various lymphomas, especially of T-cell lineage. The incidence of MAS is underestimated, as also

suggested by new reports of MAS in patients with Ebola virus, parasitic, and influenza infections (Kumar et al., 2014; van der Ven et al., 2015). In addition, patients with rheumatologic conditions, particularly SoJIA and AOSD but also systemic lupus erythematous, Kawasaki disease (KD), or systemic vasculitis can develop MAS (Grom, 2003; Grom et al., 2003; Grom and Mellins, 2011; Janka, 2012).

The pathogenesis of MAS is captivating increasing interest (Schulert and Grom, 2015), and debate is ongoing as to whether MAS is prevalently mediated by IL-1 or IL-18. In the case of familial HLH, gene expression for IL-18 is upregulated in circulating mononuclear cells (Ogilvie et al., 2007; Sumegi et al., 2011), and serum levels of IL-18 are unusually elevated (Honda et al., 2000; Emmenegger et al., 2002; Maeno et al., 2004; Mazodier et al., 2005; Nold et al., 2010). For comparison, levels of circulating IL-18 are below 1 ng/mL in inflammatory diseases such as severe sepsis, but can reach a 20–30 nm/mm range in MAS complicating systemic SoJIA (Larroche and Mouthon, 2004; Fitzgerald et al., 2005; Mazodier et al., 2005; Wada et al., 2013). However, since an IL-18 neutralizing protein [IL-18 binding protein (IL-18BP)] is present in the circulation in health and promptly increases during inflammation, it is critical to determine the levels of free, biologically active IL-18 (Novick et al., 2001). In patients with MAS, free IL-18 significantly correlated with clinical status and biologic markers of MAS, such as anemia, hypertriglyceridemia, and hyperferritinemia, but also with markers of Th1 lymphocyte or macrophage activation, such as IFNγ and soluble receptors for IL-2 and TNFα (Mazodier et al., 2005). A case of MAS due to a mutation in the NLRC4 inflammasome was successfully treated with IL-18BP, whereas anakinra therapy did not effectively reduce the severity of the disease (Canna et al., 2017).

However, IL-1 is responsible for several signs and symptoms of MAS. For example, fever and the increase in ferritin levels are IL-1-mediated, since IL-18 does not cause fever (Gatti et al., 2002; Robertson et al., 2006), does not induce prostaglandins (Lee et al., 2004), and does not induce hepatic acute phase proteins (Stuyt et al., 2005). It should also be noted that IL-1 induces IL-18, and that this mechanism likely plays a critical role in MAS. For example, IL-1 induces the release of constitutively preformed IL-18 precursor from the endothelium (Pomerantz et al., 2001). Elevated levels of IL-18 in MAS likely reflect release from IL-1-activated endothelium, rather than myeloid origin. IL-1-induced myocardial suppression is also mediated by IL-18 (Pomerantz et al., 2001; Toldo et al., 2014). IL-1 mediates fever, hyperferritimia, coagulopathy, and production of IL-18; IL-18 likely mediates hypersplenism, hypertriglyceridemia, hypotension, and elevated IFNγ. With high levels of IL-18-dependent IFNγ, there is macrophage activation in the bone marrow and hemophagocytosis, which characterizes MAS.

Anakinra for MAS

Anakinra has primarily been used in MAS due to SoIJA and occasionally to AOSD (Kelly and Ramanan, 2008; Miettunen et al., 2012; Rajasekaran et al., 2014). Although most studies describe favorable results, reports of efficacy are mixed, and include some cases of SoJIA or AOSD who developed MAS while receiving therapy with IL-1 blockade (Colafrancesco et al., 2017). It is possible that development of MAS in these patients simply reflects extreme severity or inadequate control of underlying diseases. Indeed, increasing the dose of anakinra can result in clinical improvement (Sonmez et al., 2018).

Hints of the efficacy of IL-1 blockade in MAS also come from clinical experience with septic shock. Many years ago, anakinra treatment was evaluated in three randomized, placebo-controlled trials of patients with sepsis or septic shock. In nearly 2000 patients enrolled in these trials, anakinra did not reduce overall all-cause mortality. However, recent re-analysis of data from these original studies revealed that a significant benefit of anakinra treatment could be identified in a subset of patients exhibiting a strikingly inflammatory phenotype, which was highly reminiscent of MAS and clinically characterized by cytopenia and elevated ferritin and liver enzymes levels (Shakoory et al., 2016).

Behcet's Disease

Behçet's disease is a rare vasculitis of small- and medium-sized vessels characterized by ocular and cutaneous inflammation, oral and genital ulcers, gastrointestinal or brain vasculitis, and hypercoagulable state. Ocular involvement may cause organ-threatening uveitis and retinal vasculitis. Severe, steroid-resistant disease responds to IL-1 blockers, which can afford dramatic and sustained reversal of intraocular inflammation (Cantarini et al., 2015a,b).

In an open-label pilot study of anti-IL-1β antibodies (gevokizumab) in the treatment of acute eye inflammation, a single dose prompted complete resolution of pan-uveitis and restored normal vision within 4–21 days (Gul et al., 2012).

Systemic Vasculitides: Kawasaki Disease, Takayasu Arteritis, Giant Cell Arteritis

Kawasaki disease is one of the most common systemic vasculitides and a leading cause of acquired heart disease in children. It typically affects coronary arteries, and residual vascular damage can cause complications later in life, including myocardial infarctions. Classic treatment options include intravenous immunoglobulin (IVIG) and aspirin. Of note, the beneficial anti-inflammatory and therapeutic effects of IVIG in several immune-mediated disorders include a reduction in IL-1 production with an increase in IL-1Ra (Aukrust et al., 1994). Consistently, reports on the efficacy of anakinra treatment indicate that IL-1 plays a pivotal role in the development of vascular damage in KD (Cohen et al., 2012; Dusser and Kone-Paut, 2017; Blonz et al., 2018; Gamez-Gonzalez et al., 2018; Kone-Paut et al., 2018). A prospective trial on anakinra in KD is underway (NCT02179853).

Coronary and peripheral artery inflammation also characterizes large vessel vasculitides Takayasu and giant cell arteritis (Berti et al., 2015; De Luca et al., 2017; Cavalli et al., 2018). Therapies for steroid- and DMARD-refractory large vessel vasculitides are limited. Analysis of temporal artery specimens from GCA patients revealed that IL-1 is highly expressed in inflamed vessels (Hernandez-Rodriguez et al.,

2004): accordingly, anakinra treatment dampened systemic and arterial inflammation in two cases refractory to conventional treatment (Ly et al., 2013).

Histiocytic Disorders

Erdheim–Chester disease (ECD) is a rare form of non-Langerhans histiocytosis, characterized by infiltration of foamy macrophages into multiple tissues (Campochiaro et al., 2015). The disease is typically sustained by activating mutations along the mitogen-activated protein kinase (MAPK) or related pathways in macrophages, which lead to cell activation and consequent production of high levels of pro-inflammatory cytokines and fibrosis of affected tissues (Cavalli et al., 2014; Cangi et al., 2015; Pacini et al., 2018). Clinical manifestations include bone pain, neurological symptoms, retroperitoneal fibrosis, and congestive heart failure (Cavalli et al., 2013a,b; Ferrero et al., 2016; Iurlo et al., 2016; Chiapparini et al., 2018). Anakinra is effective in ameliorating skeletal, cardiac, retroperitoneal, and systemic manifestations (Aouba et al., 2010; Killu et al., 2013; Franconieri et al., 2016; Tomelleri et al., 2018), thus substantiating the central role of macrophages in diseases responsive to IL-1 blockade (Diamond et al., 2014; Berti et al., 2017; Cavalli et al., 2017a). Interestingly, a traditional treatment option for ECD, that is, alpha interferon (IFNα), may exert beneficial effects via induction of IL-1Ra (Tilg et al., 1993) and inhibition of inflammasome activation (Guarda et al., 2011). Modulation of the IL-1 pathway may indeed explain the efficacy of IFNα in the management of ECD, as well as in a spectrum of clinical conditions similarly characterized by the BRAFV600E mutation and activation of the IL-1 pathway, including hairy cell leukemia and melanoma.

Hearing Loss

Hearing Loss in Autoinflammatory Syndromes

Sensorineural deafness is a prominent characteristic of CAPS, a spectrum of conditions caused by activating mutations in NLRP3 leading to deregulated release of active IL-1β (Aganna et al., 2002), which are effectively treated with IL-1 blockade. The first reports of reversal in sensorineural deafness with anakinra came from patients with MWS, a CAPS subtype (Rynne et al., 2006); several other reports followed (Gerard et al., 2007; Kitley et al., 2010; Ahmadi et al., 2011; Klein and Horneff, 2011; Kuemmerle-Deschner et al., 2011b,c, 2013; Eungdamrong et al., 2013; Stew et al., 2013). This reversal in sensorineural deafness with anakinra treatment delineated the unexpected concept that hearing loss in autoinflammatory diseases is due to a reversible chronic inflammatory response, rather than permanent loss of neuronal function. Nevertheless, early treatment with anakinra is more likely to be beneficial (Sibley et al., 2012). Patients with NOMID, a more severe disease also part of the CAPS spectrum, also benefit from anakinra treatment for hearing loss. In pediatric patients with CAPS, early identification in childhood and early anakinra treatment prevents or rescues sensorineural deafness and hearing loss, and results in normal intellectual development into adulthood (Rigante et al., 2006; Hedrich et al., 2008 #7023; Sibley et al., 2012).

Anakinra in Autoimmune Hearing Loss

Sensorineural deafness also occurs in vasculitis and autoimmune inner ear disease, and is clinically manifested as rapidly progressive, often irreversible hearing loss. Treatment relies on high-dose glucocorticoids, but many patients are refractory or become unresponsive over time. In these refractory cases, elevated IL-1β was demonstrated in the circulation and in monocyte cultures (Pathak et al., 2011). In an open-label, single-arm, phase I/II clinical trial of anakinra in corticosteroid-resistant autoimmune inner ear disease, 10 patients received treatment for 12 weeks. Of these, seven obtained audiometric improvement, paralleled by reduced IL-1β plasma levels (Vambutas et al., 2014).

Dry Eye Disease

Dry eye syndrome, or keratoconjunctivitis sicca, is a common, multifactorial disorder of the eye characterized by deficient tear production, excessive tear evaporation, or both. Meibomian gland dysfunction is thought to be the leading cause of this condition, which results in discomfort, visual disturbance, and ocular surface damage. Topical administration of low-dose (2.5%) anakinra proved effective in a randomized clinical trial of 75 dry eye disease patients, which achieved a significant reduction in mean severity score and symptoms (Amparo et al., 2013). It is tempting to envisage similarly favorable results in patients with Sjögren and sicca syndrome.

Pulmonary Silicosis

Pulmonary silicosis is an occupational disease caused by inhalation of silica crystals. These are not effectively cleared by alveolar macrophages and induce a chronic inflammatory response eventually leading to pulmonary fibrosis and progressive respiratory insufficiency (Leung et al., 2012). Since silica crystals activate the inflammasome and trigger release of active IL-1β (Hornung et al., 2008), a study evaluated anakinra treatment and documented progressive improvement in respiratory symptoms and pulmonary inflammation in a patient with pulmonary silicosis and severe respiratory failure (Cavalli et al., 2015a).

Organ Transplant

Previous studies identified associations between levels of IL-1β and IL-1Ra in serum and urine and negative graft outcome; studies in experimental animals and observations in humans also substantiated a possible protective effect of blocking IL-1 after solid organ transplantation (reviewed in Mulders-Manders et al., 2017). New evidence suggests that in patients undergoing solid organ transplantation, IL-1 inhibition in addition to standard immunosuppressive regimens may dampen inflammation and protect against negative graft outcome. Three patients undergoing renal transplantation were receiving treatment with anakinra in the peri-operative and post-operative period for underlying IL-1-driven autoinflammatory diseases (AOSD, CAPS, and FMF, respectively). Kidney function increased rapidly in all patients; anakinra was well tolerated and safe with the exception of minor infections (Mulders-Manders et al., 2017). The beneficial effects of treatment are likely due to dampening of ischemia-reperfusion injury, which accompanies

renal transplantation and leads to release of IL-1 and to impaired graft function.

ANAKINRA FOR CENTRAL NERVOUS SYSTEM DISEASES

Neurologic complications observed in CAPS patients reveal the effects of IL-1-mediated inflammation in the brain. Common clinical manifestations include headache or migraine, sensory-neural hearing loss, papilledema due to elevated intracranial pressure, and mental impairment (Kitley et al., 2010). IL-1 blockade with anakinra or canakinumab reverses neurologic inflammation and related symptoms, including mental and hearing impairment (Lachmann et al., 2009; Goldbach-Mansky, 2011; Kuemmerle-Deschner et al., 2011a; Lepore et al., 2011; Neven et al., 2011).

Anakinra Enters the Brain

The first evidence that anakinra administered peripherally crossed the blood–brain barrier and reduced severity of a disease primarily localized to the central nervous system came from NOMID (Goldbach-Mansky et al., 2006). Specifically, 12 children with NOMID were treated with 1–2 mg/kg of subcutaneous anakinra daily. The median cerebrospinal fluid (CSF) level of IL-1Ra was 211 pg/mL before treatment, but rose to 1136 pg/mL after 3 months of treatment (Goldbach-Mansky et al., 2006). These effects were associated with a remarkable decrease in the severity of various NOMID manifestations, including elevated intracranial pressure, leptomeningitis, and neurosensorial hearing loss, as well as reduced CSF levels of IL-6.

Intravenous anakinra was also administered to patients with subarachnoid hemorrhage due to aneurysmal rupture (Singh et al., 2014), again in a placebo-controlled setting. Within 72 h of the acute event, patients received a bolus infusion of 500 mg of anakinra followed by a steady infusion of 10 mg/kg/h for 24 h. At 24 h, CSF levels of IL-6 were reduced in the anakinra compared to the placebo group (Singh et al., 2014).

A related study investigated the dose regimen necessary to obtain a CSF concentration of anakinra 100 ng/mL. This concentration (100 ng/mL) was deemed neuro-protective based on studies of rats subjected to brain ischemia (Clark et al., 2008); as for human reference, this target concentration of 100 ng/mL is 100-fold greater than that in the CSF of children receiving subcutaneous anakinra 100 mg daily for 3 months (Goldbach-Mansky et al., 2006). In this study, patients with subarachnoid bleed received incremental doses of intravenous anakinra (Galea et al., 2011): specifically, the patients received a bolus dose of anakinra (100–500 mg) followed by a 4-h infusion of anakinra from 1 to 10 mg/kg/h. Levels of anakinra were monitored in plasma and CSF (collected through a cerebral ventricular drain). A target CSF level of 100 ng/mL was achieved with the highest regimen (a bolus of 500 mg followed by 4 h of anakinra at 10 mg/kg/h; Galea et al., 2011; Ogungbenro et al., 2016). The authors concluded that anakinra passively enters the brain in patients with a subarachnoid hemorrhage: therefore, a high-dose regimen of anakinra may reduce inflammation, infiltration of neutrophils, and edema at the site of the lesion. In a subsequent randomized study, patients with subarachnoid hemorrhage received anakinra 100 mg twice daily subcutaneously within 3 days of stroke and for the following 21 days. Again, anakinra treatment significantly reduced levels of inflammatory markers IL-6, CRP, and fibrinogen. Although these studies were not powered to determine clinical effects, scores of the Glasgow Outcome Scale at 6 months were better, albeit not significantly, among patients receiving anakinra. Whether dampening of IL-1-mediated inflammation will result in improved neurological outcomes remains to be determined in adequately powered, randomized, placebo-controlled studies.

In a different study, intravenous anakinra was administered to patients admitted to the hospital within 6 h of an acute thrombotic stroke (Emsley et al., 2005). This trial included 34 patients and was randomized and placebo controlled; anakinra was administered at a high dose of 2 mg/kg/h for 72 h, analogous to inception trials of anakinra in septic shock. Compared to placebo-treated controls, patients treated with anakinra had lower IL-6, CRP, and neutrophil levels (Emsley et al., 2005). Although the study was not powered for detecting significant improvements in neurological outcomes, the subgroup of patients with cortical infarcts receiving anakinra performed better compared to the placebo group.

Additional evidence that anakinra crosses the blood brain barrier and exerts anti-inflammatory effects in the brain comes from studies of traumatic brain injury, a major cause of death and disability worldwide, particularly in young persons. In a randomized, open-label trial, 20 patients who had suffered diffuse traumatic brain injury within the previous 24 h received either anakinra 100 mg daily for 5 days or placebo. A central microdialysis catheter was placed in each patient as part of standard of care. Prior to administration of anakinra, the mean level of IL-1Ra in the CSF was 78 pg/mL but rose to 138 pg/mL 12 h after the first dose (Helmy et al., 2014). In general, inflammatory cytokines in the CSF were lower in patients treated with anakinra; of these, macrophage-derived chemoattractant-1 (MDC-1) was remarkably lower compared to patients treated with the placebo (1.04 pg/mL in the anakinra group compared to 45.4 pg/mL in the placebo group; Helmy et al., 2014). The study was not powered to evaluate clinical improvement, although the marked decrease in CSF levels of cytokines and MDC-1 argue in favor of beneficial anti-inflammatory effects.

Epilepsy

Although IL-1α is found in brain astrocytes and microglia, available data point at IL-1β as the main contributor to epileptic seizures (Vezzani et al., 2011). Several studies have focused on febrile seizures since these are among the most common type of seizure activity. Using an animal model for febrile seizures, an agonist role for IL-1β and an antagonist role for endogenous IL-1Ra in the hippocampus have been reported (Heida et al., 2009). Other studies examined circulating cytokines in patients with recurrent seizures, and revealed elevated levels of IL-6 and IL-1Ra in the post-acute period (Uludag et al., 2013). In one study, high levels of IL-1β were also observed during acute

episodes of recurrent temporal lobe epilepsy (Uludag et al., 2015). Some studies have reported polymorphisms in IL-1α, IL-1β, and IL-1Ra in subjects who develop epilepsy as adults (Kanemoto et al., 2000; Haspolat et al., 2005; Nakayama and Arinami, 2006; Serdaroglu et al., 2009; Chou et al., 2010). In experimental animals, suppression of peripheral IL-1-mediated inflammation reduces the severity of status epilepticus (Marchi et al., 2009).

Anakinra has been administered to a young patient with a severe seizure disorder termed febrile infection-related epilepsy syndrome (FIRES; Hirsch et al., 2018). This syndrome, which often follows an infectious encephalopathy, has a high mortality rate and few treatment options. This patient had recurrent seizures each day, which progressively decreased in frequency and eventually ceased while being treated with daily subcutaneous anakinra (Kenney-Jung et al., 2016). This was mirrored by a decrease in CSF pro-inflammatory cytokines. When anakinra was stopped, seizures resumed only to decrease again upon restarting. A subsequent study confirmed the favorable outcome with anakinra described in this report. Anakinra was administered to five children with FIRES and refractory status epilepticus beginning shortly after a febrile illness. All had received anti-epileptic drugs (AEDs, ranging from two to six different medications), had required anesthetics for seizure control, and had received treatment with corticosteroids and IVIG; three underwent plasmapheresis. Anakinra was initiated on day 12–32 of illness at a dose ranging from 3 to 7 mg/kg/day, and led to rapid and remarkable clinical improvement in all but one patient. Specifically, seizure count in the week prior to anakinra initiation ranged from 8 to 170, but dropped to 0–12 after 1 week of anakinra treatment and to 0–7 after 4 weeks of treatment. Two patients had increased seizure burden upon anakinra weaning or discontinuation, again substantiating the role of IL-1 in seizure disorder (Shukla et al., 2018). In another study, an adolescent female with signs of persistent systemic inflammation and epilepsy unresponsive to multiple AEDs also promptly responded to anakinra (DeSena et al., 2018).

SAFETY OF IL-1 BLOCKADE WITH ANAKINRA

Impaired host defense against pathogens is a concern for cytokine-blocking agents. In patients treated with biologics, particularly TNFα blocking therapies, there is an increased risk of several opportunistic infections, similar to those observed in immunosuppressed persons. Host defense against opportunistic organisms as well as common bacterial infections have since become a major concern for all anti-cytokine agents because of the indolent and dangerous nature of these infections. For example, reactivation of latent *M. tuberculosis* in patients receiving anti-TNFα therapies can be 25 times higher than in untreated persons (Solovic et al., 2011) and is often in the disseminated form, similar to that observed in HIV-1 infected patients. *M. tuberculosis* also occurs in patients treated with TNFα blockers without evidence of prior exposure to the organism. Despite screening for previous exposure to *M. tuberculosis* before beginning any anti-cytokine treatment, reactivation continues to occur and can be as high as 9.3% (Chiu et al., 2011).

As with all biologic agents, an increase in infection frequency has been reported for anakinra. Nevertheless, in comparison to other biologic agents, anakinra has an unparalleled safety benefit deriving from short half-life and effect duration, and has demonstrated a remarkable record of safety (Fleischmann et al., 2006; Mertens and Singh, 2009b). Since introduction in 2002, it is estimated that over 150,000 patients have received anakinra, some treated daily for over 10 years. Opportunistic infections in patients treated with anakinra are rare (Fleischmann et al., 2003), including in populations at high risk for reactivation of *M. tuberculosis* infections (Bresnihan et al., 1998; Lopalco et al., 2016a,b). There is a single case report of a 77-year-old man with severe RA and a history of pulmonary tuberculosis who developed a reactivation 23 months after starting anakinra (Settas et al., 2007).

In addition, a large number of animal studies including primates subjected to live bacteria inoculum demonstrated greater survival in infected animals treated with anakinra compared to vehicle. In humans, anakinra has been administered to patients with active infections (Hennig et al., 2010; van de Veerdonk et al., 2011), and in over 2000 patients in trials of sepsis and septic shock without any increase in mortality despite exceedingly high dosing (30-fold higher than the current approved dose of 100 mg/day; Dinarello et al., 2012). Other safety examples include hidradenitis suppurativa, in which anakinra treatment resolves inflammation of *Staphylococcus aureus*-infected apocrine glands (Braun-Falco et al., 2011; Hsiao et al., 2011; Zarchi et al., 2013; Tzanetakou et al., 2016), and chronic granulomatous disease, an inherited condition with recurrent bouts of infections with Gram-positive and Gram-negative bacteria as well as fungi (van de Veerdonk et al., 2011): in both conditions, treatment with anakinra reduces the severity of inflammation without increasing the infection burden.

During controlled trials of anakinra, there were more viral-type upper airway infections compared to placebo-treated patients, as for most other biologics. There are two spurious reports of anakinra-related hepatotoxicity in patients with AOSD; however, withdrawal of anakinra restored normal liver function. Of note, there are several reports of the safety of increasing the dose of anakinra to 200 mg/day or above (Vitale et al., 2016; Colafrancesco et al., 2017; Grayson et al., 2017).

Subcutaneous administrations of anakinra often cause injection site reactions. Albeit uncomfortable due to the need for daily injections, these usually resolve within 2–3 weeks of treatment initiation. A fraction of patients receiving treatment with anakinra can develop antibodies against the drug (Cohen et al., 2002; Ilowite et al., 2009). As with other biologic agents, the potential for adverse effects including hypersensitivity reactions or aplasia should be carefully monitored. Conversely, these antibodies are usually non-neutralizing and do not decrease the biologic effectiveness of anakinra, nor they appear to be linked to the development of injection site reaction.

Interleukin-1 injected into humans at doses as low as 3 ng/kg induce neutrophil mobilization from the bone marrow and

neutrophilia (Dinarello, 1996; Ogilvie et al., 1996). Hence, most patients with IL-1-mediated conditions exhibit neutrophilia as a hematological manifestation of their disease. A reduction in circulating neutrophils upon anakinra administration can be observed and often heralds a clinical response; sustained neutropenia is not typically observed, but neutrophil levels occasionally fall below the normal range, only to rise rapidly upon cessation of treatment (Cavalli and Dinarello, 2015).

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REVIEW
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Potential of IL-1, IL-18 and Inflammasome Inhibition for the Treatment of Inflammatory Skin Diseases

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In 2002, intracellular protein complexes known as the inflammasomes were discovered and were shown to have a crucial role in the sensing of intracellular pathogen- and danger-associated molecular patterns (PAMPs and DAMPs). Activation of the inflammasomes results in the processing and subsequent secretion of the pro-inflammatory cytokines IL-1β and IL-18. Several autoinflammatory disorders such as cryopyrin-associated periodic syndromes and Familial Mediterranean Fever have been associated with mutations of genes encoding inflammasome components. Moreover, the importance of IL-1 has been reported for an increasing number of autoinflammatory skin diseases including but not limited to deficiency of IL-1 receptor antagonist, mevalonate kinase deficiency and PAPA syndrome. Recent findings have revealed that excessive IL-1 release induced by harmful stimuli likely contributes to the pathogenesis of common dermatological diseases such as acne vulgaris or seborrheic dermatitis. A key pathogenic feature of these diseases is IL-1β-induced neutrophil recruitment to the skin. IL-1β blockade may therefore represent a promising therapeutic approach. Several case reports and clinical trials have demonstrated the efficacy of IL-1 inhibition in the treatment of these skin disorders. Next to the recombinant IL-1 receptor antagonist (IL-1Ra) Anakinra and the soluble decoy Rilonacept, the anti-IL-1α monoclonal antibody MABp1 and anti-IL-1β Canakinumab but also Gevokizumab, LY2189102 and P2D7KK, offer valid alternatives to target IL-1. Although less thoroughly investigated, an involvement of IL-18 in the development of cutaneous inflammatory disorders is also suspected. The present review describes the role of IL-1 in diseases with skin involvement and gives an overview of the relevant studies discussing the therapeutic potential of modulating the secretion and activity of IL-1 and IL-18 in such diseases.

Keywords: IL-1, IL-1alpha, IL-1beta, IL-18, inflammatory skin conditions, inflammasome

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INTERLEUKIN-1 FAMILY

Cytokines comprise a variety of molecules secreted by immune and non-immune cells that regulate important cellular functions and physiological processes especially in the hematopoietic and immune systems. One important class of cytokines are the interleukins, a large family of small secreted proteins that bind to specific membrane receptors on target cells. The history of

interleukins, and particularly of interleukin-1 (IL-1), began in 1948 when Paul B. Beeson discovered an active unknown substance obtained from rabbit leukocytes that was able to cause fever (Beeson, 1948). Decades later, Dinarello et al. (1974, 1977) identified two chemically and biologically distinct pyrogenic molecules produced by neutrophils and monocytes incubated with heat-killed *Staphylococcus albus*; he named them human leukocytic pyrogens (LP). Before him, Gery et al. (1972) reported that the stimulation of murine and human lymphocytes with lipopolysaccharide (LPS), an essential component of Gram-negative bacteria, led to the release of a soluble factor that was able to enhance the response of T lymphocytes to lectins (phytohemagglutinin and concanavalin A). In 1979, the molecules with inflammatory properties reported by Charles Dinarello and Igal Gery revealed to be the same, namely IL-1 (Rosenwasser et al., 1979). Following progress in sequencing technologies, it turns out that the IL-1 family comprises a total of eleven members with similar or distinct biological effects. In addition to IL-1α and β, IL-18, IL-33, IL-36α, β and γ are pro-inflammatory, whilst, IL-1 receptor agonist (IL-1Ra), IL-36Ra, IL-37, and IL-38 are anti-inflammatory. Genes encoding IL-1 family members are mostly located on human chromosome 2 with two exceptions, namely the genes encoding IL-18 and IL-33 that are located on chromosomes 11 and 9, respectively.

IL-1β is not only secreted by immune cells such as monocytes/macrophages, dendritic cells, neutrophils, B lymphocytes and NK cells but also by non-immune cells such as keratinocytes (Dinarello, 2009; Feldmeyer et al., 2010). IL-1β is able to act on a broad range of cell types (Dinarello, 2009, 2011). It is a key mediator of the acute phase of inflammation inducing local and systemic responses. Its effects are numerous and include the secretion of downstream pro-inflammatory mediators such as cyclooxygenase type-2 (COX-2), IL-6, Tumor Necrosis Factor (TNF) and IL-1 itself (Dinarello, 1996; Weber et al., 2010). In the body, the inflammatory effects of IL-1 manifest as fever, vasodilation and hypotension as well as an increased sensitivity to pain. The pyrogenic activity of IL-1 is due to the activation of NF-κB and the resulting expression of COX-2, an enzyme involved in the synthesis of prostaglandins (Lee et al., 2004).

IL-1 cytokines bind to and act through specific receptors, which are characterized by intracellular Toll/Interleukin-1 receptor (TIR) domains and an extracellular immunoglobulin-like binding domain (Boraschi and Tagliabue, 2013). The IL-1 receptor family comprises several members including IL-1R1, the decoy receptor IL-1R2, IL-1R accessory protein (IL-1RaP or IL-1R3), IL-1R4 (T1 or ST2), IL-18Rα (IL-1R5), IL-36R (IL-1R6), IL-18R accessory protein (IL-18Rβ or IL-1R7), IL-1R8 (TIR8), IL-1R9 (IL-1RAPL2), and IL-1R10 (TIGIRR).

IL-1α/IL-1β, IL-18, and IL-36 initiate immune and inflammatory responses by binding to IL-1R1, IL-18Rα, and IL-36R, respectively. The co-receptor IL-1RaP interacts with IL-1R1, IL-1R2, IL-1R4, and IL-36R while IL-18Rβ is a unique accessory chain for IL-18Rα. The decoy receptor IL-1R2 lacks the cytoplasmic TIR domains and is therefore unable to initiate a signaling cascade even in the presence of its accessory receptor. IL-1R2 binds IL-1β with high affinity and IL-1α or IL-1Ra with low affinity (Symons et al., 1995). The biological activity of IL-1 family cytokines is tightly regulated not only by decoy receptors but also by soluble receptor antagonists such as IL-1Ra and IL-36Ra that can specifically antagonize IL-1α, IL-1β, and IL-36. In addition, IL-1R1 can be released into the extracellular space where, in its soluble form (sIL-1R1), it can also function as a soluble decoy receptor and prevent the binding of IL-1α, IL-1β, and IL-1Ra to membrane IL-1R1 (Burger et al., 1995). Furthermore, IL-1R2 can also be cleaved and solubilized by metalloproteinases resulting in an increased segregation of IL-1β due to its higher affinity (Symons et al., 1995).

First identified and described as interferon-γ-inducing factor (IGIF) (Nakamura et al., 1989, 1993), IL-18 received its current name 3 years later (Ushio et al., 1996). In contrast to the strong pyrogenic activity of IL-1α and IL-1β, IL-18 is only able to induce fever at higher concentrations. IL-18 activates primarily p38 MAPK and AP-1, but fails to activate NF-κB (Lee et al., 2004). IL-18 activity is mainly regulated by a soluble protein called IL-18 binding protein (IL-18BP). IL-18BP differs from the other soluble IL-1 receptors because it retains a unique binding sequence composed by a single immunoglobulin domain (Dinarello et al., 2013). Similar to the IL-1Rs, IL-18Rs can also be found in a soluble form that is used as a biomarker for inflammatory diseases such as rheumatoid arthritis (RA) and adult-onset Still's disease (AoSD) (Takei et al., 2011).

Both IL-1β and IL-18 are first synthesized as precursors which need to be processed into their biologically active form by a cytoplasmic protein complex known as the inflammasome. In contrast, both pro and cleaved forms of IL-1α are biologically active and induce, via IL-1R1 signaling, the production of TNFα and IL-6 in human A549 epithelial cells and peripheral blood mononuclear cells (PBMCs) (Kim et al., 2013). The transcription of the IL-1α gene is regulated by a variety of stimuli including proinflammatory or stress-associated stimuli and growth factors (Di Paolo and Shayakhmetov, 2016). Pro-IL-1α lacks a signal secretion peptide, however, its release from dying cells is able to trigger acute inflammation (Chen et al., 2007). IL-1α can be translocated to the plasma membrane where it signals in an intra and paracrine manner but can also be secreted in its mature form via both IL-1β-dependent or independent pathways (Fettelschoss et al., 2011; Gross et al., 2012). Since pro-IL-1α contains a nuclear localization signal, it can induce the expression of proinflammatory genes independently of IL-1R1 signaling (Werman et al., 2004). Because of the multiplicity of mechanisms of action of IL-1α, it plays an important role in the maintenance of homeostasis and the pathology of several human diseases (Di Paolo and Shayakhmetov, 2016).

PATHOGEN RECOGNITION RECEPTORS

Pathogen recognition is fulfilled by a distinct set of receptors, the so-called pathogen-recognition receptors (PRRs). These include C-type lectin receptors (CLRs), Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like

receptors (NLRs), AIM2-like receptors (ALRs), and partially the complement system. Such PRRs have different localizations and ligands. For example: the complement system acts as an extracellular sensor for conserved pathogen motives (carbohydrates) and host antibodies; TLRs are found on cellular membrane and endosomes and bind to a variety of molecules including nucleic acids (TLR3, TLR8, TLR9, and TLR13), small proteins (TLR2, TLR4, TLR11, and TLR12), lipopeptides and lipoproteins (TLR1, TLR2, and TLR6), glycolipids (TRL2, TLR4) and small drugs (TLR4, TLR7) (Leifer and Medvedev, 2016). CLRs are either localized at the cell surface or in endosomes and primarily bind to carbohydrates (mannose, fucose, GlcNAc, and β-1,3-glucan) in a Ca^{2+}-dependent manner but the recognition of proteins, lipids and inorganic compounds like $CaCO_3$ has also been reported (Zelensky and Gready, 2005).

Retinoic acid-inducible gene I (RIG-I)-like receptors and NLRs are exclusively located in the cytosol. RLRs include RIG-I, MDA5 and the co-receptor LGP2. They are specialized in the sensing of viral double-stranded RNA (Reikine et al., 2014). NLRs constitute an expanding family of receptors able to detect a variety of molecules. They are composed of several domains: the central NOD or nucleotide-binding domain (NBD) that includes a NTPase NACHT domain controlling self-oligomerization, and the leucine-rich repeat (LRR) domain involved in ligand sensing (Schroder and Tschopp, 2010). On their N-terminal extremity, NLRs have either a pyrin domain (PYD), a caspase-recruitment domain (CARD) or a baculoviral inhibition of apoptosis protein repeat domain (BIR) and are consequently named NLRPs, NRLCs or NAIPs, respectively.

THE INFLAMMASOMES

In 2002, the group of Prof. Tschopp described a multiprotein complex able to oligomerize and activate inflammatory caspases leading to the processing of IL-1β and IL-18 (Martinon et al., 2002). This complex was named **NLR PYD-containing protein 1 (NLRP1)-inflammasome** and was shown to contain the scaffold NLRP1 interacting via PYD with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC, also known as PYCARD) which can then recruit the inflammatory procaspase, caspase-1(also known as IL-1β-converting enzyme or ICE). Upon sensing of appropriate ligands and subsequent inflammasome activation, procaspase-1 is autocatalytically cleaved and activated (Wilson et al., 1994). Active caspase-1 can then process IL-1β and IL-18 and the biologically active cytokines are secreted in an unconventional golgi/endoplasmic reticulum-independent manner (Keller et al., 2008). The NLRP1 inflammasome is able to sense bacterial peptidoglycan muramyl dipeptide (MDP) (Hsu et al., 2008) and can be activated in keratinocytes by ultraviolet B (UVB) irradiation (Feldmeyer et al., 2007). Mutations in the *Nlrp1* gene have been linked to susceptibility to vitiligo-associated autoimmune diseases (Jin et al., 2007), systemic lupus erythematosus (SLE) and RA (Masters, 2013). Gain-of-function mutations of the *NLRP1* gene were recently described in two skin disorders, namely multiple self-healing palmoplantar carcinoma (MSPC) and familial keratosis lichenoides chronica (FKLC). *NLRP1* mutations result in the blockade of the autoinhibitory effect of NLRP1 PYD domain and lead to an increased activation of the inflammasome (Zhong et al., 2016). NLRP1 also contains a C-terminal CARD domain which mediates direct interaction with caspase-1. A recent study has demonstrated that anthrax lethal factor can cleave the PYD domain of murine but not human NLRP1 causing its activation. This identifies proteolysis as an alternative activation mechanism for NLRP1 (Chavarria-Smith et al., 2016).

The **NLRP3 inflammasome** is the best characterized inflammasome to date, and a broad range of stimuli can induce its activation. These include PAMPs such as LPS, fungal zymosan, bacterial toxins, and also the bacteria *Listeria monocytogenes* (Meixenberger et al., 2010), *S. aureus* (Munoz-Planillo et al., 2009), and *Propionibacterium acnes* (Kistowska et al., 2014b; Qin et al., 2014), as well as yeasts like *Candida albicans* (Hise et al., 2009) and of the *Malassezia* spp. (Kistowska et al., 2014a). NLRP3 can also be activated by danger-associated molecules that are not derived from pathogens but often associated with cellular stress, the so-called DAMPs, including extracellular ATP (Mariathasan et al., 2006), asbestos (Dostert et al., 2008), amyloid-β (Halle et al., 2008), DNA:RNA hybrids (Kailasan Vanaja et al., 2014), and crystals such as gout-causing monosodium urate (MSU) (Martinon et al., 2006), silica (Dostert et al., 2008), or cholesterol (Duewell et al., 2010). Interestingly, the study of patients with autosomal dominant cold-induced urticaria, later termed familial cold autoinflammatory syndrome (FCAS), allowed the identification of mutations in the *CIAS1*/cryopyrin/*NLRP3* gene (Hoffman et al., 2001). These studies permitted major advances in the identification and understanding of autoinflammatory diseases but also resulted in a gain of interest in IL-1β biology and its role in inflammatory disorders.

Since such a broad range of stimuli can activate the NLRP3 inflammasome, it is believed that a common mechanism triggered by diverse activators leads to NLRP3 activation. Several events such as the release of oxidized mitochondrial DNA (Shimada et al., 2012), production of reactive oxygen species (ROS) (Dostert et al., 2008), mitochondrial stress (Zhou et al., 2011), lysosomal rupture with cathepsin B release (Hornung et al., 2008), changes in intracellular calcium (Ca^{2+}) levels (Murakami et al., 2012) and potassium (K^+)-efflux (Petrilli et al., 2007) have been reported to be associated to inflammasome activation (**Figure 1**). Whether all or only a part of these events are required for NLRP3 inflammasome activation is not clear. Munoz-Planillo et al. (2013) suggested that the sole reduction of intracellular K^+ was sufficient for NLRP3 inflammasome activation but recent reports have suggested that, in certain circumstances, inflammasome activation can occur independently of K^+-efflux (Gross et al., 2016) or phagocytosis of bacteria (Chen et al., 2016). Moreover, the activity of the NRLP3 inflammasome has also been reported to be controlled by kinases such as Bruton's tyrosine kinase (BTK) interacting with NLRP3 and ASC thus favoring the recruitment of caspase-1 (Ito et al., 2015), and JNK or Syk kinases regulating ASC oligomerization (Hara et al., 2013; Okada et al., 2014). ROS were shown to activate NEK7, a kinase involved in the control of mitosis, causing its direct binding to the LRR domain of NLRP3 and modulating

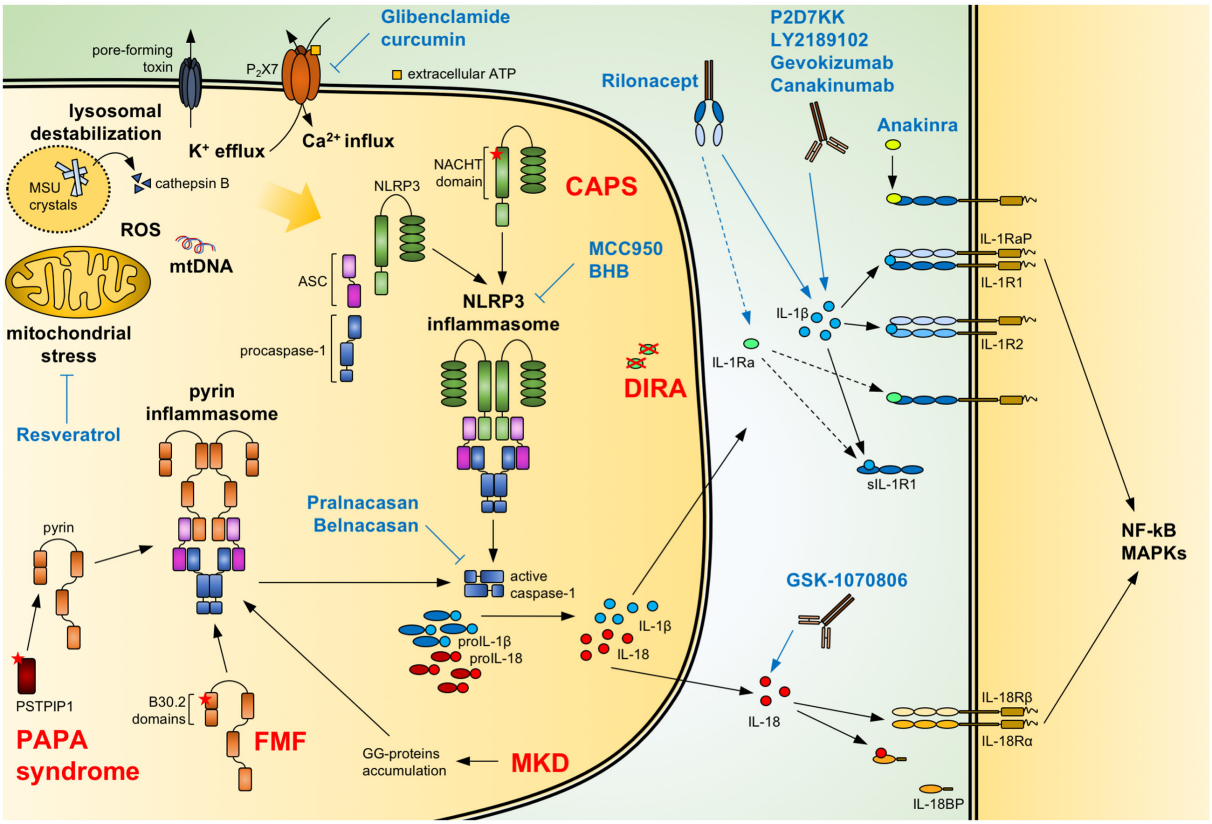

FIGURE 1 | Regulation of IL-1/18 production and current IL-1/18 antagonists. Pathogen- and danger-associated molecular patterns can induce the formation of a functional inflammasome via events including mitochondrial stress with release of oxidized mtDNA, ROS, lysosomal destabilization with cathepsin B release, changes in intracellular calcium (Ca^{2+}) and potassium (K^{+}) efflux. Also, mutations of NACHT-PYD domains of NLRP3 in cryopyrin-associated periodic syndromes (**CAPS**) lead to the activation of the NLRP3 inflammasome. Similarly, mutations of B30.2 domains in familial Mediterranean fever (**FMF**) and of PSTPIP1 in **PAPA syndrome** or accumulation of geranylgeranylated proteins (GG-proteins) in mevalonate kinase deficiency (**MKD**) lead to activation of the pyrin inflammasome. Activated inflammasomes recruit procaspase-1 via the adaptor ASC. Autocatalytically cleaved and activated caspase-1 can then process IL-1β and IL-18. The secreted cytokines bind to the IL-1 receptor (IL-1R1:IL-1RaP) and IL-18 receptor (IL-18Rα:IL-18Rβ), respectively, resulting in NF-κB and MAPKs signaling. Soluble IL-1R1 (sIL-1R1), IL-1R2 and IL-18 binding protein (IL-18BP) can block the signaling pathway. Caspase-1 activity can be modulated by the specific inhibitors **Pralnacasan** and **Belnacasan**. The activation of the NLRP3 inflammasome can be inhibited with **Glibenclamide** and **curcumin** inhibiting or downregulating the ATP-sensitive K^{+}-channels P_2X7, respectively; with **Resveratrol** increasing autophagy and inhibiting mitochondrial stress; and with the specific NLRP3 inhibitors **MCC950** and **BHB**. **Anakinra** and the secreted IL-1 receptor antagonist (IL-1Ra), absent in deficiency of IL-1 receptor antagonist (**DIRA**) patients, compete with IL-1β and IL-1α (not shown) for binding to the IL-1 receptor. **Rilonacept** acts as a soluble decoy binding IL-1β and with lower affinity IL-1α (not shown) and IL-1Ra. The recombinant antibodies **Canakinumab**, **Gevokizumab**, **LY2189102,** and **P2D7KK** specifically target and neutralize IL-1β. Similarly, **GSK-1070806** and **MABp1** inhibits IL-18 and IL-1α (not shown), respectively. Blue arrows: inhibitory effect; dotted arrow: low affinity; ASC, apoptosis associated speck-like domain protein containing a CARD; MSU, monosodium urate; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

its function (He et al., 2016; Shi et al., 2016). The consensual and unifying mechanism leading to NLRP3 inflammasome is currently a matter of intense debate and investigation.

The **absent in melanoma 2 (AIM2) inflammasome** recognizes viral and bacterial double-stranded DNA (dsDNA) via its PYHIN domain (Muruve et al., 2008). AIM2, like NLRP3, recruits caspase-1 via the adaptor protein ASC. Increased levels of AIM2 were found in keratinocytes of patients with psoriasis and atopic dermatitis, causing acute and chronic skin barrier disruption-related inflammation (Ito et al., 2015).

The **NLRC4 inflammasome** is activated by bacterial flagellin (Mariathasan et al., 2004) and type 3 secretion system proteins (Miao et al., 2010). NLRC4 contains a CARD domain and is therefore able to recruit and activate caspase-1 without the adaptor ASC. *Salmonella typhimurium* is reported to activate NLRC4 by inducing its phosphorylation by protein kinase C δ-type (PKCδ) (Qu et al., 2012). Moreover, NLRC4 can recruit NLRP3 resulting in increased caspase-1 processing (Qu et al., 2016). Reported *NLRC4* gene mutations cause recurrent fever flares and macrophage activation syndrome (MAS) (Canna et al., 2014), neonatal-onset enterocolitis and fatal or near-fatal episodes of autoinflammation (Romberg et al., 2014). A missense mutation of NLRC4 was also associated with FCAS (Kitamura et al., 2014).

The **pyrin inflammasome** is encoded by the *MEFV* gene and contains PYD, TRIM, and B30.2 domains. This inflammasome is activated by bacterial toxins like *Clostridium difficile* toxin B (TcdB) and C3 toxins (Park et al., 2016). Mutations in

this gene are the cause of familial Mediterranean fever (FMF) (Chae et al., 2006) and the recently described disease-entity pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) (Masters et al., 2016). Mevalonate kinase deficiency (MKD) was also linked to the activation of the pyrin inflammasome (Park et al., 2016).

Other less characterized inflammasomes include NLRP2, NLRC2, NLRP6, NLRP7, and NLRP12. Besides their role in caspase-1 activation, they possess roles in activating/inhibiting NF-κB as well as MAPK pathways and autophagy (Lupfer and Kanneganti, 2013).

The involvement of caspase-5 in the processing of IL-1 was already described in the original publication describing the inflammasome (Martinon et al., 2002), but it is only a decade later that its role in cell death was reported (Kayagaki et al., 2011, 2015). Human caspase-4, caspase-5 and the orthologous murine caspase-11 are activated by cytosolic LPS (Shi et al., 2014) and can cleave the substrate gasdermin D (GSDMD). GSDMD creates pores in the cell membrane resulting in pyroptosis, an inflammatory form of programmed cell death (He et al., 2015; Shi et al., 2015). Activated GSDMD can also induce the formation of the NLRP3 inflammasome and subsequent IL-1β and IL-18 secretion. This process known as the **non-canonical inflammasome** activation, can occur either in a caspase-1/inflammasome-dependent or independent manner (Man and Kanneganti, 2016). Indeed, caspase-8, an initiator caspase mainly involved in apoptosis, can be involved in the activation of the NF-κB pathway and IL-1β/IL-18 processing. Recognition by dectin-1 of extracellular fungi such as *Candida albicans* results in the formation of a complex with CARD9, Bcl-10, MALT1, ASC and caspase-8 which, once activated, can directly process IL-1β. Interestingly, dectin-1 dependent internalization of fungi drives instead the NLRP3 inflammasome (Gringhuis et al., 2012). Moreover, activation of caspase-8 through the Fas-signaling pathway can also lead to the direct processing of IL-1β and IL-18 independently of caspase-1 and ASC (Bossaller et al., 2012).

Other enzymes that are described to process IL-1 family members include neutrophil-derived elastase, cathepsin G and proteinase 3 (myeloblastin), mast cell-derived chymase and granzyme B from cytotoxic lymphocytes and natural killer cells (Afonina et al., 2015).

INHIBITION OF IL-1 SIGNALING

IL-1β Antagonists

Given the key role of IL-1β in inflammatory and autoinflammatory disorders, several IL-1 inhibitors have been developed and evaluated especially in life-threatening autoinflammatory syndromes. To date, the most efficient way to block IL-1 signaling consists of biologics that specifically target IL-1 or IL-1R1 (**Figure 1** and **Table 1**).

Anakinra (Kineret®; Sobi, Inc.) is a recombinant non-glycosylated homolog of IL-1Ra that competes with both IL-1α and IL-1β for the binding to IL-1R1 thus impairing the recruitment of IL-1RaP and downstream NF-κB/MAPKs signaling. It is the first biologic developed to specifically target IL-1. Anakinra was first approved in 2001 for the treatment of RA. A decade later, its use was extended to the treatment of cryopyrin-associated periodic syndromes (CAPS) in Europe and for the severest form of CAPS, namely chronic infantile neurological cutaneous and articular syndrome (CINCA) in the USA. Anakinra has a short half-life of 4–6 h and therefore common posology requirements are daily subcutaneous injections of 100 mg/day for RA and 1–2 mg/kg/day for CINCA.

Rilonacept (Arcalyst®; Regeneron) is a long-acting dimeric fusion protein consisting of portions of IL-1R1 and IL-1RaP linked to the Fc portion of human immunoglobulin G1 (IgG1). Rilonacept acts a soluble decoy binding IL-1β, but also IL-1α and IL-Ra, therefore inhibiting their association with cell surface receptors (IL-1Trap). Rilonacept binds three times stronger to IL-1β than to IL-1α and 12 times stronger to

TABLE 1 | Biologics and inhibitors of IL-1, IL-18, and inflammasome activation.

Name	Trade name	Company	Class	Target	$h_{1/2}$	Status
Anakinra	Kineret®	Sobi, Inc.	recIL-Ra	IL-1α, IL-1β	4–6 hours	Marketed
Rilonacept	Arcalyst®	Regeneron	srR (IL-1Trap)	IL-1α, IL-1β, IL-Ra	∼7.5 days	Marketed
Canakinumab (ACZ855)	Ilaris®	Novartis	mAb (IgG1/κ)	IL-1β	23–26 days	Marketed
Gevokizumab (XOMA 052)		XOMA	mAb (IgG2/κ)	IL-1β	22 days	Phase 3† discontinued
LY2189102		Eli Lilly and Co	mAb (IgG4)	IL-1β	16.8 days	Phase 2
P2D7KK		A*STAR	mAb (IgG1)	IL-1β	∼2 weeks*	Preclinical
Pralnacasan (VX-740)		Vertex	SMI	Caspase-1	nd	Phase 2†
Belnacasan (VX-765, HMR3480)		Vertex	SMI	Caspase-1	nd	Phase 2†
MCC950			SMI	NLRP3	nd	Preclinical
BHB			SMI	NLRP3	nd	Preclinical
Glibenclamide (glyburide)	Generic		SMI	K_{ATP}	10 hours	Marketed
MABp1	Xilonix™	XBiotech	mAb (IgG1/κ)	IL-1α	8 days	Phase 3‡ Phase 2
GSK-1070806		GlaxoSmith-Kline	mAb (IgG1/κ)	IL-18	23–30 days	Phase 2

*srR, soluble recombinant receptor; SMI, small molecule inhibitor; K_{ATP}, ATP-sensitive potassium channels; $h_{1/2}$, half-life; nd, not determined. *Estimation. †Clinical trial was terminated. ‡Only for the treatment of colorectal and non-small cell lung cancers.*

IL-1β than to IL-1Ra. It was approved by the FDA in 2008 for the treatment of CAPS including FCAS and Muckle-Wells syndrome (MWS). Rilonacept has a half-life of 6.3–8.6 days which allows a weekly subcutaneous administration of 320 mg (loading dose) followed by weekly injections of half the loading dose.

Canakinumab (ACZ885, Ilaris®; Novartis) is a human anti-IL1β monoclonal IgG1/κ isotype antibody with a terminal half-life of 23–26 days and can be therefore administered as a single subcutaneous injection every 2 months.

Canakinumab was approved by the FDA in 2009 for the treatment of CAPS and active systemic juvenile idiopathic arthritis. Recently, it received approval from the FDA as first-line treatment for TNF receptor associated periodic syndrome (TRAPS), MKD and FMF (FDA, 2016).

Gevokizumab (XOMA 052; XOMA) is a recombinant humanized anti-IL-1β antibody. In contrast to Canakinumab, which neutralizes IL-1β by competing for binding to IL-1R, Gevokizumab modulates IL-1β bioactivity by reducing its affinity for IL-1R1:IL-1RAcP signaling complex (Blech et al., 2013).

The clinical development of this antibody was interrupted in 2016 after a phase 3 clinical trial evaluating Gevokizumab for the treatment of uveitis in patients with Behçet's disease did not meet the primary endpoint criteria (Xoma, 2015, 2016).

Gevokizumab showed promising results in phase 2 trial on acne vulgaris (Xoma, 2013) but failed to show benefits in the treatment of pyoderma gangrenosum (Xoma, 2016).

LY2189102 (Eli Lilly and Co) is a high affinity anti-IL-1β humanized monoclonal immunoglobulin G4 with a terminal half-life of 16.8 days (Bihorel et al., 2014). A weekly treatment of patients suffering from type 2 diabetes mellitus (T2DM) with LY2189102 for 3 months resulted in modest reductions in glycated hemoglobin and blood glucose (Sloan-Lancaster et al., 2013). No further studies have been conducted to date.

P2D7KK is another neutralizing monoclonal antibody against IL-1β developed by A*STAR researchers in Singapore. It shares the same mechanism of action as Canakinumab but with an *in vitro* neutralization potency that is 11 times higher. P2D7KK has not been evaluated in human subjects yet but has shown promising effects in three different inflammatory animal models (Goh et al., 2014).

Virus-like particles (VLPs)-based vaccination constitutes a novel approach to target cytokines (Assier et al., 2017). Recombinant mutated IL-1β chemically cross-linked to bacteriophage Qβ VLPs (**hIL1βQβ**) was investigated in a phase 1 clinical trial for T2DM resulting in safe production of specific IL-1β antibodies in the treated patients (Cavelti-Weder et al., 2016).

Inflammasome Inhibitors

Other possibilities to block IL-1β include the targeting of caspase-1 and NLRP3. Two caspase-1 inhibitors have been developed, namely **Pralnacasan** (VX-740) and **Belnacasan** (VX-765, also HMR3480) (Vertex Pharmaceuticals). These orally absorbed compounds are synthetized as prodrugs which are then converted into the active principle, VRT-018858 and VRT-043198, respectively.

Pralnacasan has been evaluated in clinical trials for the treatment of RA and osteoarthritis but due to safety issues its development has been interrupted (Braddock and Quinn, 2004; Vertex, 2007).

Belnacasan was shown to inhibit IL-1β and IL-18 release from PBMCs of FCAS patients *in vitro* (Stack et al., 2005). It induces anti-inflammatory effects in a mouse model of delayed-type hypersensitivity (DTH) (Wannamaker et al., 2007) and it has been evaluated in phase 1 and 2a clinical trials in the setting of epilepsy and psoriasis (Vertex, 2011).

NLRP3 inhibitors include MCC950, β-hydroxybutyrate and glibenclamide. **MCC950** is a small-molecule able to block canonical and non-canonical NLRP3-induced ASC oligomerization without interfering with NLRC4 and AIM2 activity or TLR signaling (Coll et al., 2015). MCC950 has been shown to be effective for the treatment of CAPS in mice harboring activating *Nlrp3* mutations (Coll et al., 2015) and in mouse models of dermal and airway inflammation (Primiano et al., 2016). β-**hydroxybutyrate** (BHB) is an anti-inflammatory molecule that specifically targets NLRP3 activity. In murine models of FCAS and MWS, BHB inhibited constitutive NLRP3 inflammasome activation (Youm et al., 2015). **Glibenclamide** (glyburide), is an anti-diabetic drug used in the treatment of T2DM. It inhibits the ATP-sensitive K^+ channel and was shown to block the NLRP3 inflammasome activation induced by LPS, ATP, nigericin and silica (Lamkanfi et al., 2009). These inhibitors present a potential advantage in the treatment of CAPS since they specifically target the NLRP3 inflammasome impacting both IL-1β and IL-18 secretion.

Resveratrol and **curcumin** are natural polyphenols found in several plants and are able to block IL-1β secretion. Resveratrol was described to inhibit NLRP1, NLRP3, and NLRC4 activation by preventing mitochondrial damage and augmenting autophagy (Chang et al., 2015). Moreover, Resveratrol was shown to directly bind and block COX-2 activity (Zykova et al., 2008) which is known to be involved in NLRP3 activation (Hua et al., 2015). Curcumin has been shown to impair IL-1β secretion in PMA-treated macrophages by downregulating $P2X_7$ receptor and thus inhibiting the TLR4/MyD88/NF-κB pathway (Kong et al., 2016).

IL-1α and IL-18 Blockers

In contrast to IL-1β, the development of IL-1α and IL-18 inhibitors is less advanced. The monoclonal antibody against IL-1α, **MABp1** (Xilonix™; XBiotech) is the only biologic that specifically target this cytokine and it is currently under investigation for the treatment of advanced cancer (Hong et al., 2014; Hickish et al., 2017).

MABp1 has been evaluated for the treatment of psoriasis (Coleman et al., 2015), acne vulgaris (Carrasco et al., 2015), T2DM (Timper et al., 2015) and is currently being evaluated in patients with hidradenitis suppurativa (HS) who were refractory to anti-TNFα treatment (discussed below).

The only agent targeting the cytokine IL-18 is the **GSK-1070806** antibody (GlaxoSmithKline). In a study for

the treatment of T2DM, GSK-1070806 did not reveal any improvement in glucose control (McKie et al., 2016). However, neutralization of IL-18 was shown to reduce the severity of dextran sulfate sodium-induced colitis in mice (Siegmund et al., 2001; Sivakumar et al., 2002).

SKIN DISEASES WITH IL-1 INVOLVEMENT

Keratinocytes are the most abundant cells in the skin and act as a barrier against water loss and entry of pathogens and irritants. Human keratinocytes constitutively express IL-1α, IL-β, and IL-18 and possess all inflammasome components (Feldmeyer et al., 2007).

Inflammation in the skin with extensive release of IL-1β is often associated with neutrophilic infiltration as first line of defense. In the absence of infection, neutrophils can become detrimental for the host by causing tissue damage (Navarini et al., 2016).

Monogenic Autoinflammatory Diseases

Monogenic autoinflammatory diseases are a rare group of hereditary syndromes with early manifestation in childhood. They present as inflammatory recurrent flares of fever and skin lesions. Neutrophilic dermatosis is the most common pathological hallmark of these syndromes (Lipsker et al., 2016).

Cryopyrin-associated periodic syndromes are disorders caused by mutations in the *NLRP3* gene, previously known as cold-induced autoinflammatory syndrome 1, which results in uncontrolled processing of IL-1β and IL-18 (**Figure 1**). CAPS is a spectrum of three syndromes of increasing severity: **familial cold autoinflammatory syndrome** (FCAS, OMIM #120100), **Muckle-Wells syndrome** (MWS, OMIM #191900) and **chronic infantile neurological cutaneous and articular syndrome** (CINCA, OMIM #697115) also known as neonatal-onset multisystem inflammatory disease (NOMID). They phenotypically share episodes of recurring fever, urticaria-like skin-lesions, conjunctivitis and inflammatory joint pain. In MWS and CINCA, progressive hearing loss and eye inflammation occur; in CINCA, the most severe form of CAPS, central nervous system inflammation is the most devastating symptom leading to increased intracranial pressure and aseptic meningitis (Goldbach-Mansky, 2011). The mutations in the *NLRP3* gene causing FCAS, MWS and CINCA were identified long before the discovery of the inflammasome (Hoffman et al., 2001; Aksentijevich et al., 2002). To date, 182 mutations in the *NLRP3* gene have been reported in the online *registry of hereditary autoinflammatory disorders mutations* (Infevers, 2017).

Mouse models for FCAS and MWS were generated by knocking-in NLRP3 with a L351P and A350V mutation, respectively. Mating of these mice with *Il1r1*$^{-/-}$ mice confirmed the pivotal role of IL-1β in the pathogenesis of these diseases but did not completely rescue the phenotype suggesting a possible IL-18 involvement (Brydges et al., 2009). Generation of FCAS mice lacking both IL-1 and IL-18 receptors did not prevent the mice from succumbing to the disease; this could be explained by residual inflammation due to increased pyroptosis (Brydges et al., 2013).

Although mutations in *NLRP3* gene are the major cause of CAPS, mutation in NLRC4 and NLRP12 have also been reported in few cases (Jeru et al., 2008; Kitamura et al., 2014).

Biologics against IL-1β have revealed successful in the treatment of CAPS. In 2003, a remarkable response after 6 months treatment with 100 mg/day of Anakinra, as was reported in two MWS patients (Hawkins et al., 2003). Anakinra was later revealed to be successful for the treatment of FCAS (Hoffman et al., 2004) and CINCA (Lovell et al., 2005). In a first clinical trial, 18 patients with CINCA received injections of 1-2 mg/kg/day Anakinra resulting in a rapid response in all patients (ClinicalTrials.gov Identifier: NCT00069329) (Goldbach-Mansky et al., 2006). Withdrawal of treatment resulted in relapse of the disease within days and re-administration recovered the drug's effects. Another open-label study proved the efficacy of Anakinra treatment in 5 FCAS patients over a period of 16 months (ClinicalTrials.gov Identifier: NCT00214851) (Ross et al., 2008).

In an open-label study, five patients with FCAS received a 300 mg loading dose of Rilonacept, resulting in improvement of all symptoms within days of drug administration (ClinicalTrials.gov Identifier: NCT00094900) (Goldbach-Mansky et al., 2008). Under treatment with 100 mg/week (max. 320 mg/week) symptoms were under control in all patients for 24 months. In a randomized double-blind, placebo controlled clinical trial, 47 patients with FCAS and MWS were enrolled and injected weekly with 160 mg Rilonacept for 6 weeks. Ninety-six percent of the patients receiving Rilonacept experienced at least a 30% reduction in the mean key symptom score in contrast to 29% of patients receiving placebo (ClinicalTrials.gov Identifier: NCT00288704) (Hoffman et al., 2008).

Neutralization of IL-1β with Canakinumab for the treatment of CAPS was first described in a phase 3 study involving 35 patients. In the first open-label part, all patients received a single subcutaneous 150 mg Canakinumab injection: 34 individuals had a complete response at day 29. In the second, double-blind, placebo-controlled, randomized withdrawal part of the study, all patients receiving the drug remained in remission whereas 13 out of 16 patients receiving placebo experienced a disease flare. In the third and final open-label part, all enrolled patients received Canakinumab and 97% sustained clinical and biochemical remission at the end of the study (ClinicalTrials.gov Identifier: NCT00465985) (Lachmann et al., 2009). Several other studies support the efficacy of Canakinumab in the treatment of CAPS. In an open-label, phase 3 study with 166 patients, 78% of Canakinumab-naïve patients had a complete response and 90% of the assessed patients were relapse-free over the study period (ClinicalTrials.gov Identifier: NCT00685373) (Kuemmerle-Deschner et al., 2011a). In another study, pediatric MWS and CINCA patients achieved complete response within 1 week after the first Canakinumab injection (ClinicalTrials.gov Identifier: NCT00487708) (Kuemmerle-Deschner et al., 2011b). An additional report revealed that treatment with Canakinumab

of CINCA resulted in clinical improvement in five out of six patients but none experienced a full remission (ClinicalTrials.gov Identifier: NCT00770601) (Sibley et al., 2015). Recently, a long-term (26 months) open-label study of 19 CAPS patients, revealed that 95% of the patients were relapse-free at the end of the study and the treatment was well tolerated (ClinicalTrail.gov Identifier: NCT00991146) (Yokota et al., 2016). Finally, results from the β-*confident register* enrolling 288 CAPS patients showed sustained safety of Canakinumab over a follow up period of up to 5 years. Eighty-six patients experienced severe adverse reactions but only five discontinued the treatment (Hoffman et al., 2016, meeting abstract) (ClinicalTrail.gov Identifier: NCT01213641).

Extensive studies on the pathogenesis of CAPS have revealed how mutant NLRP3 has defective interaction with the IL-1β maturation inhibitor cyclic AMP (Lee et al., 2012) or with its negative regulator CARD8 (Ito et al., 2014). Small molecule inhibitors were shown to be beneficial in mice models of FCAS and MWS; the anti-inflammatory molecule BHB inhibits constitutive NLRP3 inflammasome activity (Youm et al., 2015). Additionally, the specific NLRP3 inhibitor MCC950 showed efficacy in mouse models of CAPS harboring activating NLRP3 mutations (Coll et al., 2015), and in mouse models for dermal and airway inflammation (Primiano et al., 2016).

Familial Mediterranean fever (FMF, OMIM #249100) is an autosomal recessive disorder caused by gain-of-function mutations in the *MEFV* gene encoding pyrin (French FMF Consortium, 1997). Pyrin contains a 14-3-3 binding motif which, when phosphorylated, regulates the compartmentalization (Jeru et al., 2005) and inhibits the activity of pyrin (Park et al., 2016). Pyrin mutations or inactivation of effector kinases by bacterial toxins leave the protein unphosphorylated and free to form a pyrin-inflammasome and activate caspase-1 (Chae et al., 2006; **Figure 1**). The current first-line treatment for FMF is colchicine, which, via RhoA effector kinases, can lead to pyrin phosphorylation and result in its inactivation (Park et al., 2016). Symptoms of FMF include periodic fever attacks, abdominal and chest pain, serositis, amyloidosis and cutaneous inflammation (Jesus and Goldbach-Mansky, 2014). Recently, a specific dominantly inherited S242R mutation in the 14-3-3 binding motif has been identified and shown to result in pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), an autoinflammatory disease with distinct clinical features such as severe recurrent neutrophilic dermatosis, fever and absence of serositis and amyloidosis (Masters et al., 2016).

In 2008, treatment of FMF using IL-1β antagonists was first reported in patient that received 50 mg/day Anakinra subcutaneously without interrupting colchicine. Fever attacks and chest pain were reduced during Anakinra treatment but reappeared upon discontinuation (Calligaris et al., 2008). Recent results of a double-blind, placebo-controlled, randomized study involving 14 colchicine-resistant FMF patients showed that those who received Anakinra daily at a subcutaneous dosage of 100 mg had significantly less fever attacks per month (1.7 vs. 3.5 in the placebo group) (ClinicalTrials.gov Identifier: NCT01705756) (Ben-Zvi et al., 2017).

Previously, Rilonacept was also shown to be a possible treatment option for colchicine-resistant or -intolerant FMF patients: in a small randomized, double-blind, alternating treatment study, Rilonacept given at 2.2 mg/kg weekly reduced the attack frequency to 0.77 per month in comparison to 2 per month in the placebo-treatment group. (ClinicalTrials.gov Identifier: NCT00582907) (Hashkes et al., 2012).

IL-1β inhibition with Canakinumab was also reported to be effective in colchicine-resistant FMF patients. In a 6-month, phase 2, open-label, single-arm study, seven children who experienced FMF attacks under daily colchicine treatment received three monthly subcutaneous injections of Canakinumab (2 mg/kg). The median attack rate per month decreased from 2.7 to 0.3 during the treatment period (ClinicalTrials.gov Identifier: NCT01148797) (Brik et al., 2014). In a second study, nine patients received three consecutive injections of 150 mg Canakinumab every 4 weeks. During the treatment period, only one patient had an attack (peritonitis) and five patients experienced an attack in the 2-months follow up period (ClinicalTrials.gov Identifier: NCT01088880) (Gül et al., 2015). In a retrospective longitudinal outcome study, the effects of long-term Canakinumab treatment in 14 colchicine-resistant FMF patients were assessed. All patients responded to the treatment but four relapsed during the follow-up. The shortening of Canakinumab administration intervals from 8/6 weeks to 4 weeks resulted in partial to full clinical remission (Laskari et al., 2017).

Deficiency of IL-1 receptor antagonist (DIRA, OMIM #612852) is very rare autoinflammatory disease with onset in the neonatal period and presents as systemic inflammation, pustular skin lesions, joint swelling, periostitis and multifocal osteomyelitis (Altiok et al., 2012). DIRA is caused by homozygous mutations in the *IL1RN* gene. It was first described in nine children harboring mutations leading to the synthesis of a truncated non-functional form of IL-1Ra (Aksentijevich et al., 2009; **Figure 1**). Around the same time, another group reported the case of a 49-day-old baby presenting a 175-kb homozygous deletion in chromosome 2 which was spaced over six IL-1 family members including *IL1RN*. This patient completely recovered after Anakinra treatment (Reddy et al., 2009). Another case report described the positive response to Anakinra in a 3 month-old child with confirmed DIRA (Schnellbacher et al., 2013).

More recently, treatment of a 12 year-old child suffering from DIRA due to a novel IL1RN mutation with 150 mg Canakinumab given every 6 weeks led to complete remission without side effects (Ulusoy et al., 2015).

A pilot open-label study to assess the efficacy of Rilonacept treatment for DIRA was completed in April 2016 but results are yet to be published (ClinicalTrials.gov Identifier: NCT01801449). Preliminary data on safety and efficacy in six patients suggests that a weekly injection of 4.4 mg/kg Rilonacept is required to achieve remission (Neal et al., 2014, meeting abstract).

Tumor Necrosis Factor Receptor Associated Periodic Syndrome (TRAPS, OMIM #142680) is an autosomal dominant inherited disorder linked to mutations of *TNFRSF1A* gene encoding the TNFα receptor 1 (McDermott et al., 1999; Hull et al., 2002). These mutations produce a misfolded receptor defective in shedding that accumulates in the cytoplasm and results in enhanced NF-κB activation, ROS production and

impaired autophagy (Bachetti and Ceccherini, 2014). TRAPS symptoms include long-lasting (more than 1 week) fever associated with abdominal pain, skin lesions, and serositis. Various types of skin lesions occur, most frequently erythematous patches and plaques that can be migratory and associated with underlying myalgia.

Anti-TNF treatments were shown to be partially beneficial in TRAPS but may also cause paradoxical inflammatory attacks (Drewe et al., 2007). In contrast, IL-1 blockade seems to be more beneficial. Indeed, remarkable improvement was reported in TRAPS patients treated with Anakinra (Simon et al., 2004; Gattorno et al., 2008; Greco et al., 2015). Specifically targeting IL-1β was also shown to be successful for the treatment of TRAPS. In 2012, it was first reported that a woman who was taken off anti-TNF treatment and received 150 mg Canakinumab every 8 weeks instead, had complete remission (Brizi et al., 2012). Recently, the results of an open-label, proof-of-concept, phase 2 study were released: 20 patients received 150 mg Canakinumab every 4 weeks for 4 months. 19/20 patients achieved clinical remission at day 15 and all relapsed after withdrawal of the drug (ClinicalTrials.gov Identifier: NCT01242813) (Gattorno et al., 2017). Interestingly, a mutation or duplication of the *TNFRSF11A* gene coding for the receptor RANK was associated in three patients with recurrent episodes of fever. Analysis of serum from one patient revealed increased levels of inflammatory cytokines and particularly an eightfold increase for IL-18 (Jeru et al., 2014).

Mevalonate kinase deficiency (MKD) is an autosomal recessive metabolic disorder caused by mutations in the *MVK* gene (Haas and Hoffmann, 2006). Mevalonate kinase is an enzyme involved in the synthesis of cholesterol and isoprenoids. Mutations in this gene lead to shortage of geranylgeranylated proteins which cause the activation of the pyrin inflammasome and subsequent secretion of IL-1β (Mandey et al., 2006; van der Burgh et al., 2013; Park et al., 2016; **Figure 1**). Two forms of the disease exist. The less severe **hyperimmunoglobulinemia D syndrome** (HIDS; OMIM #260920) is characterized by sporadic fever episodes with skin lesions (widespread erythematous macules and papules), lymphadenopathy, abdominal and joint pain, diarrhea and headache (van der Meer et al., 1984). The rare, more severe form of the disease **mevalonic aciduria** (MVA; OMIM #610377) presents all above symptoms chronically (Berger et al., 1985).

In 2005, the case of a 38-year-old HIDS patient with recurrent fever episodes with symptom normalization following 100 mg/day Anakinra treatment was reported (Bodar et al., 2005). As fever episodes in HIDS occur at irregular intervals of 2–8 weeks (van der Burgh et al., 2013), Anakinra treatment "on-demand" appears to be an optimal mode of management with significant clinical response in 8 out 12 attacks (Bodar et al., 2011). Treatment of HIDS with Canakinumab was first reported in a 7-year-old child where 4 mg/kg administered every 4 weeks resulted in the prevention of fever attacks (Tsitsami et al., 2013). Recently, a retrospective study of 144 MKD patients described the response to different therapeutic approaches, including IL-1 antagonists. Anakinra given only during attacks resulted in three complete and five partial responses whereas out of the 19 patients who received Anakinra as maintenance therapy, 3 exhibited a complete remission, 13 a partial remission and 3 did not respond. Canakinumab led to complete remission in four patients and partial remission in a patient resistant to all other therapies (Ter Haar et al., 2016). In an open-label, single treatment arm study, 9 HIDS patients received Canakinumab every 6 weeks for 6 months followed by a withdrawal phase lasting up to 6 months and a 24 month-long-term treatment period. Canakinumab treatment reduced the frequency of flares from a median of 5 flares to 0 (unpublished results; Aróstegui et al., 2015, oral presentation) (ClinicalTrials.gov Identifier: NCT01303380).

PAPA syndrome (pyogenic arthritis, pyoderma gangrenosum, and acne, OMIM #604416) is a hereditary autosomal dominant autoinflammatory syndrome caused by gain-of-function mutations in the *PSTPIP1* gene (Lindor et al., 1997; Wise et al., 2002). The resulting mutated protein interacts with, and activates pyrin, causing dysregulated processing of IL-1β and IL-18 (Shoham et al., 2003; **Figure 1**). PAPA syndrome is characterized by early onset of recurrent sterile arthritis with neutrophilic infiltrates, with variable skin involvement including pyoderma gangrenosum and severe nodulo-cystic acne in adolescence and beyond.

In the first report of PAPA, Anakinra was administered to control arthritis flares in patients presenting the PAPA mutation while not presenting PG symptoms (Dierselhuis et al., 2005). Efficacy of Anakinra was subsequently reported again in a patient presenting the triad of symptoms; after 5 days of daily Anakinra administration at 100 mg per day, the skin lesions improved and after 1 month the ulcer due to PG completely healed with concomitant disappearance of arthritis and acne (Brenner et al., 2009).

The use of Canakinumab for the treatment of PAPA has also been reported: 150 mg Canakinumab given every 8 weeks led to complete healing of PG and disappearance of acne lesions in a single patient (Geusau et al., 2013). IL-18 levels were reported to be elevated in a PAPA syndrome patient treated with cyclosporine. Although PG was treated, acne and splenomegaly were not, suggesting a possible role for IL-18 (Kanameishi et al., 2016). The treatment of PAPA syndrome is challenging, however, since the response to therapy varies largely between patients.

Blau syndrome (BS, OMIM # 186580) is an autosomal dominant granulomatous disease caused by a mutation in *CARD15/NOD2* gene (Miceli-Richard et al., 2001). NOD2 is an intracellular receptor able to sense the bacterial peptidoglycan MDP (McDermott et al., 1999), and signals via the NF-κB pathway (Franchi et al., 2009). Mutations of *NOD2* are also linked to Crohn's disease (CD; OMIM #266600) and early-onset sarcoidosis (EOS, OMIM #609464); in EOS and BS, mutations in the nucleotide-binding oligomerization domain results in increased NF-κB activity (Maekawa et al., 2016).

IL-1β inhibition was shown in individual case reports to be useful for the treatment of BS. Indeed, a patient treated with Anakinra exhibited inflammatory symptom improvement and normalization of plasma cytokine levels (Aróstegui et al., 2007). The use of Canakinumab was also reported for the treatment of Blau syndrome-related uveitis in a young patient and resulted in

disease remission and stabilization of proinflammatory cytokine expression comparable to that seen in healthy controls (Simonini et al., 2013).

Polygenic Autoinflammatory Diseases and Chronic Inflammatory Diseases

PASH syndrome (pyoderma gangrenosum, acne, and suppurative hidradenitis) is an autoinflammatory syndrome similar to but distinct from PAPA. It was first described in two patients presenting both pyoderma gangrenosum and acne without suffering from pyogenic arthritis (Braun-Falco et al., 2012). The genetic background of PASH syndrome is very heterogeneous; the absence of a *PSTPIP1* gene mutation was first reported, however, researchers recently found a *PSTPIP1* gene mutation in a PASH patient (Calderon-Castrat et al., 2016). Moreover, mutations in other genes involved in this autoinflammatory disease including *NLRP3*, *MEFV, NOD2,* and *NCSTN* have also been described in PASH (Marzano et al., 2014a; Duchatelet et al., 2015).

Treatment of PASH with Anakinra was reported in only one patient and resulted in partial remission (Braun-Falco et al., 2012).

In addition to PAPA and PASH, other phenotypically related syndromes are emerging. They include PASS (pyoderma gangrenosum, acne, suppurative hidradenitis, and axial spondyloarthritis), PAPASH (pyogenic arthritis, pyoderma gangrenosum, acne, and HS) and PsAPASH (psoriatic arthritis, pyoderma gangrenosum, acne, and HS) (Bruzzese, 2012; Marzano et al., 2013; Saraceno et al., 2015). Recently, a PASS patient treated with 100 mg/day Anakinra and improvement of all symptoms was reported, after Anakinra discontinuation relapse occurred within 3 days (Leuenberger et al., 2016).

Schnitzler syndrome (SchS) is a rare late-onset inflammatory disease considered as a sporadic acquired autoinflammatory disorder characterized by recurrent fever, urticarial skin lesions, arthritis and lymphadenopathy accompanied with IgM gammopathy. Treatment with Anakinra was reported to completely abrogate the symptoms within 24 h in several case reports (de Koning et al., 2006; Dybowski et al., 2008; Sonnichsen et al., 2016).

In a prospective, open-label study, all patients receiving Rilonacept for up to 1 year showed a rapid clinical response over the treatment duration with nearly complete remission in four of eight patients (ClinicalTrials.gov Identifier: NCT01045772) (Krause et al., 2012).

Canakinumab treatment in SchS was first positively reported in a patient switching from Anakinra (de Koning et al., 2011). In an open-label, single-treatment arm trial, eight additional patients switched from Anakinra to monthly injections of 150 mg Canakinumab for 6 months. Clinical remission was observed in all patients at day 14 and lasted up to 6 months (full duration of the trial) in seven out of eight patients (ClinicalTrials.gov NCT01276522) (de Koning et al., 2013). In another case, injection of Canakinumab every 8 weeks resulted in the disappearance of the symptoms until drug withdrawal (ClinicalTrials.gov Identifier: NCT01245127) (Vanderschueren and Knockaert, 2013). Recently, a phase 2, randomized placebo-controlled, multi-center trial including 20 patients confirmed the potential of Canakinumab for the treatment of SchS. 7 days after initial injection, 5/7 patients who received the drug showed significant improvement when compared to placebo treated patients (0/13). In the open-label trial phase, all patients received Canakinumab, and after 14 days, 15/20 exhibited complete remission and 5 partial remission (ClinicalTrials.gov Identifier: NCT01390350) (Krause et al., 2017).

Hidradenitis suppurativa (HS; OMIM #142690, #613736, #613737), also known as acne inversa, is a chronic skin disease of the hair follicles affecting the axillary, inguinal and anogenital regions with formation of nodules and abscesses (Kurzen et al., 2008). Mutations in the γ-secretase genes *NCSTN, PSENEN,* and *PSEN1* impairing the Notch signaling in hair follicles have been found in some HS patients (Pink et al., 2012). TNF-α, IL-1β, and IL-10 levels were frequently increased in HS lesions (van der Zee et al., 2011). Moreover, IL-17, caspase-1 and NLRP3 are elevated in lesions of HS skin (Lima et al., 2016).

The use and success of Anakinra for the treatment of HS has been a matter of controversy (van der Zee and Prens, 2013; Zarchi et al., 2013; Leslie et al., 2014; Menis et al., 2015; Russo and Alikhan, 2016). However, in a recent double-blind, randomized, placebo controlled trial, 20 patients were treated with Anakinra or placebo daily for 12 weeks. HS clinical response after 12 weeks was 78% in Anakinra-treated patients versus 30% in the placebo group (ClinicalTrials.gov Identifier: NCT01558375) (Tzanetakou et al., 2016).

Treatment with Canakinumab was reported in one HS patient with concomitant PG. HS healed after the first injection and PG after 4 months of treatment (Jaeger et al., 2013). Recently a double-blind, randomized, placebo-controlled clinical trial investigating the efficacy of anti-IL-1α therapy in HS patients who were refractory to anti-TNF drugs was completed. Patients receiving MaBp1 every 2 weeks for 12 weeks showed a 60% response rate in comparison to 10% in the placebo group (ClinicalTrials.gov Identifier: NCT02643654) (Xbiotech, 2017).

SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis, osteitis) is a chronic inflammatory disorder targeting bones, skin and joints (Chamot et al., 1987). Skin manifestations include palmoplantar pustulosis, psoriasis, severe acne and HS (Firinu et al., 2016).

Dysregulation of the ATP receptor $P2X_7$ in SAPHO PBMCs causes in increased processing of IL-1β suggesting a possible therapeutic approach: 100 mg/day Anakinra treatment in a 47-year-old female resulted in the disappearance of the symptoms within 3 months (Colina et al., 2010). In a short-term open study 6 SAPHO patients received 100 mg/day Anakinra, with clinical response reported in 5/6 patients (Wendling et al., 2012).

Behçet's disease (BD) is a chronic multisystem disease that features vasculitis leading to clinical symptoms comprising bipolar aphtosis (oral and genital), uveitis, polyarthritis and skin lesions including sterile non-follicular pustules on the skin and erythema nodosum (Mazzoccoli et al., 2016). The etiology of BD is still unclear but there is an association with genetic factors like human leukocyte antigen (HLA)-B51 or the extrinsic

factor heat shock protein from *Streptococcus sanguinis* which could activate the innate immune system via TLR signaling (Alpsoy, 2016). Anti-IL-1β therapy is an effective treatment of Behçet's disease. Treatment with Anakinra of nine BD patients refractory to anti-TNF resulted in a response in eight individuals (Cantarini et al., 2015). Treatment of three BD patients with 150 mg Canakinumab every 6–8 weeks resulted in complete remission of all clinical manifestations and relapse was not observed at long-term follow up (Vitale et al., 2014). A recent retrospective study showed that the treatment of patients with Canakinumab or Anakinra for at least 12 months led to complete and sustainable remission of the disease (Emmi et al., 2016) and was also effective for BD-related uveitis (Fabiani et al., 2016).

A phase 3 clinical trial evaluating Gevokizumab for the treatment of patients with BD uveitis was terminated because it did not meet the primary endpoint criteria. However, decreased disease severity was observed in the setting of this trial (ClinicalTrial.gov Identifier: NCT01965145) (Xoma, 2015).

Neutrophilic Dermatoses

Pyoderma gangrenosum (PG) is a rare non-infectious neutrophilic dermatosis characterized by sterile pustular skin lesions that rapidly evolve into tender skin ulcers with undermined borders of varying size and depth, sometimes exposing underlying tendons or muscles. PG is frequently associated with systemic diseases. Increased expression of IL-1β was found in lesional skin of PG patients compared to healthy skin (Marzano et al., 2014b; Kolios et al., 2015). In an open-label, proof of concept study evaluating the anti-IL-1β monoclonal antibody Gevokizumab, six patients with active ulcers received three subcutaneous injections once every 4 weeks and four out of six patients had a complete clearance of the target ulcer, 1 a partial (90%) closure of the ulcer and 1 did not respond (ClinicalTrials.gov Identifier: NCT01882504) (Huang et al., 2014, poster). A phase 3 trial of the same drug was prematurely terminated after the company's decision to interrupt clinical development of Gevokizumab. However, preliminary results with 25 patients treated with gevokizumab did not apparently reveal any significant benefit (ClinicalTrials.gov Identifier: NCT02326740 and NCT02315417) (Xoma, 2016). Canakinumab treatment was first reported in a PG patient with concomitant HS. Ulceration disappeared after 4 months and complete remission was achieved after 12 months of treatment (Jaeger et al., 2013). In an open-label study, five steroid-refractory PG patients were treated with Canakinumab once with 150 mg at onset and then optionally at weeks 2 and 8 if response was suboptimal. At the week 16 study endpoint 80% of the patients showed decreased size of target ulcers and 60% were in complete remission (ClinicalTrials.gov Identifier: NCT01302795) (Kolios et al., 2015). A case report described complete healing of a PG patient that received a Canakinumab monthly at a 150 mg dose for 3 months (Galimberti et al., 2016). The involvement of IL-1α in the pathogenesis of PG is currently being investigated in a phase 2 open label study of MABp1 (ClinicalTrials.gov Identifier: NCT01965613) (unpublished data).

Sweet's syndrome (SwS) or acute febrile neutrophilic dermatosis, is a neutrophilic dermatosis with systemic symptoms characterized by fever, tender red cutaneous nodules or papules, occasionally covered with vesicles, pustules or bullae, usually affecting the upper limbs, face and neck. SwS is frequently observed in patients with leukemia or connective tissue diseases. Overexpression of proinflammatory genes including IL-1β is reported in lesional skin of SwS patients (Marzano et al., 2014b; Imhof et al., 2015).

Anakinra (100 mg/day) resulted in symptom resolution within 4 days and subsequent remission for 19 months in one case reported (Delluc et al., 2008). Another case report described disappearance of skin lesions within a month of Anakinra treatment and reappearance of symptoms upon withdrawal (Kluger et al., 2011).

Amicrobial pustulosis of the skin folds (APF) is a rare, chronic cutaneous disease presenting aseptic pustular lesions in cutaneous folds and usually occurring in young women affected by autoimmune diseases such as SLE (Marzano et al., 1996).

Treatment of APF with Anakinra was described in one patient who had increased levels of IL-1α expression in lesional skin, was refractory to steroid therapy and TNF antagonists. Daily subcutaneous injection of Anakinra for 1 month resulted in clearance of the lesions (Amazan et al., 2014).

Other Diseases with Skin Involvement

Acne Vulgaris

Acne vulgaris is a common inflammatory and potentially severe skin disease associated with colonization of the pilo-sebaceous unit by the commensal bacterium *P. acnes*. *P. acnes* is considered to contribute to inflammation in acne and has been shown to activate the NLRP3 inflammasome in human monocytes (Kistowska et al., 2014b; Qin et al., 2014) and in sebocytes (Li et al., 2014). Therefore, IL-1β is thought to play an important role in acne pathogenesis. Gevokizumab was evaluated in a double-blind, randomized, placebo-controlled phase 2 trial for the treatment of inflammatory facial lesions. Patients who received 0.6 mg/kg Gevokizumab once a month for 3 months showed a significant clinical response associated with reduction of inflammatory acne lesions in comparison to the control group (ClinicalTrial.gov Identifier: NCT01498874) (Xoma, 2013). On the other hand, an open label, phase 2 study testing the anti-IL-1α antibody MABp1 on 11 patients showed a 36% decrease in lesion counts (Carrasco et al., 2015).

Malassezia-Associated Skin Diseases

The fungal genus *Malassezia* is linked to several inflammatory skin diseases such as seborrheic dermatitis (seborrheic eczema), pityriasis versicolor (tinea versicolor), atopic eczema, psoriasis, *Malassezia* folliculitis and Onychomycoses (Gaitanis et al., 2012). The etiological agent of pityriasis versicolor, *Malassezia* was shown to activate the NLRP3 inflammasome via the dectin-1 and Syk signaling cascade, causing the release of IL-1β (Kistowska et al., 2014a). To date, no IL-1 blocker has been evaluated for the treatment of *Malassezia*-associated skin diseases.

Psoriasis is an immune-mediated inflammatory disease that affects 2–3% of the global population. It affects primarily the skin and the joints. Psoriasis vulgaris manifests as red, scaly patches of the skin. In lesions, keratinocytes express IL-1α, IL-1β, and IL-18 which regulate the expression of genes involved in the pathogenesis of psoriasis including S100A7 and LL-37 (Perera et al., 2012). By binding to cytosolic DNA, LL-37 has been shown to impair the activation of the AIM2 inflammasome, which is highly expressed in psoriatic lesions (Dombrowski et al., 2011). However, LL-37 is able to induce secretion of IL-18 from keratinocytes independently of caspase-1 (Niyonsaba et al., 2005). IL-1α inhibition for the treatment of plaque psoriasis was investigated in a small size (eight patients), open-label, single-arm trial. 200 mg of MABp1 injected subcutaneously every 3 weeks until evaluation at day 56 showed an average of 13% decrease of the PASI score (Coleman et al., 2015), which is lower than that obtained with the current approved therapies using anti-TNF, -IL-17A, and -IL-12/IL-23 (Mansouri and Menter, 2015).

Generalized pustular psoriasis (GPP; OMIM # 614204, # 602723) is a rare, severe form of psoriasis caused by mutations in the *IL36RN* and *CARD14* genes. Treatment of GPP with Anakinra (Viguier et al., 2010) and Gevokizumab (Mansouri et al., 2015) resulted in a reduction in GPP area and severity index.

Moreover, the caspase-1 inhibitor Belnacasan (VX-765) was evaluated in a phase 2a trial against psoriasis but patients

TABLE 2 | Selected clinical trials targeting IL-1 in inflammatory skin diseases.

Syndrome	Drug	CTI	*n*	Ph	Design	Reference
CINCA	Anakinra	NCT00069329	18	1	O, W	Goldbach-Mansky et al., 2006
FCAS	Anakinra	NCT00214851	8	1	O	Ross et al., 2008
FCAS	Rilonacept	NCT00094900	5	2	O	Goldbach-Mansky et al., 2008
FCAS/MWS	Rilonacept	NCT00288704	47	3	R-B-P, W	Hoffman et al., 2008
CAPS	Canakinumab	NCT00465985	35	3	O, R-B-P, W	Lachmann et al., 2009
MWS/CINCA	Canakinumab	NCT00487708	34	2	O	Kuemmerle-Deschner et al., 2011b
CAPS	Canakinumab	NCT00685373	166	3	O	Kuemmerle-Deschner et al., 2011a
CINCA	Canakinumab	NCT00770601	6	3	O	Sibley et al., 2015
CAPS	Canakinumab	NCT00991146	19	3	O	Yokota et al., 2016
CAPS	**Canakinumab**	**NCT01213641**	**288**	**pr**	**O**	Hoffman et al., 2016
FMF	Anakinra	NCT01705756	25	3	R-B-P	Ben-Zvi et al., 2017
FMF	Rilonacept	NCT00582907	14	2	R-B-AT	Hashkes et al., 2012
FMF	Canakinumab	NCT01148797	7	2	O	Brik et al., 2014
FMF	Canakinumab	NCT01088880	9	2	O	Gül et al., 2015
DIRA	**Rilonacept**	**NCT01801449**	**6**	**2**	**O**	Neal et al., 2014
TRAPS	Canakinumab	NCT01242813	20	2	O, W	Gattorno et al., 2017
MKD/HIDS	**Canakinumab**	**NCT01303380**	**9**	**2**	**O, W**	Aróstegui et al., 2015
SchS	Rilonacept	NCT01045772	8	2	O	Krause et al., 2012
SchS	Canakinumab	NCT01276522	8	2	O	de Koning et al., 2013
SchS	Canakinumab	NCT01245127	1	2	O	Vanderschueren and Knockaert, 2013
SchS	Canakinumab	NCT01390350	20	2	R-B-P, O	Krause et al., 2017
HS	Anakinra	NCT01558375	20	2	R-B-P	Tzanetakou et al., 2016
HS	**MABp1**	**NCT02643654**	**20**	**2**	**R-B-P**	Xbiotech, 2017
BD (uveitis)	Gevokizumab	NCT01965145†	83	3	R-B-P	Xoma, 2015
PG	**Gevokizumab**	**NCT01882504**	**6**	**2**	**O**	Huang et al., 2014
PG	Gevokizumab	NCT02326740†	9	3	R-B-P, O	Xoma, 2016
PG	Gevokizumab	NCT02315417†	16	3	R-B-P, O	Xoma, 2016
PG	Canakinumab	NCT01302795	5	2	O	Kolios et al., 2015
PG	**MABp1**	**NCT01965613**	**10**	**2**	**O**	na
Acne vulgaris	Gevokizumab	NCT01498874	127	2	R-B-P	Xoma, 2013
Acne vulgaris	MABp1	na	11	2	O	Carrasco et al., 2015
Psoriasis	Belnacasan	NCT00205465	64	2	R-B-P	Vertex, 2011
sJIA	Anakinra	NCT00339157	24	2	R-B-P	Quartier et al., 2011
sJIA	Rilonacept	NCT01803321	24	1	R-B-P	Lovell et al., 2013
sJIA	Canakinumab	NCT00886769	84	3	R-B-P	Ruperto et al., 2012
AoSD	Anakinra	NCT01033656	22	2	R-O-CD	Nordstrom et al., 2012

CTI, ClinicalTrial.gov Identifier; n, number of enrolled individuals; Ph, phase; pr, prospective; R, randomized; O, open-label; B, double-blind; P, placebo-controlled; W, withdrawal phase; AT, alternating treatment; CD, comparator drug; na, not available. In bold: studies having only preliminary or not peer-reviewed results. †Clinical trial was terminated.

did not respond to this therapy (ClinicalTrial.gov Identifier: NCT00205465) (Vertex, 2011).

Systemic juvenile idiopathic arthritis (sJIA) is a juvenile form of polyarticular arthritis that also presents with systemic symptoms including fever and skin lesions. Standard treatment includes non-steroidal anti-inflammatory drugs, corticosteroids, anti-IL-1 and anti-IL-6 (Cimaz, 2016).

IL-1 blockade with Anakinra, Canakinumab, and Rilonacept has shown positive results in three clinical trials by Quartier et al. (2011) (ClinicalTrials.gov Identifier: NCT00339157), and Ruperto et al. (2012) (ClinicalTrials.gov Identifier: NCT00886769) and Lovell et al. (2013) (ClinicalTrials.gov Identifier: NCT01803321), respectively. These studies are extensively discussed in the review by Giancane et al. (2016) in this issue.

Adult-onset Still's disease (AoSD) is a rare form of inflammatory arthritis that shares symptoms with sJIA but presents during adulthood.

Treatment of AoSD with Anakinra has shown efficacy in several studies (Castaneda et al., 2016). In an open, randomized study involving 22 patients, Anakinra treatment was compared to disease-modifying anti-rheumatic drugs (DMARDs). Patients receiving $\geq$10 mg/day of Anakinra showed a better overall response in comparison to DMARDs (ClinicalTrials.gov Identifier: NCT01033656) (Nordstrom et al., 2012). In another report, three patients with refractory AoSD that were switched from Anakinra to Rilonacept showed prolonged complete remission (Petryna et al., 2012). Treatment of AoSD with Canakinumab was first reported in two patients resistant to Anakinra and resulted in improvement of both systemic symptoms and polyarthritis (Kontzias and Efthimiou, 2012). In another report, a patient receiving 150 mg Canakinumab every 8 weeks showed long-term improvement of systemic symptoms but active arthritis persisted up to 14 months follow-up (Lo Gullo et al., 2014).

Graft-versus-host disease (GvHD) is a severe complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Acute GvHD occurs in 35–50% of transplanted patients and about half of them will eventually develop chronic GvHD (Jacobsohn and Vogelsang, 2007). Skin manifestations include erythema, morbilliform exanthema, and confluent erythroderma, but in the severest forms (Grade IV) widespread skin detachment (Lipsker et al., 2016). First-line treatment for GvHD consists of corticosteroids and calcineurin inhibitors followed by anti-TNF, anti-IL-2 or mTOR inhibitors (Dignan et al., 2012).

The efficacy of Anakinra in the treatment of GvHD was assessed in 1994 in an open-label, phase 1/2 trial of 17 steroid-resistant GvHD patients. Anakinra was continuously administered per infusion for 1 week. In 63% of the patients, acute GvHD improved by at least one grade (Antin et al., 1994). A second double-blind, placebo-controlled randomized trial on 181 patients investigated the role of IL-1 in the initial T-cell mediated development of the disease by giving Anakinra during conditioning (4 days) and for 10 days after allo-HCT. There was no difference between IL-1ra- and placebo-treated patients with 61 and 59% of them, respectively, developing moderate to severe GvHD (Antin et al., 2002).

Recently, in a murine model of acute GvHD, it was demonstrated that conditioning therapy before allo-HCT resulted in NLRP3 activation in the recipient. Microflora translocation and uric acid released by dying cells were able to activate the inflammasome. Inhibition of NLRP3 with glibenclamide, the IL-1β antagonist Anakinra or gene deletion of *Nlrp3* or *Asc* in mice resulted in delayed and reduced mortality (Jankovic et al., 2013).

Similarly, the blockade of IL-18R in mice has been shown to prevent the early phase of GvHD pathogenesis (Li et al., 2015).

CONCLUSION

Thanks to the discovery of the inflammasome and to major advances in the understanding of biological properties and clinical relevance of IL-1 family members,' the use of IL-1 antagonists has been quite intensely investigated for the treatment of inflammatory and autoinflammatory diseases (**Table 2**). The introduction of IL-1 antagonists represents a major breakthrough in the management of several autoinflammatory diseases, including not only cryopyrinopathies but also other inflammatory conditions refractory to standard therapies where neutrophils play an important pathogenic role. Clinical responses to IL-1β antagonists suggest that this cytokine plays a critical role in the pathogenesis of autoinflammatory disorders. Indeed, many studies have demonstrated that there is no loss in therapeutic efficacy when Anakinra is substituted with the IL-1β-specific antagonist Canakinumab, suggesting that in comparison to IL-1α and/or IL-18, IL-1β likely plays a predominant role in a substantial number of diseases described in this review.

Due to the rarity of autoinflammatory syndromes, sample size represents a major limitation of clinical studies. Nevertheless, retrospective studies, including online registers such as the β-*confident register* for CAPS, that collect data from several reports have definitively helped establishing solid data on the efficacy and safety of IL-1 antagonists for these pathologies.

AUTHOR CONTRIBUTIONS

GF collected and reviewed the literature, drew the figure and wrote the manuscript. EC and LF gave valuable and professional suggestions and revised the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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IL-1 Inhibition in Systemic Juvenile Idiopathic Arthritis

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Systemic juvenile idiopathic arthritis (sJIA) is the form of childhood arthritis whose treatment is most challenging. The demonstration of the prominent involvement of interleukin (IL)-1 in disease pathogenesis has provided the rationale for the treatment with biologic medications that antagonize this cytokine. The three IL-1 blockers that have been tested so far (anakinra, canakinumab, and rilonacept) have all been proven effective and safe, although only canakinumab is currently approved for use in sJIA. The studies on IL-1 inhibition in sJIA published in the past few years suggest that children with fewer affected joints, higher neutrophil count, younger age at disease onset, shorter disease duration, or, possibly, higher ferritin level may respond better to anti-IL-1 treatment. In addition, it has been postulated that use of IL-1 blockade as first-line therapy may take advantage of a "window of opportunity," in which disease pathophysiology can be altered to prevent the occurrence of chronic arthritis. In this review, we analyze the published literature on IL-1 inhibitors in sJIA and discuss the rationale underlying the use of these medications, the results of therapeutic studies, and the controversial issues.

Keywords: systemic juvenile idiopathic arthritis, IL1-inhibitors, anakinra, canakinumab, rilonacept

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INTRODUCTION

Systemic juvenile idiopathic arthritis (sJIA) is the most severe form of childhood arthritis and the most difficult to treat. Until recently, sJIA was considered a therapeutic orphan, since the most effective treatment was corticosteroids, whose long-term administration is associated with a wide range of side effects, including an increased risk of vertebral fractures, cataracts, growth retardation, and susceptibility to infection. Traditional disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate, have limited efficacy for the joint disease and virtually no impact on the systemic features. Poor responses have also been reported with the newer anti-tumor necrosis factor (TNF) agents (Quartier et al., 2003; Horneff et al., 2004; Kimura et al., 2005; Solari et al., 2013), although these medications may be effective in the later afebrile disease phase, characterized by chronic arthritis (Lovell et al., 2008; Giannini et al., 2009). Recently, anti-TNF therapy was found to restore normal levels of vasculoprotective and proangiogenic endothelial progenitor cells in children with JIA (Martini et al., 2015). Several experimental studies have suggested a major pathogenetic role for cytokines such as interleukin (IL)-6 (de Benedetti and Martini, 2005) and, more recently, IL-1 (Pascual et al., 2005). These findings have opened the way to the successful treatment of sJIA with biologic agents that antagonize selectively these cytokines.

In the present review, we provide a brief overview of the main clinical features of sJIA and summarize the recent advances in therapy with IL-1 inhibitors.

CLINICAL CHARACTERISTICS OF sJIA

sJIA accounts for 5–15% of all children with chronic arthritis in Europe and North America and is rather distinct from the other forms of JIA, owing to the association of arthritis with a severe systemic illness (Martini, 2012a; De Benedetti and Schneider, 2016). It is considered the childhood-onset equivalent of adult-onset Still's disease. Children with sJIA typically present with a quotidian, high-spiking fever, often accompanied by an erythematous, salmon pink, macular rash, which tends to be migratory and is strikingly evanescent (**Figure 1**). Myalgias and abdominal pain may be intense during fever peaks. Other systemic manifestations include diffuse lymphoadenopathy, hepatosplenomegaly, and serositis, especially pleuritis and pericarditis. Arthritis is more often symmetrical and polyarticular, but may be absent at onset and develop during the disease course weeks, months, or, rarely, years after the occurrence of extra-articular symptoms. At disease presentation, particularly when arthritis is not yet present, children often require an accurate diagnostic work-up to exclude other potential diagnoses, such as infections and malignancy. Characteristic laboratory features include anemia (usually hypochromic and microcytic), leukocytosis, thrombocytosis, elevated immunoglobulins, increased erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), and hypoalbuminemia. The International League for Associations of Rheumatology (ILAR) criteria for the classification of sJIA are shown in **Table 1**.

It has recently been argued that there are patients not classifiable as sJIA by current criteria who present with the same systemic features seen in classic sJIA, but never develop arthritis (Martini, 2012a). The similarity of clinical manifestations suggest that their illness is closely related to sJIA, despite the absence of arthritis. This subgroup of patients, which nowadays lacks a taxonomic designation, would meet the criteria for adult-onset Still's disease, which do not require the presence of arthritis for diagnosis (Yamaguchi et al., 1992). These considerations have led to propose to include these patients in the sJIA category, and to rename sJIA as Still's disease in order to harmonize the terminology with that of the adult counterpart (Martini, 2012b). A recent analysis of initial clinical features of 136 children with sJIA through a Web-based registry has shown that the ILAR criteria identified only 30% of sJIA patients at disease presentation (Behrens et al., 2008).

The course and prognosis of sJIA are variable (Martini, 2012a; De Benedetti and Schneider, 2016). Around 40% of patients have a good long-term outcome, with a monocyclic course that enters a permanent remission with time. A small proportion of patients have an intermittent course, with relapses followed by periods of quiescence. In the remaining half of the patients, the disease pursues a more severe, persistent disease course. Among this unremitting subset, the sickest children have ongoing systemic symptoms, early destructive polyarthritis (**Figure 2**), growth failure, and are exposed to the serious side effects of corticosteroids. This particular disease phenotype represents the most disabling of all the different forms of JIA.

Children with sJIA are uniquely susceptible to develop a potentially fatal complication known as macrophage activation syndrome (MAS). MAS is characterized by an overwhelming inflammatory reaction due to an uncontrolled and dysfunctional immune response involving the continued activation and expansion of T lymphocytes and macrophages, with resultant massive hypersecretion of proinflammatory cytokines (Ravelli et al., 2012; Grom et al., 2016). Distinctive clinical features of MAS are high, non-remitting fever, hepatosplenomegaly, generalized lymphadenopathy, central nervous system dysfunction, hemorrhagic manifestations, and, in its most extreme forms, multiorgan failure. Characteristic laboratory abnormalities include pancytopenia, increased levels of ferritin, liver enzymes, lactate dehydrogenase, triglycerides, D-dimers, and soluble IL-2 receptor a (also known as soluble CD25), and decreased fibrinogen levels. A characteristic histopathologic feature of MAS is the accumulation of well-differentiated

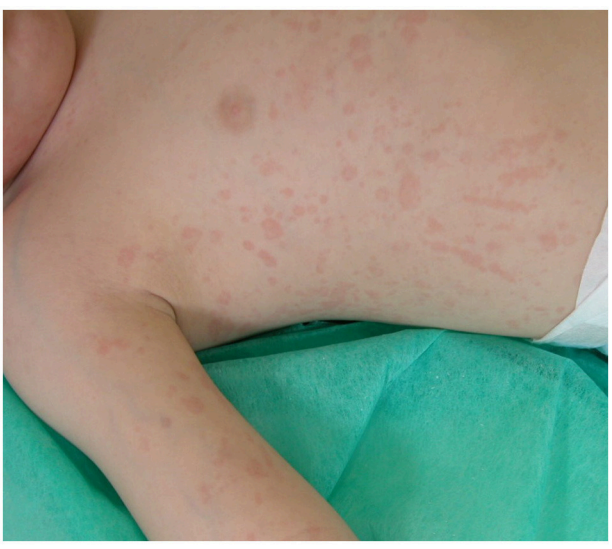

FIGURE 1 | Salmon-macular rash in systemic juvenile idiopathic arthritis.

TABLE 1 | ILAR criteria for sJIA.

Arthritis with, or preceded by, daily fever of at least 2 weeks' duration that is documented to be quotidian for at least 3 days, and accompanied by one or more of the following:
(1) evanescent, non-fixed, erythematous rash
(2) generalized lymphadenopathy
(3) hepatomegaly or splenomegaly
(4) pericarditis, pleuritis and/or peritonitis
Exclusion criteria
- Psoriasis or a history of psoriasis in patient or first-degree relative
- Arthritis in HLA-B27–positive male >6 years of age
- HLA-B27 associated diseases such as ankylosing spondylitis, enthesitis-related arthritis, sacroiliitis with inflammatory bowel disease, reactive arthritis, or acute anterior uveitis; or history of these in a first-degree relative
- Positive rheumatoid factor test on two occasions ≥ 3 months apart

Adapted from Petty et al. (2004).

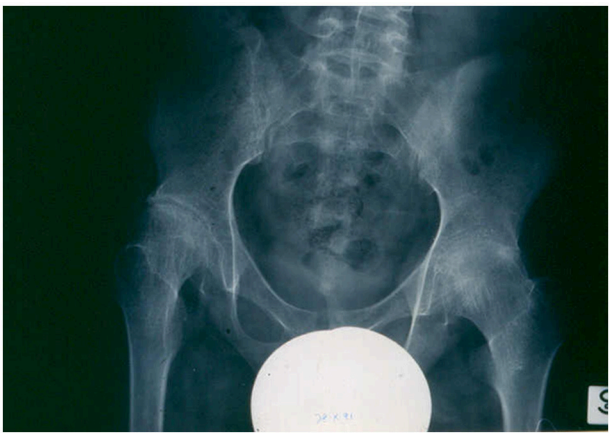

FIGURE 2 | X-ray showing advanced destructive changes in the hips of a systemic JIA patient.

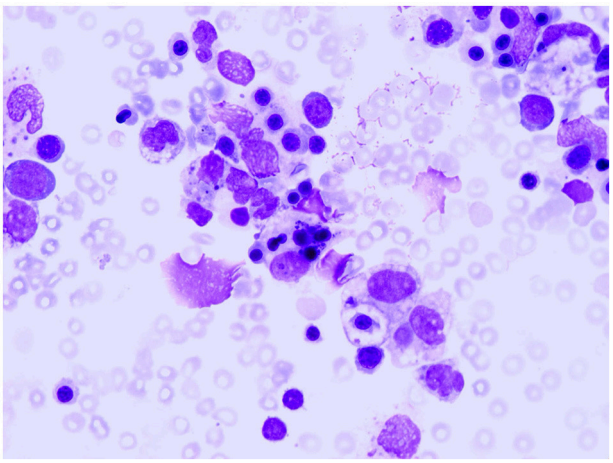

FIGURE 3 | Bone marrow specimen showing macrophage hemophagocytosis in a patient with systemic arthritis and macrophage activation syndrome.

macrophages exhibiting hemophagocytic activity in bone marrow biopsy specimens or aspirates (**Figure 3**; Ravelli, 2002). Although ∼10% of sJIA patients develop overt MAS, up to 30% of children have evidence of subclinical MAS (Behrens et al., 2007; Bleesing et al., 2007). MAS can result in progressive multi-organ failure and eventually a fatal outcome if unrecognized. Recent studies indicate a mortality rate of 8% (Minoia et al., 2014, 2015). In 2016, classification criteria for MAS complicating sJIA have been published (**Table 2**) (Ravelli et al., 2015, 2016).

TABLE 2 | 2006 classification criteria of MAS.

A febrile patient with known or suspected systemic juvenile idiopathic arthritis is classified as having macrophage activation syndrome if the following criteria are met:
Ferritin > 684 ng/ml and any two of the following:
Platelet count ≤ 181 × 10^9/liter
Aspartate aminotransferase > 48 units/liter
Triglycerides > 156 mg/dl
Fibrinogen ≤ 360 mg/dl

Adapted from Ravelli et al. (2015, 2016).

INTERLEUKIN-1 INHIBITORS IN THE MANAGEMENT OF sJIA

Anakinra

The first observation of successful treatment of sJIA with IL-1 inhibition dates back to 2004, when a remarkable response to the recombinant interleukin (IL)-1 receptor antagonist anakinra in two patients with severe and refractory disease manifestations was described (Verbsky and White, 2004).

In a landmark study published in 2005, Pascual et al. (2005) reported that the administration of anakinra to 9 children with active sJIA refractory to other therapies led to striking improvement in clinical symptoms and inflammatory markers. Seven patients achieved complete remission and the other 2 patients had a partial response. The rationale for the treatment was provided by the demonstration that patients' serum induced the transcription of innate immunity genes, included those of IL-1α and IL-1β, in healthy peripheral-blood mononuclear cells, and that patients' peripheral-blood mononuclear cells produced an excess of IL-1β upon activation.

A less impressive effectiveness was seen in a French multicenter, randomized, double-blind, placebo-controlled trial (ANAJIS trial), whose primary outcome was the achievement of an American College of Rheumatology Pediatric (ACR Pedi) 30 response at 1 month. At treatment endpoint, 8 of 12 patients (67%) in the anakinra group and only 1 of 12 patients (8%) in the placebo group were responders ($p = 0.003$). However, no patient in both groups achieved a more robust improvement (i.e., a modified ACR Pedi 100 response). Furthermore, loss of response was observed in most patients over time. The authors attributed the frequent lack of sustained efficacy to the presence of severe polyarthritis and the absence of fever in most patients at enrolment, to the possible insufficient dosage in younger patients, and to the study design, which precluded the concomitant use of DMARDs and allowed early tapering of corticosteroids. Notably, a *de novo* type I interferon signature, which is not a feature of untreated sJIA, was induced in the majority of anakinra-treated patients, regardless of clinical response (Quartier et al., 2011).

That anakinra could be less effective on arthritis symptoms than on systemic and laboratory features of inflammation was highlighted in a retrospective study by Gattorno et al. (2008). By examining the pattern of response to anakinra in 22 children with sJIA, they identified two groups of patients: one group exhibited a dramatic response, with rapid improvement of arthritis and normalization of the CRP within the first week of treatment; the other group had no response or experienced only transient improvement of joint disease and CRP. The only difference between responders and non-responders or incomplete responders was a lesser extension of arthritis and an increased absolute neutrophil count in the former group. *In vitro* secretion of IL-1β and IL-18 by patient monocytes was not

increased and was independent of both treatment outcome and disease activity. Other case series published around the same time also showed remarkable benefit among many, but not all, users of anakinra (Lequerré et al., 2008; Ohlsson et al., 2008; Zeft et al., 2009).

Recent observations suggest that initiation of anakinra early in the disease course may improve outcome. A multicenter retrospective cohort study of 46 patients who had received anakinra as part of initial corticosteroid-sparing regimen showed that around 60%, including 8 of 10 receiving anakinra monotherapy, attained a complete response without escalation of therapy. Almost all patients had rapid improvements in fever and rash, whereas a slower response of arthritis to treatment was seen, with persistently active synovitis in 39% of patients at 1 month, 27% of patients at 3 months, and 15% of patients at 6 months. Inflammatory markers normalized in most patients within 1 month. Evidence that early intervention with anakinra could prevent the development of persistent synovitis was obtained for 91% of 35 patients followed up for at least 6 months. Disease characteristics and treatment were similar in patients with partial or absent response and patients with complete response, except that that the former patients were markedly younger at disease onset (median age 5.2 years vs. 10.2 years; $P = 0.004$). Notably, however, the median peak ferritin level was higher in complete responders than in partial or non-responders (3008 vs. 1329 ng/ml). Although the difference was not significant, perhaps owing to the small size of the study population, this observation suggests that patients with more prominent activation of the monocyte/macrophage system are more responsive to IL-1 inhibition (Nigrovic et al., 2011).

Vastert et al. (2014) conducted the first prospective study of the use of an IL-1 antagonist as first-line therapy in sJIA. They started anakinra in 20 patients with new-onset sJIA who were corticosteroid-naïve. At 3 months, 85% of patients achieved an adapted ACR Pedi 90 response or had inactive disease; 75% of patients achieved this response while receiving anakinra monotherapy. In the majority of responding patients (73%), treatment could be stopped within 1 year, with remission being preserved during follow-up. However, in around one third of patients, concomitant therapy was required for maintenance of clinical response. IL-18 as well as the myeloid-related proteins (MRP) S100A12 and S100A8/9 were found to be potential biomarkers for guiding the strategy of stopping treatment with IL-1 inhibitors.

A recent single-center experience with anakinra therapy in 25 patients with sJIA showed that 56% of patients attained inactive disease. The only baseline variable significantly associated with response was the time interval disease onset and treatment start, with earlier treatment being associated with better outcome. Once more, however, the median ferritin level tended to be higher in patients who reached inactive disease than in those who did not (1506 vs. 360 ng/ml). Importantly, the comparison of the dose administered with the ideal dose of anakinra in each individual patient did not show any relation with therapeutic response (Pardeo et al., 2015).

In spite of the demonstrations of its effectiveness, anakinra is not currently registered for the treatment of sJIA.

Canakinumab

A preliminary phase II, multicenter, open-label study evaluated dosing, efficacy, and safety of the fully human anti-IL-1β antibody canakinumab in 23 children with sJIA and active systemic features. This analysis showed that the administration of 4 mg/kg was associated with rapid and sustained improvement in clinical response and enabled reduction or discontinuation of corticosteroids. In keeping with the findings of the aforementioned study by Gattorno et al. (2008), responders to canakinumab had fewer active joints and a higher white blood cell count at baseline than did non-responders (Ruperto et al., 2010).

The results of this pilot study provided the basis for performing two double-blind placebo-controlled trials of canakinumab in a larger population of sJIA patients with active systemic features (Ruperto et al., 2012). In the first trial, 84% of patients receiving a single injection of canakinumab compared with only 10% of those receiving placebo achieved an ACR Pediatric 30 response with no fever ($p < 0.001$). The frequency of inactive disease in the canakinumab group was as high as 33% after only 15 days. In the second trial, conducted with a withdrawal design, 73% of the patients demonstrated at least an ACR Pediatric 50 response and no fever and 31% had inactive disease at the end of the open-label phase, after a median of 113 days. In the randomized withdrawal phase, the frequency of flare was markedly lower in the canakinumab group than in the placebo group (74% of patients in the canakinumab group had no flare, vs. 25% in the placebo group; $P = 0.003$). At the end of the withdrawal phase, 62% of canakinumab-treated patients and 34% of patients in the placebo group had inactive disease. The average corticosteroid dose was reduced from 0.34 to 0.05 mg/kg/day and corticosteroids were discontinued in 33% of patients. Medication safety was overall good, although infections were more frequent with canakinumab than with placebo and 7 patients had MAS.

Canakinumab has been approved for the treatment for the treatment of active sJIA in children aged 2 years and older both in Europe and in the US.

Rilonacept

The efficacy and safety of the anti-IL-1 soluble decoy receptor protein, rilonacept, were evaluated in a pilot 3-phase trial consisting in a 23 months of open-label treatment preceded by a 4 week, double-blind, placebo-controlled phase. Although no significant differences in efficacy were observed between the rilonacept- and placebo-treated patients during the initial double-blind phase, fever and rash completely resolved by month 3 in all patients during the open-label treatment period and did not recur. The adapted ACR Pedi 30, 50, and 70 response rates at 3 months were 78.3, 60.9, and 34.8%, respectively, and were generally maintained over the study duration. In addition to declines in high-sensitivity CRP, reductions were seen in the levels of MRP-8/MRP-14 and D-dimer. In 22 of 23 patients, prednisone was tapered or discontinued. Treatment was not associated with serious adverse events (Lovell et al., 2013).

A larger 24 week randomized trial of the same agent in 71 children with active arthritis in ≥2 joints, which incorporated

a 4 week double-blind placebo phase, found a shorter time to response in the rilonacept arm than in the placebo arm ($P = 0.007$). In a secondary analysis, 57% of the patients in the rilonacept arm had a response at week 4 compared with 27% of the patients in the placebo arm ($P = 0.016$). No statistically significant association was observed between a poorer response at week 4 and absence of systemic manifestations or longer disease duration. However, the median disease duration tended to shorter among patients who responded at week 4 compared to those who did not. The medication was generally well-tolerated (Ilowite et al., 2014).

Thus far, rilonacept has not been approved for use in children with sJIA.

OPEN ISSUES AND FUTURE OUTLOOK

The advent of biologic agents that specifically inhibit IL-1 has dramatically improved clinical outcomes for many children with sJIA and confirmed the pathogenic role of this cytokine in disease processes. The demonstration of the prominent involvement of IL-1, together with the lack of HLA associations and autoantibodies and the strong implication of cells of the innate immune system, has led to the suggestion that sJIA is a distinct disease entity, with more similarities with autoinflammatory syndromes than with classic autoimmune diseases (Masters et al., 2009; Vastert et al., 2009; Mellins et al., 2011; Martini, 2012b).

However, not all patients respond to IL-1 blockade (Gattorno et al., 2008; Lequerré et al., 2008; Swart et al., 2010; Quartier et al., 2011). The varying susceptibility to anti-IL-1 therapy may be explained by the heterogeneity of sJIA. The aforementioned analysis of the pattern of response to anakinra identified two patient subsets, one with dramatic response, similar to that observed in cryopirin-associated autoinflammatory syndromes, and the other resistant or with an intermediate response. Patients responding to anti-IL-1 therapy had fewer affected joints and a higher neutrophil count (Gattorno et al., 2008). This observation has led to postulate that the group with bright response represents a separate entity in which autoinflammatory mechanisms play the leading pathogenetic role, whereas the group with more severe arthritis may also have autoimmune components (Martini, 2012a). Other investigators have found evidence that anti-IL-1 treatment may be more effective for systemic features than for articular manifestations of the disease (Lovell et al., 2013). However, in the canakinumab study, the response to treatment of children with polyarthritis was similar to those without polyarthritis. A differential therapeutic response based on the presence or absence of systemic features could not be evaluated in this trial because all children enrolled had ongoing fever (Ruperto et al., 2012).

The heterogeneous nature of sJIA has been further highlighted by Shimizu and co-workers (Shimizu et al., 2013), who delineated two distinct sJIA patient subsets based on their serum IL-6 and IL-18 levels: an IL-6 dominant and an IL-18 dominant. The IL-6-dominant subset had a more severe polyarthritis and higher serum levels of matrix metalloproteinase (MMP-3), whereas the IL-18-dominant subset was more prone to develop MAS. Whether the differences in the predominant cytokine expression or in the susceptibility to anti-cytokine therapies dissect the spectrum of systemic JIA into clinically or pathogenetically distinct disease entities, remains to be established.

As noticed above, the tendency for ferritin level to be higher in responders to anakinra in some series suggests that patients with more pronounced activation of the monocyte system, which may predispose them to the progression to overt MAS, may be more susceptible to benefit from IL-1 inhibition. This hypothesis is in keeping with the recent reports of the effectiveness of anakinra in cases of MAS refractory to conventional therapies (Ravelli et al., 2012).

Another explanation for the inconsistent effectiveness of IL-1 inhibition could be the timing of therapy. Nearly all patients included in earlier open studies and in randomized clinical trials had long-standing disease and were still receiving systemic corticosteroids when treatment with IL-1 blocking agents was initiated. These characteristics may account for the partial or absent responses seen in a significant minority of patients. More favorable outcomes were obtained with the use of IL-blockade as first-line therapy, particularly in patients with new-onset disease and not yet exposed to corticosteroids or other DMARDs (Nigrovic et al., 2011; Vastert et al., 2014). Many patients achieved inactive disease rapidly and were able to stop anti-IL-1 therapy within 1 year, with sustained remission during follow-up (Vastert et al., 2014). Of equal importance was the observation of a significant reduction in the proportion of children who developed the chronic polyarthritis manifestation of their disease (Nigrovic et al., 2011).

The differential clinical responses in early vs. late disease, coupled with data from animal models, have led to theorize a biphasic model of sJIA, in which the disease begins with a highly inflammatory febrile phase that, in more than half of the patients, converts over time to an afebrile phase characterized by chronic arthritis. The predominance of innate immune mechanisms in the early systemic stage, as opposed to the involvement of autoreactive T-cells in the later induction of chronic arthritis, would explain why antagonism of IL-1 in new-onset disease is associated with better outcomes than those observed when this therapy is initiated later in the disease course. Thus, early treatment with IL-1 inhibitors may take advantage of this "window of opportunity," in which disease pathophysiology can be altered to avoid the occurrence of chronic arthritis (Nigrovic, 2014).

However, although this hypothesis is logical and attractive, its clinical background should be regarded in the light of some caveats. Because around 40% of patients with sJIA have a monocyclic course with spontaneous remission, results of open studies on patients with early disease may be biased toward patients destined to a milder course. Conversely, most patients enrolled in clinical trials had already had years of disease and, therefore, are unlikely to include patients with a monophasic course. In addition, the majority of these patients had proven refractory to other therapies. Thus, the observed different efficacy of IL-1 blockade between early and established sJIA could simply reflect the fact that the latter patient subset may be more challenging to treat. Nevertheless, although the hypothesis of a window of opportunity is far from proven, it should become

TABLE 3 | Characteristics of the IL-1 inhibitors used for the treatment of sJIA.

	Dosage	Route of administration	Half-life
Anakinra	1–4 mg/kg/day	Subcutaneous	4–6 h
Canakinumab	≥2 years: 4 mg/kg/dose q 4 weeks Maximum dose: 300 mg	Subcutaneous	23–26 days
Rilonacept	Starting dose 4.4 mg/kg, then 2.2 mg/kg/week Maximum loading dose: 320 mg Maximum weekly dose: 160 mg/week	Subcutaneous	1 week

the focus of further research into the pathophysiology of sJIA and, possibly, the objective of further multicenter trials in large populations, ideally combined with biomarker analyses.

Since there are now three IL-1 inhibitors on the market, the question arises about which of them is preferable. Not only they differ in the molecular structure, but the mechanism of action is slightly different: anakinra blocks both IL-1α and IL-1β, canakinumab inhibits only IL-1β, and rilonacept binds IL-1α, IL-1β, and IL-1 receptor antagonist. However, it is still unknown whether the different biding properties translate into differential clinical effects (Beukelman, 2014). Anakinra has been the first agent tested and is, thus, the one for which more experience has been gained (although it is not registered for the treatment of sJIA). It has a short half-life of 4–6 h, which is advantageous for handling a major adverse event and provides a greater flexibility for the management of a medical emergency like MAS. However, the need of daily subcutaneous administrations, which are often associated with injection site reactions, may make it difficult to conduct therapy over long-term, particularly in younger children (Lequerré et al., 2008; Quartier et al., 2011). The longer half-life of canakinumab, which enables its administration every 4 weeks, together with its blockage limited to IL-1β, makes this medication potentially better accepted and tolerated. Rilonacept could offer an alternative with its circulating half-life of 8.6 days, in contrast to the long biologic activity of canakinumab (236 days), which could be a disadvantage in the setting of a serious toxic effect. Importantly, significant responses to canakinumab and rilonacept were seen in many patients who had previously been treated with anakinra, which suggests that failure of one anti-IL-1 therapy does not necessarily preclude use of another (Lovell et al., 2013). Last but not least, the issue of cost may have a major impact on the choice of a particular molecule. The dosage, route of administration and half-life of the IL-1 inhibitors used in the management of sJIA is reported in **Table 3**.

Overall, all anti-IL-1 agents have proven safe and well-tolerated. However, concerns have been raised regarding the risk of infection, neutropenia, and liver dysfunction (Canna et al., 2009; Sandborg and Mellins, 2012; Buckland, 2013). Furthermore, several instances of MAS during treatment with IL-1 inhibitors, some of which with a fatal outcome, have been seen in clinical practice, randomized controlled trials, and post-marketing experience (Grom and Mellins, 2010; Ruperto et al., 2012). The same phenomenon was reported during treatment with the IL-6 blocker tocilizumab (De Benedetti et al., 2012; Yokota et al., 2012). As discussed elsewhere, the occurrence of MAS during treatment with medications that inhibit proinflammatory cytokines implicated in its pathogenesis is a paradoxical phenomenon. Possible explanations include the increased rate of infections (which, in turn, may trigger MAS) associated with biologic therapies or the induction of an imbalance between up- and down-regulation of the various molecules that are part of the cytokine network (Ravelli et al., 2012; Minoia et al., 2014). However, these episodes of MAS often abated after increasing the dose of biologic medications, which suggests a lack of causality and a real associative relationship in only a few instances.

Treatment targeting another cytokine implicated in the pathogenesis of sJIA, such as the IL-6 blocker tocilizumab, has also demonstrated efficacy in clinical trials (De Benedetti et al., 2012; Yokota et al., 2012). So far, however, there are no clinical data that allow either to compare the effectiveness and safety of IL-1 and IL-6 antagonists or to establish their relative indications in sJIA.

Additional investigations are needed to define the exact role of the currently available agents in the management of sJIA. Future studies will likely optimize the care of children with sJIA and further elucidate the disease pathogenesis.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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IL-1 Inhibition May Have an Important Role in Treating Refractory Kawasaki Disease

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Kawasaki disease (KD) is an acute inflammatory vasculitis occurring in young children before 5 years and representing at this age, the main cause of acquired heart disease. A single infusion of 2 g/kg of intravenous immunoglobulins along with aspirin has reduced the frequency of coronary artery aneurysms from 25 to 5%. However, 10–20% of patients do not respond to standard treatment and have an increased risk of cardiac complications and death. The development of more potent therapeutic approaches of KD is an urgent need. Phenotypical and immunological similarities between KD and systemic juvenile idiopathic arthritis led to the hypothesis that KD could be considered as an autoinflammatory disease. New insights regarding KD's pathogenesis have merged from the combination of genetic and transcriptomic data revealing the key role of interleukin-1 (IL-1) signaling in the pathogenesis of the vasculitis. Once activated, IL-1α and IL-1β trigger a local proinflammatory environment-inducing vasodilatation and attracting monocytes and neutrophils to sites causing tissue damage and stress. Both IL-1α and IL-1β have been shown to induce myocarditis and aneurysm formation in *Lactobacillus casei* cell-wall extract mouse model of KD; both being successfully improved with IL-1 blockade treatment such as anakinra. Treatment failure in patients with the high-risk inositol-triphosphate 3-kinase C genotype was associated with highest basal and stimulated intracellular calcium levels, increased cellular production of IL-1β, and IL-18, and higher circulating levels of both cytokines. Three clinical trials of IL-1 blockade enrolling KD patients are currently being conducted in Western Europe and in USA, they could change KD outcome.

Keywords: Kawasaki disease, vasculitis, pediatric, interleukin-1, coronary artery aneurysms, pediatrics, autoinflammatory disease

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INTRODUCTION

Kawasaki disease (KD) is an acute inflammatory vasculitis of the medium- and small-sized arteries generally occurring in children under 5 years old. It was first described by Kawasaki, 1967 associated with the development of coronary artery aneurysms (CAA) or ecstasies in 15–25% of untreated children. Coronary lesions may lead to ischemic heart disease and sudden death. The etiopathology of KD remains unknown though it is widely accepted that it results in an important inflammation cascade triggered by unknown infectious or other stress trigger in a genetically predisposed individual. A single infusion of 2 g/kg of intravenous immunoglobulins (IVIGs) along with aspirin is the standard treatment for KD but not all children may respond, especially the youngest ones and those predisposed

to develop CAA. Interleukin 1 (IL-1) cytokine has been shown to play a key role in the development of CAA leading to a potential use of IL-1 blockade in patients with KD.

KAWASAKI DISEASE

Classically, KD is diagnosed in the presence of high fever lasting for at least 5 days associated to at least four principal features (**Table 1**). No blood tests are available for diagnosis of KD, therefore, a clinical algorithm has been established and validated by the American Academy of pediatrics (**Table 1**) (Newburger et al., 2004). In some cases, KD diagnosis can be made at day 4 of illness in the presence of ≥4 principal criteria. Some patients have incomplete KD, especially infants ≤6 months. In this situation, KD diagnosis is challenging and should be looked for in infants with ≥7 days of fever without explanations, even though no KD clinical criteria are found. In this case, children should, therefore, undergo laboratory testing and, if any systemic inflammation is found, an echocardiogram should be performed (Newburger et al., 2004). Echocardiographic evaluation should be performed at the time of diagnosis, at 2 weeks and at 6–8 weeks after onset of the disease. More frequent echocardiographic evaluation is needed in children at higher risk (Newburger et al., 2004). Kawasaki disease vasculitis may occur outside the heart in other medium-sized vessel such as axillary, renal and femoral arteries somewhat difficult to distinguish from infantile periarteritis nodosa (Burns and Glodé, 2004).

Treatment in KD

A single infusion of 2 g/kg of IVIG along with aspirin has reduced CAA frequency from 25 to 5%. However, 10–20% of patients do not respond to standard treatment and have an increased risk of cardiac complications and death.

Corticosteroids (CS) as well as anti-tumor necrosis factor (TNF) agents are the two main treatments used in IVIG-resistant patients (Eleftheriou et al., 2014). Although there are no formal recommendations regarding optimal CS doses and duration (Chen et al., 2013), CS has not shown significant differences compared to an additional IVIG treatment in terms of preventing the development of CAA (Miura et al., 2008; Ogata et al., 2009). The elevated level of TNF-α in the sera of KD patients correlated with CAA development has led to the use of anti-TNF agent (Eleftheriou et al., 2014). The most frequently used is infliximab (IFX), a chimeric murine/human IgG1 monoclonal antibody that binds to TNF-α. This treatment has been administered in IVIG-resistant patients with success regarding fever and inflammatory parameters, however, with no differences regarding cardiac disease (Burns et al., 2005, 2008; Son et al., 2011). Other immunosuppressive agents have occasionally been used such as: cyclosporine, cyclophosphamide, methotrexate, and plasma exchange in resistant KD patients to IVIG, steroids, and anti-TNFα (Galeotti et al., 2016).

Although the use of different treatments has changed KD outcome, this disease is still lethal in certain cases. The individual prognostic factors are still poorly defined, and resistance to standard therapy represents a major risk of cardiac complications. Developing more efficient treatments and with a better action on cardiac involvement seems a priority. The phenotypical similarities between KD and systemic autoinflammatory disease (SAID) led researches to look at the role of inflammatory cytokines namely IL-1 in KD.

Why to Use IL-1 Blockade in KD?

KD and Systemic Juvenile Idiopathic Arthritis (SJIA): Is There a Missing Link?

Kawasaki disease and SJIA represent a major cause of fever of unknown origin in young children and share intriguing

TABLE 1 | Kawasaki disease (KD) clinical algorithm (Newburger et al., 2004).

Typical KD	Positive echocardiogram (1/3 conditions)
Fever persisting at least 5 days associated to at least four of the five principal features	LAD or RCA ≥ 2.5 *Z*-score
Changes in the peripheral extremities:	Any coronary segment with an internal lumen diameter *Z*-score ≥ 2.5
– Palm and soles erythema	
– Feet and hand edema	
– Peeling of the hands and feet at week 2 or 3	
Bilateral non-exudative conjunctivitis	3/6 features • Perivascular brightness
Changes in the oral cavity:	• Lack of taperin
– Lips dryness	• Decreased LV function
– Erythema	• Mitral regurgitation
– Strawberry tongue	• Pericardial effusion
– Diffuse injection of mouth and throat mucosa	• LAD or RCA: 2–2.5 *Z*-score
Cervical lymphadenopathy >1.5 cm diameter and usually unilateral	Supplementary laboratory criteria
Polymorphous exanthema	• Albumin ≤3 g/dl
	• Anemia for age
	• Elevation of alanine aminotransferase
	• Platelets 450,000/mm^3 after 7 days of fever
	• White blood cell count ≥15000/mm^3
	• Urine white blood cells >10 cells per high-power field

Proximal right coronary artery (RCA) or left anterior descending coronary artery (LAD).

similarities. Clinically, both diseases present with high fever, macular rashes, myalgia, arthralgia, and adenopathy although arthritis seems to be specific to SJIA (Lefèvre-Utile et al., 2014); for this reason, it is difficult to differentiate KD from early SJIA especially when KD is incomplete. Early age of presentation seems to favor KD. Moreover, cardiac abnormalities have been described, especially serositis as in many SAID. Unlike KD, SJIA coronary lesions are mild (essentially hyper echogenic coronaries) with favorable evolution; no CAA are described. Because of CAA risk in incomplete KD and need for early treatment, many patients with SJIA may be initially treated as KD, with IVIG and aspirin, but without efficacy (Lefèvre-Utile et al., 2014). Looking at laboratory findings, no differences can be seen. Both present elevated C-reactive protein, leukocytosis, thrombocytosis, hypoalbuminemia, anemia, and even macrophage activation syndrome (MAS) (Lefèvre-Utile et al., 2014). Assumptions have been made that these two systemic inflammatory disorders could share common triggering agents, susceptibility factors, or immunopathogenic pathways.

When looking at sera of KD and SJIA patients, inflammatory cytokines such as IL-1, IL-6, TNF-α, and interleukin-18 (IL-18) are increased. IL-18 being specifically higher in SJIA compared to KD patient (Mizuta et al., 2016). These phenotypical similarities between KD and SJIA along with the immunological features led to the hypothesis that KD could be considered as an SAID as SJIA and cryopyrin-associated periodic syndrome (CAPS) (Alphonse et al., 2016).

IL-1 Signature in SJIA and KD:

Inflammatory cytokines, especially IL-1β, has first been described as markedly increased in SAID such as CAPS and SJIA (Goldbach-Mansky, 2012). CAPS has allowed to understand the key role of IL-1 in the disease pathogenesis and showed striking response to IL-1-blocking therapies (Ter Haar et al., 2013). NLRP3 is a nod-like receptor (NLR) that is part of an inflammasome, which activates the caspase-1 (CASP1) and consequently the secretion of active IL-1β and IL-18 (Baroja-Mazo et al., 2014). *NLRP3* gene mutations result in constitutive activation of the NLRP3 protein and in an amplification loop of inflammation in which normal regulatory systems, i.e., ATP and second signal requirement are debrided, and where the pro IL-1β may act itself as a danger signal (Koné-Paut and Galeotti, 2015).

More recently, IL-1 has been shown to play a critical role in the pathogenesis of SJIA. Pascual et al. (2005) showed three major results. First, serum from SJIA patients induces the transcription of innate immunity genes including IL-1 in peripheral blood mononuclear cells (PBMCs) from healthy volunteers. Second, when activating PBMCs of SJIA patients, a large amount of IL-1β is released. Finally, they showed that, the use of recombinant IL-1 receptor antagonist (IL1-RA) (anakinra) allowed complete clinical remission in seven of the nine refractory-treated patients thus, emphasizing the central role of the innate immune system (IIS), and specifically, inflammasome-derived cytokines, in the pathogenesis of SJIA (Pascual et al., 2005).

As in systemic diseases, IL-1 seems to play a key role in the physiopathology of KD and more importantly in cardiac involvement for various reasons. Alphonse et al. (2016) showed a significant increased level of IL-1β, IL-18 and of their antagonists (IL-1RA and IL-18BP) in acute KD patients compared with age-matched control patients with viral or bacterial infections. Moreover, IL-1-induced inflammation has been shown to play a role in acute myocardial infarction and contributes to acute ischemic diseases. Indeed, IL-1 is known to enhance the expansion, differentiation and migration of antigen-specific CD8+ T cells as well as the induction of matrix enzymes source of major tissue damage. In the heart and brain, this inflammation can be fatal (Martinon and Tschopp, 2004). In KD, antigen-driven CD8+ T cells are known to infiltrate the coronary artery wall and contribute to the pathogenesis of CAA (Brown et al., 2001). The assumption appears all the more justified when looking at IVIG mechanism on inflammatory cytokines. In responsive KD patients treated with IVIG therapy, the level of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) are decreased emphasizing immunoglobulin's (IG) effect on the modulation of inflammatory cytokines namely on IL-1. Although the way IVIG acts is not perfectly understood, it is known to reduce CAA prevalence (Galeotti et al., 2010).

Interleukin-1 polymorphisms could be associated either to response or resistance to IVIG treatment (Weng et al., 2010). Interestingly elevated transcripts have been shown in IVIG-resistant KD patients, which carry the highest risk for coronary aneurysms (Fury et al., 2010). Increased transcript abundance of the neutrophil-associated calcium-binding proteins, S100A8 and A9, confirms the role of activated neutrophils in acute KD, as these proteins regulate adhesion of neutrophils and monocytes to the endothelial cell, a critical process in KD vasculitis. S100A8/9 proteins are elevated in patients who develop coronary aneurysms. The S100A8/9 heterodimer is known to activate the IL-1 receptor-associated kinase and the NF-$_{\kappa}$B. S100A8/9 appears to be useful biomarkers for identifying IVIG-resistant patients. Other markers of endothelial cell activation CEACCAM1 (carcino embryonic antigen-related cell adhesion) and VEGF (vascular endothelial growth factor) have been detected in acute KD and may correlate with IGIV resistance and coronary vasculitis (Weng et al., 2010).

The role of IIS in the histopathology of KD has also been shown *in vivo* in mice.

Mouse Model of CAA- and IL-1-signaling Pathways

A mouse model of coronary arteritis has been developed using intraperitoneal injection of *Lactobacillus casei* cell-wall extract (LCCWE). This mouse develops a focal, localized coronary arteritis that histopathologically mimics the coronary artery lesions found in human KD (Lehman et al., 1985). As in human CAA, the coronary lesions of LCCWE contains macrophages, activated dendritic cells, and T cells (Schulte et al., 2009). Moreover, the CAA in LCCWE mice responds to IVIG therapy as in KD children (Lehman, 1993). Although both innate and adaptive immunity have been shown as essential for the development of CAA in the LCCWE mouse model, IIS seems to play a key role. Two cytokines have been described as important in the development of CAA: NF-κB and IL-1. Rosenkranz et al.

(2005) have pointed out the role of toll-like receptors (TLRs), a major sensor of IIS, in KD inflammation and therefore in CAA. In LCCWE, NF- NF-κB, an inflammatory cytokine, is activated and synthesized after activation of TLR-2 using a MyD88-dependent pathway (Rosenkranz et al., 2005). NF-κB activation coordinately controls both the innate and adaptive immune responses. To induce vasculitis in LCCWE mice, TLR2 are required as IL-1R signaling highlighting, amongst others, the importance of IL-1-signaling pathway in vasculitis (Rosenkranz et al., 2005). Both IL-1α and IL-1β have been shown to induce aneurysm formation in LCCWE mouse model of KD; aneurysm that are successfully improved with IL-1 blocker treatment such as anakinra (Schett et al., 2016). Similar successful results were reported in recalcitrant KD children using IL-1 blockade (Alphonse et al., 2016).

Lee et al. (2012) presented a mouse model of a knock-out LCCWE mouse (CASP1-/- and IL-1R-/-) in whom KD finally developed after injection of recombinant IL1-β protein. This mouse developed coronary arteritis, which could be prevented by injection of the IL-1 receptor antagonist (IL-1RA): anakinra, during 3–5 days. Using the LCWE mouse model, a logical progression of experiments demonstrated that (i) bone marrow-derived macrophages secrete high levels of IL-1β and TNFα; (ii) IL-1β is processed from pro-IL-1β by CASP1 through the NLRP3 inflammasome; (iii) exogenous treatment with IL-1β recreates the inflammatory phenotype in CASP1 deficient mice; and (iv) IL-1R-deficient mice or mice treated with the recombinant IL-1RA, anakinra fail to develop the arteritis lesions. Of particular note, only blockade of IL-1β, but not blockade of TNF-α, reduced the myocarditis in the LCWE-injected mice (Burns, 2012; Lee et al., 2012). A recent case report showed a dramatic effect on rescuing a life-threatening case of relapsing KD (Cohen et al., 2012).

Genetics: IL-1 Pathway and Calcium Signaling

Finally, analysis of the whole-genome expression profile of acute KD patients has pointed out the importance of IL-1β activation in KD inflammatory profile by showing the link between calcium concentration and inflammasome.

Inositol-triphosphate 3-kinase C (ITPKC) is a candidate gene located on chromosome 19q13.2 whose CC genotype is implicated as a determinant of both disease susceptibility and outcome in KD. ITPKC phosphorylates inositol 1, 4, 5-triphosphate (IP3) to inositol 1, 3, 4, 5-tetraphosphate (IP4), therefore, regulating the calcium response to extracellular signals. At the same time, NLRP3 inflammasome has been shown to be dependent of both extracellular and intracellular calcium concentration ($[Ca^{2+}]i$). Amazingly, ITPKC CC genotype is associated with both highest basal and stimulated $[Ca^{2+}]i$ levels and increased amounts of NLRP3 protein compared with other genotypes at baseline. These findings, allowed making the hypothesis of a link between the calcium level and the activation of NLRP3 in ITPKC CC genotype leading to an excess of IL-1 secretion as in SAID. Moreover, ITPKC CC genotype is associated with failure of IVIG therapy (Alphonse et al., 2016). This emphasizes the fact that phenotypic similarities between

TABLE 2 | Clinical trials of IL-1 blockade enrolling KD patients conducted in Western Europe and in USA (Burns et al., 2016).

Name	Type of trial	IL-1 blockade/Doses	Population KD patients	Objective	Time
Kawakinra trial (Europe) (Eudract Number: 2014-002715-41)	Phase IIa, multi-centered trial	Anakinra: 2 mg/kg/day Dose can be increased by 2 mg/kg/24 h if persistent or recrudescent fever (max 6mg/kg/d)	Children: 8 months (≥10 kg)–18 years Screened: 4th–13th day of fever	Primary end points Efficacy and safety of anakinra Secondary objectives Effects of anakinra on coronary artery Disease activity and inflammation biomarkers	14 days of treatment
Anakid trial (USA) (clinicaltrials.gov#NCT02179853)	Phase I/IIa study: Two-centered and dose escalation trial	Anakinra: 2, 4 or 8 mg/kg Persistent or recrudescent fever after ≥ 36 h and <7 days following the end of intravenous immunoglobulin (IVIG) infusion	Children (≥8-M -old) with acute KD and with coronary artery (Z-score ≥3.0 in the RCA or LAD) abnormalities	Safety, tolerability, and pharmacokinetics of anakinra	2–6 weeks
Canakinumab trial (Europe)	Phase II trial: Two-arm, multi-centered and carried out in seven European countries	Canakinumab: 6 mg/kg IV Group 1: Complete fever resolution Canakinumab (1 or 2 SC injections) at 4 and 8 weeks Depending on the clinical and CRP course Group 2 Fever remains after 48–72 h of canakinumab IVIG	Naïve KD patients or IVIG-resistant KD patient	The presence or absence of fever will be looked-for.	

IVIG, intravenous immunoglobulin; RCA: right coronary artery; LAD, left anterior descending coronary artery; SC, subcutaneous.

KD and AID are anchored by the common immunobiological processes associated with inflammasome activation.

Experience of IL-1 Blockade in KD Patients

Nowadays, three IL-1 blockades have been approved: anakinra, rilonacept, and canakinumab. Anakinra (Kineret®) was the first IL-1 blockade agent administered initially in rheumatoid arthritis (1993) and is now used in numerous diseases such as hereditary SAIDs (Schett et al., 2016). It is an IL1-RA blocking both IL-1α and IL-1β (Carter et al., 1990). In 2008, rilonacept (Hoffman et al., 2008), a soluble IL-1 decoy receptor, that neutralizes either IL-1α or IL-1β, received US Food and Drug Administration (FDA) approval in CAPS patients, and Canakinumab (Ilaris®) in 2009 (Chakraborty et al., 2012). The latter is a humanized monoclonal antibody that specifically blocks IL-1β (Dinarello et al., 2012). In pediatrics, only anakinra (≥8 months and 10 kg) and canakinumab (≥24 months and ≥7.5 kg) have FDA and European Medicines Agency (EMA) approval for CAPS disease. These IL-1 blockades are safe and well tolerated with a low-adverse event rate (Rossi-Semerano et al., 2015). Anakinra is preferred for it has a remarkable record of safety with over 150,000 patients treated daily for over 10 years (Bresnihan et al., 2004; Fleischmann et al., 2006). In addition, drug level significantly drops 1 h after discontinuation of treatment (Dinarello et al., 2012).

In SJIA, the three IL-1 blockers have been tested so far and were proven as effective and safe, although only canakinumab is currently approved for use (Giancane et al., 2016). In addition, anakinra has been demonstrated as efficient in severe SJIA with MAS (Miettunen et al., 2011) a severe complication that can occur in up to half of SJIA patients. In KD, MAS is probably a frequently under-recognized complication situation which could benefit from IL-1 blockers (Wang et al., 2015).

For now, two case reports showing promising results with anakinra in severe KD patient have been published. The first one is an 11-week-old Caucasian female that presented with severe KD complicated by MAS. Diffuse enlargement of the entire coronary artery system was revealed by echocardiogram. IVIG, aspirin and CS were inefficient. High doses of anakinra (3 mg/kg/dose, twice daily for 3 days) were introduced at day 6 because of bad clinical outcome and biological signs of MAS. IFX and methylprednisolone were added at day 9 because of cardiac failure despite favorable clinical and biological course. The evolution was favorable allowing CS to be tapered off over 10 days following discharge and under anakinra over the next 5 months. At 8 months, the echocardiogram was normal (Shafferman et al., 2014).

The second one is a 2-year-old boy diagnosed with KD who developed secondarily cardiac failure (shortening fraction of 20%) without CAA under IVIG (2 g/kg) treatment. A second IVIG perfusion was administered as well as multiple methylprednisolone pulses with little effect and worsening of cardiac involvement. Extracorporeal membrane oxygenation was performed from day 14 until day 17 and subcutaneous anakinra (1 mg/kg/day) was introduced at day 18 until day 24 with success. Relapse was seen three days after anakinra's last injection with progression to giant CAA. Anakinra was therefore reinitiated for 6 weeks with normalization of the coronary lesions at 6 months (Cohen et al., 2012).

What Could Be the Place of Anti-IL1 in the Current Standard of KD Treatments?

Considering current knowledge, it seems reasonable to use IL-1 blockade in resistant KD with CAA before IFX which has not proven its efficiency in coronary disease. A new approach could be the early use of IL-1 blockade associated with CS in patients at high risks of severe KD depending on validated risk scores, in Japanese patients. Apart from IVIG's, anti-IL1 are the only therapies that have proven their effect on CAA. It should be considered whether their use should not be generalized to all patients. Indeed, IL-1 blockers seem to better prevent CAA development than IVIG, especially if used at diagnosis. In this idea three clinical trials of IL-1 blockade enrolling KD patients are currently being conducted in Western Europe and in the US (**Table 2**) (Burns et al., 2016). Their conclusion may help to better define, in the future, the place of IL-1 blockade in KD treatment in association or in replacement of IGIV and CS.

CONCLUSION

Kawasaki disease clinical and immunological features mimic SAID. These similarities have allowed looking at new inflammatory cytokines such as IL-1. Better understanding of IL-1 involvement in KD and specifically in CAA with the use of IL-1 blockers, has brought hope for resistant and severe patients. Doses and time to introduce IL-1 therapy has still to be defined. Another challenge is the need to better define patients with a higher risk of CAA, allowing better medical care and the use of new treatment strategies. We hope that results of clinical trials using IL-1 blockade will allow to better understand the respective roles of IL-1α and β, and to pursue with phase III trials.

AUTHOR CONTRIBUTIONS

Both the authors had a substantial contribution to the work. PD wrote the first draft. PD and IK-P were involved in drafting the article or critically revising it for important intellectual content and approved the final version to be published.

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Response to Interleukin-1 Inhibitors in 140 Italian Patients with Adult-Onset Still's Disease: A Multicentre Retrospective Observational Study

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Background: Interleukin (IL)-1 plays a crucial role in the pathogenesis of Adult onset Still's disease (AOSD).

Objectives: To evaluate the efficacy and safety of anakinra (ANA) and canakinumab (CAN) in a large group of AOSD patients.

Methods: Data on clinical, serological features, and concomitant treatments were retrospectively collected at baseline and after 3, 6, and 12 months from AOSD patients (Yamaguchi criteria) referred by 18 Italian centers. Pouchot's score was used to evaluate disease severity.

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Results: One hundred forty patients were treated with ANA; 4 were subsequently switched to CAN after ANA failure. The systemic pattern of AOSD was identified in 104 (74.2%) of the ANA-treated and in 3 (75%) of the CAN-treated groups; the chronic-articular type of AOSD was identified in 48 (25.8%) of the ANA-treated and in 1 (25%) of the CAN-treated groups. Methotrexate (MTX) was the most frequent disease modifying anti-rheumatic drug (DMARD) used before beginning ANA or CAN [91/140 (75.8%), 2/4 (50%), respectively]. As a second-line biologic DMARD therapy in 29/140 (20.7%) of the patients, ANA was found effective in improving all clinical and serological manifestations ($p < 0.0001$), and Pouchot's score was found to be significantly reduced at all time points ($p < 0.0001$). No differences in treatment response were identified in the ANA-group when the patients were stratified according to age, sex, disease pattern or mono/combination therapy profile. ANA primary and secondary inefficacy at the 12-month time point was 15/140 (10.7%) and 11/140 (7.8%), respectively. Adverse events (AEs) [mainly represented by in situ (28/47, 59.5%) or diffuse (12/47, 25.5%) skin reactions and infections (7/47, 14.8%)] were the main causes for discontinuation. Pouchot's score and clinical and serological features were significantly ameliorated at all time points ($p < 0.0001$) in the CAN-group, and no AEs were registered during CAN therapy. Treatment was suspended for loss of efficacy only in one case (1/4, 25%).

Conclusion: This is the largest retrospective observational study evaluating the efficacy and safety of IL-1 inhibitors in AOSD patients. A good response was noted at 3 months after therapy onset in both the ANA- and CAN-groups. Skin reaction may nevertheless represent a non-negligible AE during ANA treatment.

Keywords: Adult-onset Still's disease, treatment, interleukin (IL)-1, anakinra, canakinumab

INTRODUCTION

Adult-onset Still's disease (AOSD) is a rare multisystemic inflammatory disorder predominantly affecting young adults, with an estimated annual incidence of 0.16–0.4 per 100,000 persons globally (Jamilloux et al., 2015; Sfriso et al., 2016). Various genetic, infectious and environmental factors seem to interact triggering a systemic autoinflammatory response in predisposed individuals. A dysregulation of cytokine-mediated pathways, in particular those linked to interleukin (IL)-1, IL-6, IL-18, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (Fujii et al., 2001; Giampietro and Fautrel, 2012) has been hypothesized.

Adult-onset Still's disease is clinically characterized by daily high spiking fever, evanescent maculopapular skin rash, arthritis, musculoskeletal symptoms, sore throat and hepatosplenomegaly. Cardiopulmonary manifestations and significant liver dysfunctions are only rarely present. Central nervous system (CNS) and renal involvement has, likewise, been described only in very few case reports (Gerfaud-Valentin et al., 2014a). Typical laboratory findings include marked leukocytosis with neutrophilia, hyperferritinemia, high liver enzymes, and elevated acute-phase reactants such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

The clinical presentation of AOSD can be distinguished into two phenotypes: a highly symptomatic, feverish, systemic pattern and a chronic articular profile showing features of polyarthritis. AOSD treatment, which essentially remains empirical, is based on small retrospective case series studies (Jamilloux et al., 2015). Non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are generally used as a first-line treatment, in particular for musculoskeletal manifestations and fever. Disease-modifying anti-rheumatic drugs (DMARDS), such as methotrexate, azathioprine, and leflunomide, are often used in the attempt to reduce the quantity of corticosteroids being administered. Intravenous immunoglobulin, anti-TNF-α drugs, such as etanercept, infliximab and adalimumab, as well as anti-IL-6 agents, i.e., tocilizumab, also appear to adequately control the disease in non-responders to conventional therapy (Cefle, 2005; Fautrel et al., 2005; Kim et al., 2012). These treatments may nevertheless show variable efficacy and/or may be linked to potentially severe side effects.

Increasing evidence regarding the global efficacy of IL-1 inhibitors (IL-1-INH), such as Anakinra (ANA; IL-1 receptor antagonist) and Canakinumab (CAN; monoclonal anti-IL-1β antibody) has been collected from refractory systemic and articular AOSD patients (Kontzias and Efthimiou, 2012; Ortiz-Sanjuán et al., 2015). ANA is a recombinant, non-glycosylated form of human IL-1 receptor antagonist; since it has a very short half-life, daily subcutaneous administrations are necessary. CAN, which is a human anti-IL-1β monoclonal antibody, has a longer half-life.

In August 2016, the European Commission extended the license approval of CAN to treat active Still's disease including AOSD and Systemic Juvenile Idiopathic Arthritis (SJIA). The

decision reached in the light of evidence supporting the concept of a Still's disease continuum including both the juvenile and adult onset forms (Nirmala et al., 2015) and as the scientific comunity awaits the results of an ongoing trial (NCT02204293).

The current study aimed to examine the use of IL-1-INH in a large number of Italian patients with AOSD, found to be refractory to other therapies. A nationwide cross-sectional observational study promoted by the Italian Group of Study on Autoinflammatory Diseases and endorsed by the Italian Society of Rheumatology (SIR) was thus conducted to gather information about their efficacy and their effect on clinical features and inflammatory markers during treatment.

MATERIALS AND METHODS

Patients and Data Collection

Demographic, clinical, and therapeutic data were retrospectively collected from AOSD patients attending 18 Italian University-Hospital centers. The patients were considered eligible if they were adults with AOSD diagnosed in accordance with Yamaguchi's criteria (Yamaguchi et al., 1992) (**Table 1**) and being treated with IL-1-INH after failure of therapy based on NSAIDs and immunosuppressive drugs, such as steroids and DMARDs, and in some cases other biologic agents.

Attending physicians provided retrospective anonymous information from medical records, which were entered into a database, regarding the patients' clinical and laboratory data, the therapies prescribed to manage AOSD including all those prior to IL-1-INH, response to ANA or CAN, adverse events (AEs), and the effect on clinical symptoms 3, 6, or 12 months after IL-1-INH therapy was begun.

The study was carried out in accordance with Good Clinical Practice, the Declaration of Helsinki and the recommendations of the local Ethical Committee rules of all participating centers.

Definition of Clinical and Laboratory Criteria

In accordance with the established definition, the disease was considered the systemic form if the patient primarily showed marked increase in inflammatory markers, hyperferritinemia, and multi-organ involvement. The disease was considered the chronic form if involvement was prevalently polyarticular and the patient presented low levels of inflammatory markers and erosive damage. Disease severity was determined using a modified Pouchot's score (range 0–12) (Rau et al., 2010), which considers 12 disease-related manifestations [fever, evanescent rash, pleuritis, pneumonia, pericarditis, hepatomegaly, serum ferritin levels (>3000 mg/L), lymphadenopathy, white blood cells count (>15,000/mm), sore throat, myalgias, and arthritis]. Fever was defined by temperatures ≥39°C; cutaneous rash was considered positive if the patients presented an evanescent salmon-pink, macular, or maculopapular rash predominantly on the trunk and limbs. Pleuritis was defined as pleural effusion linked with pleuritic pain; pericarditis was defined by chest pain, pericardial friction rub, and effusion documented by echocardiogram. Pneumonia was diagnosed in presence of pulmonary consolidations documented by X-rays or chest computed tomography (CT) scan. Lymphoadenopathy was confirmed by ultrasound and/or CT scan in at least two different sites. Diagnosis of hepatomegaly was confirmed by ultrasound and/or CT and/or nuclear magnetic resonance spectroscopy (NMR) scan findings. Leukocytosis was defined as a white blood cell count ≥15,000/mm^3; hyperferritinemia was defined as serum ferritin ≥3000 ng/mL. ESR and CRP levels were considered elevated when values fell beyond the laboratory reference limit.

Response to anti IL-1 treatment was scored as complete, partial, or failure. Response was considered complete or provoking remission when signs of active disease were absent and inflammatory markers were normalized. It was considered partial when complete response was not achieved although there were clear signs of clinical improvement according to the attending physician.

Statistical Analysis

D'Agostino–Pearson's test for normality was used. The normally distributed variables were described by the mean ± standard deviation (SD), and the non-normally distributed variables using the median and range. Wilcoxon's matched-pairs test and paired *t*-tests were performed. Pearson's and Spearman's tests were carried out to analyze the correlations where appropriate. Univariate analysis of nominal variables was carried out using the chi-square (χ^2) test or Fisher's exact-test where appropriate. The *P*-values of two-tailed tests were calculated; *p*-values less than or equal to 0.05 were considered significant.

The statistical calculations were performed using the Statistical Package for Social Sciences 13.0 (SPSS, Chicago, IL, United States).

TABLE 1 | Yamaguchi criteria (Yamaguchi et al., 1992).

Major criteria	Minor criteria
(1) Fever ≥39°C (≥1 week)	(1) Sore throat
(2) Arthralgia (≥2 weeks)	(2) Lymphadenopathy and/or splenomegaly
(3) Typical rash	(3) Liver dysfunction
(4) Leukocytosis (≥10 000/mm^3) with ≥ 80% of granulocytes	(4) Negative RF and ANA

Diagnosis requires ≥5 criteria including at least 2 or more major criteria. Infections, malignancies, and other rheumatic diseases must be excluded. RF, rheumatoid factor; ANA, antinuclear antibody.

RESULTS

The data from 140 AOSD patients (93 females and 47 males; mean age at disease onset = 35.4 ± 17 years, mean age at diagnosis = 37.4 ± 16.1 years) were evaluated. All the patients were treated with ANA; four were later switched to CAN after ANA failed. The mean disease duration before starting treatment with ANA was 50.33 ± 81.67 months. Most of the patients presented a systemic disease pattern (104/140, 74.2%) and the rest presented a chronic articular one (36/140, 25.8%).

Anakinra Treatment

Previous or Concomitant Therapies

Most of the patients were treated with NSAIDs, glucocorticoids and/or DMARDs before starting ANA (**Table 2**). In the majority of cases, steroids represented the first-line therapy (mean initial dosage of prednisone (PDN) equivalent of 77.6 ± 186.3 mg daily). DMARDs were employed in 120/140 (85.7%). Methotrexate (MTX) was used in 91/120 (75.8%) and cyclosporine A (CyA) in 50/120 cases (41.6%). ANA was adopted as a second-line biological therapy (bDMARD) in 29/140 patients (20.7%); it was the first-line biological treatment in 111/140 patients (79.3%). As far as bDMARDs are concerned, anti-TNF-α therapies represented the prevalent strategy and etanercept (ETN) and infliximab (IFX) were the most frequently used drugs (79.3 and 44.8%, respectively). In the majority of cases (106/140, 75.8%), ANA was prescribed in combination with other DMARDs; in 24.2% (34/140) it was used as a monotherapy (**Figure 1**). MTX represented the first choice DMARD used in association with ANA (**Figure 1**); in some cases (13/140, 9.2%), a combination of more than one DMARD was utilized.

ANA Dosage

At baseline, 100 mg per day ANA was administered to 127/140 patients (90.7%); higher or lower doses were prescribed to 13/140 cases (9.3%). Four patients presented with a very aggressive systemic form of the disease; 3 (23.1%) of these were originally treated with 200 mg/day ANA and 1 (7.6%) was treated with 150 mg/day. In view of intolerability issues, nine patients were prescribed lower, non-conventional dosages, i.e., 50 mg per day (one case) or 100 mg every other day (eight cases).

TABLE 2 | Previous therapies prescribed to the Adult onset Still's disease (AOSD) patients before they began Anakinra (ANA) therapy.

Therapies	% of patients (n = 140)
Non-steroidal Anti-Inflammatory Drugs (NSAIDs)	69.2
Steroids	97.8
Disease-Modifying Anti-Rheumatic Drugs (DMARDs)	85.7
Methotrexate (MTX)	75.8
Cyclosporine A (CyA)	41.6
Hydroxychloroquine (HCQ)	22.1
Colchicine	9.2
Azathioprine (AZA)	6.4
Salazopyrine (SSZ)	5.7
Leflunomide (LEF)	3.5
Biological therapies (bDMARDs)	20.7
Etanercept (ETN)	79.3
Infliximab (IFX)	44.8
Adalimumab (ADA)	20.6
Tocilizumab (TOCI)	6.8
Abatacept (ABA)	6.8
Golimumab (GOL)	6.8
Rituximab (RTX)	6.8
Certulizumab (CTZ)	6.8

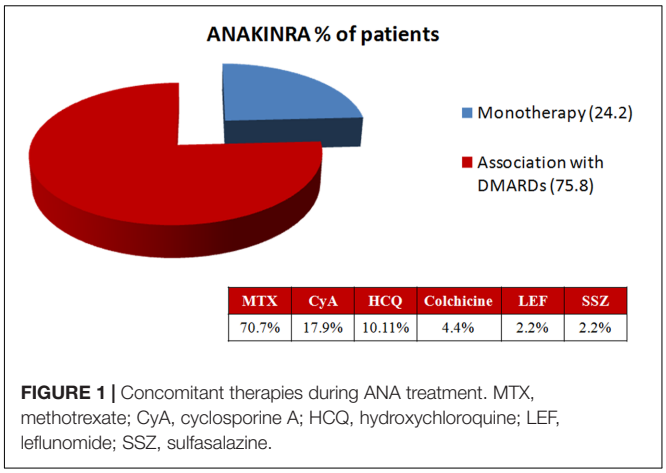

FIGURE 1 | Concomitant therapies during ANA treatment. MTX, methotrexate; CyA, cyclosporine A; HCQ, hydroxychloroquine; LEF, leflunomide; SSZ, sulfasalazine.

Dosage was adjusted for 33/140 patients (23.5%) over the course of treatment. In 29/33 cases showing marked improvement, the dosage was reduced from 100 mg per day to 100 mg every other day. In 3/33 cases therapy was upgraded to the standard 100 mg/day dose. In a single case, therapy was increased from 100 mg to 200 mg per day in view of an incomplete clinical response.

Duration of Therapy, Discontinuation, and AEs

After 12 months of treatment, 97/140 patients (69.2%) were still receiving ANA. Overall, the mean duration of therapy was 35.7 ± 36.1 months. At the time, we analyzed the data, 69 out of 140 patients (49.3%) were still being treated with ANA, and 71 (50.7%) had discontinued therapy (**Table 3**).

The main reason for treatment discontinuation was linked to the development of AEs (24/71 patients, 33.8%) followed by remission (sustained disappearance of all clinical and serological manifestations) in 20/71 cases (28.1%) (**Figure 2**). Discontinuation for primary inefficacy was documented in 16/71 subjects (22.5%). ANA was discontinued in 11/71 cases (15.4%) due to loss of efficacy during the follow-up.

AEs occurred in 47/140 patients (33.5%). The principal AE reported was the appearance of *in situ* (28/47 cases, 59.5%) or diffuse (12/47 cases, 25.5%) local reactions, usually in the form of cutaneous urticarial lesions. Most of the patients abandoning therapy because of AEs (18/24, 75%) did so because of severe skin reactions. Infectious events (7/47 patients, 12.7%) were quite rare: they consisted in three cases of pneumonia, three cases of urinary tract infections and one case of recurrent dental abscesses; infectious events caused withdrawal in two out of seven cases.

The Clinical Efficacy of ANA

Anakinra proved to be effective in reducing all AOSD-linked clinical and serological manifestations. Primary and secondary inefficacy after 12 months was reported in 15/140 (10.7%) and 11/140 (7.8%) patients, respectively. Pouchot's score, which was calculated at baseline and then at 3, 6, and 12 months, demonstrated a significant improvement; there was a drop in the mean score already at 3 months (5.5 ± 1.9, range 2–10, at

TABLE 3 | The number of patients receiving ANA during the study period.

	Baseline	3 months	6 months	12 months	Time of this study
N° of patients (%)	140 (100%)	118 (84.2%)	109 (77.8%)	97 (69.2%)	69 (49.2%)

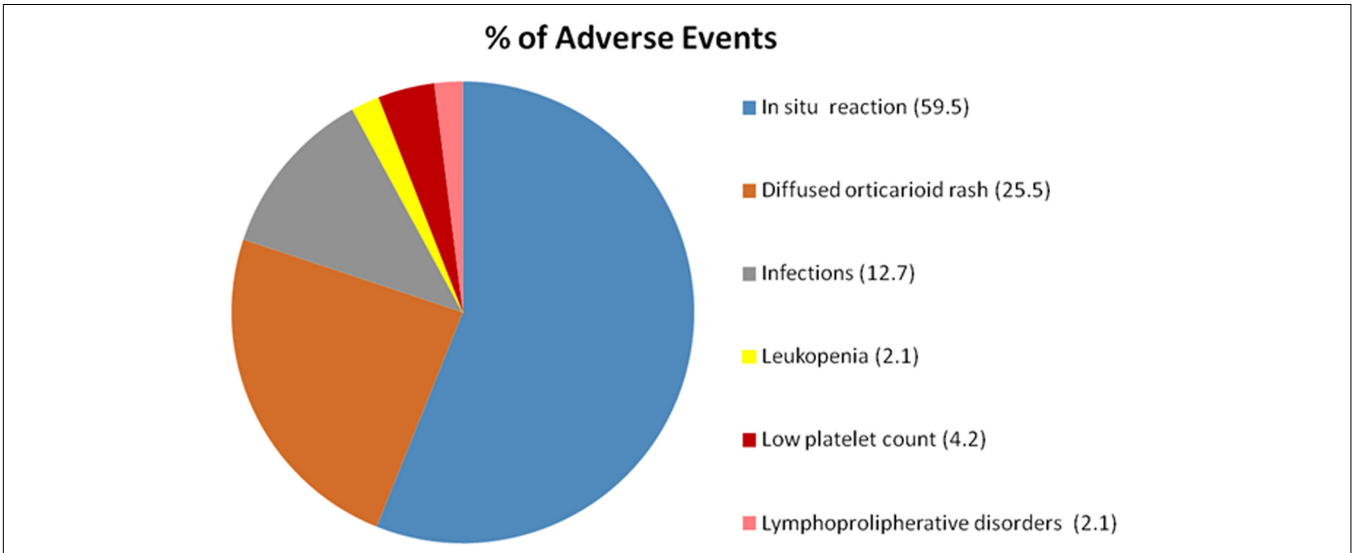

FIGURE 2 | Adverse events (AEs) linked to ANA.

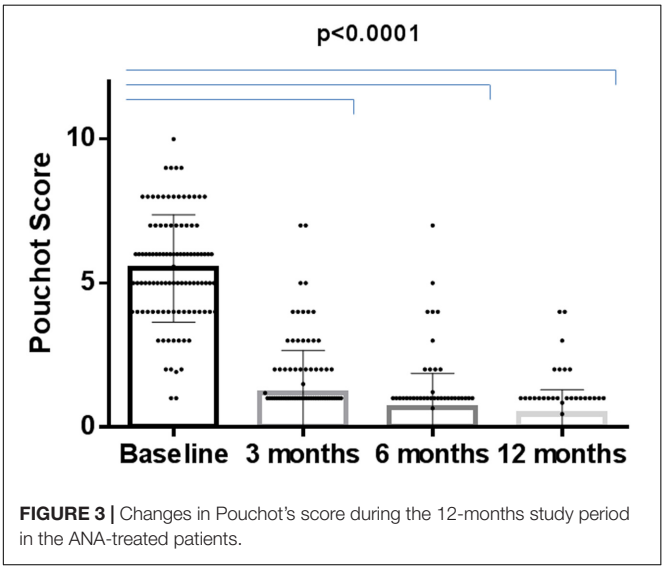

FIGURE 3 | Changes in Pouchot's score during the 12-months study period in the ANA-treated patients.

baseline versus 1.1 ± 1.4, range 0–7, after 3 months; $p < 0.0001$) (**Figure 3**).

An analysis of the prevalence of the disease's main clinical symptoms [fever, rash, pneumonia, pericarditis, pleuritis, sorethroat, lymphadenopathy, hepatomegaly, myalgia, arthritis, macrophage activation syndrome (MAS)] and laboratory features (increased liver enzymes, hyperferritinemia, leucocytosis) at 3, 6, and 12 months uncovered a significant reduction in all of these ($p < 0.0001$) beginning as early as 3 months into the follow-up (**Table 4**). There were 98/140 (70%) patients who were experiencing arthritis symptoms at baseline with a mean Disease Activity Score 28 (DAS28) score of 4.7 ± 1.2 (range 1.4–7.29) which fell significantly 3 months into therapy to 2.4 ± 1.08 (range 0.96–6.01) ($p < 0.0001$). After 12 months, the mean DAS28 score fell even further reaching 1.7 ± 0.9 (0.49–6.8).

No differences in the clinical or serological response to treatment were identified when the patients were stratified according to age, sex or disease pattern (systemic or chronic articular). Nor were differences observed when the patients receiving ANA monotherapy were compared to those receiving ANA combined with DMARDs or when the patients previously treated with other biological drugs were compared with those naïve to biological therapy.

Twelve (8.5%) patients showed signs of MAS before they were prescribed ANA treatment. After therapy was begun, five cases of MAS presented: one after 3 months (the patient recovered without discontinuing ANA), one after 6 months (the patient fully recovered without discontinuing therapy), 2 after 12 months (one patient recovered and continued ANA therapy; the other died). Another case of fatal MAS occurred after 16 months of therapy and the patient underwent disseminated intravascular coagulation (DIC); then the patient was successfully reanimated after cardiac arrest. However, a new episode of MAS led to the death of the patient who was still receiving ANA treatment 30 months later.

In one case a patient who was one of the 20 (14.2%) who showed complete disease remission needed to begin ANA therapy again. The other 19 cases are still in remission (mean follow-up 56.8 ± 54 months): 9 (47.3%) are receiving only DMARDs therapy, while 10 (52.7%) are completely drug-free.

TABLE 4 | Clinical and laboratory features at baseline and during the 12-months study period in the ANA-treated patients.

Clinical and Laboratory	Baseline ($n = 140$) (%)	3 months ($n = 118$) (%)	6 months ($n = 109$) (%)	12 months ($n = 97$) (%)
Fever	96.4	12.7	10	1
Rash	73.5	9.3	4.2	3
Pleuritis	14.2	1.6	3.6	2
Pneumonia	7.1	0	0	0
Pericarditis	17.8	0.8	0	0
Lymphadenopathy	51.4	12.7	4.5	3
Hepatomegaly or increased liver enzymes	47.1	9.3	5.5	5
Hyperferritinaemia	67.8	10.1	2.7	2
Leucocytosis	70	8.4	3.6	2
Sore throat	54.2	5	2.7	3
Myalgia	75	33	18.3	13.4
Arthritis	69.2	33	15.5	14.4
Macrophage Activation Syndrome (MAS)	8.5	0.8	0.9	1

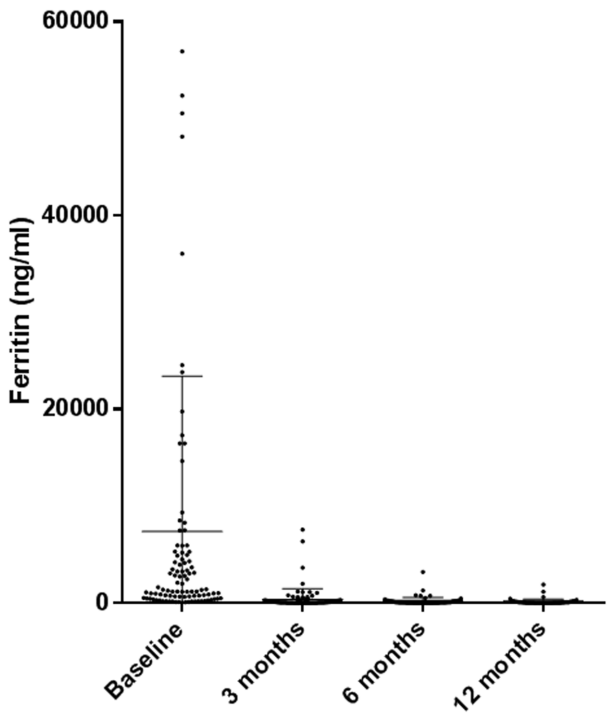

FIGURE 4 | Modifications in ferritin levels during the 12-months study period in the ANA-treated patients.

The Laboratory Efficacy of ANA

Laboratory parameters were significantly modified by therapy. Ferritin serum levels were lower after 3 months of ANA with respect to baseline data [397.5 ± 1072.68 ng/ml (range 15–7581 ng/ml) versus 5965.97 ± 14677.48 ng/ml (range 43–105000 ng/ml); $p < 0.0001$] and other inflammatory markers including ESR and CRP showed improved levels (**Figure 4** and **Table 5**). Almost 33% of the patients experienced liver involvement which was confirmed by higher liver enzyme levels. Improvement was noted already after 3 months of therapy (4.2% patients, $p < 0.0001$).

The Impact of ANA on Concomitant Therapies during the Follow-Up

Three months after beginning ANA therapy, the prevalence of patients also receiving steroids was not significantly different (97.8% patients at baseline versus 86.4% at the end of the third month, $p > 0.05$). The mean dosage was significantly lower (77.6 ± 186.3 mg of Prednisone (PDN) at baseline versus 8.8 ± 11.2 mg of PDN at the end of the third month; $p < 0.0001$). After 12 months, the percentage of patients receiving steroid therapy, which had fallen to 55.6%, was significantly different ($p < 0.001$) (**Table 6**). The percentage of patients receiving DMARD therapy had significantly fallen at the end of 12 months (85.7% patients at baseline versus 59.7% at the end of the 12th month, $p < 0.001$) and it fell even further (to 50.7%) at study completion (**Tables 6**, **7**).

Canakinumab Treatment

Four patients in whom ANA proved inefficacious were switched to CAN. The mean age of these patients at onset was 34.2 ± 15.4 years; the mean age at diagnosis was 34.7 ± 13.3 years. The mean duration of disease before starting CAN treatment was 58.33 ± 48.4 months. Three of these presented a systemic disease pattern and one a chronic articular profile.

Previous or Concomitant Therapies

Two patients were receiving CAN in association with other DMARDs, the other 2 were receiving monotherapy. The latter were treated previously with other DMARDs including MTX, hydroxychloroquine (HCQ) and CyA. Before starting ANA treatment, three patients were unsuccessfully treated with other bDMARDs: one was prescribed IFX, ETN, adalimumab, and tocilizumab; another tocilizumab; the third adalimumab.

CAN Dosage, Therapy Duration, Reasons for Discontinuing and AEs

Canakinumab was administered at the standard dose of 150 mg every 8 weeks without dose adjustment neither at the beginning

TABLE 5 | Laboratory features at baseline and during the 12-months study period in the ANA-treated patients.

Laboratory	Baseline ($n = 140$) (%)	3 months ($n = 118$) (%)	6 months ($n = 109$) (%)	12 months ($n = 97$) (%)
Ferritin				
>200 ng/ml	67.8	21.6	8.4	7.3
>1000 ng/ml	47.1	9.2	2.4	2.9
>3000 ng/ml	28.5	3	1.2	0
>10000 ng/ml	10	0	0	0
Eritrocyte sedimentation rate (ESR)	85	32.1	9.1	8.2
C-reactive protein (CRP)	90	30.5	12.8	8.2
Augmented liver enzymes	32.8	5	6.4	3

TABLE 6 | Concomitant therapy change during the 12-months study period in the ANA-treated patients.

Therapy	Baseline ($n = 140$)	3 months ($n = 118$)	6 months ($n = 109$)	12 months ($n = 97$)
Steroids	97.8%	86.4%	68.8%	55.6%
	77.6 ± 186.3 mg	8.8 ± 11.2 mg	5.2 ± 6.9 mg	3.4 ± 4.8 mg
DMARDs	85.7%	66.1%	59.6%	59.7%

TABLE 7 | Anakinra and canakinumab (CAN) therapy status after 12 months of follow-up.

	ANA	CAN
Last Follow-up	32.2 ± 41.5 months	65.75 ± 76.34 months
Ongoing Therapy	49.2% (69/140) patients	50% (1/4) patients
Mean therapy duration	35.7 ± 36.1 months	22.1 ± 16.5 months
Ongoing steroids	31.8% (22/69) patients	100% (3/3) patients
Ongoing DMARDs	50.7% (35/69) patients	33.3% (1/3) patients

nor during the follow-up period. The mean duration of therapy was 22.1 ± 16.5 months. Treatment is still ongoing in two patients. In the other two cases, it was discontinued; in one case,it was discontinued after 9 months because of loss of efficacy, in the other it was discontinued after 45 months because of remission. No AEs were registered in the CAN-treated patients.

CAN Clinical Efficacy

After 3 months of CAN therapy, Pouchot's score fell significantly from 4.25 ± 2.6 (range 2–8) to 1.25 ± 1.8 (range 1–4) ($p < 0.0001$) (**Figure 5**). Despite a general improvement in symptoms, therapy was discontinued in the chronic articular AOSD patient after 9 months because fever, arthritis and lymphadenopathy persisted over time. We were thus able to evaluate clinical parameters at 12 months in only three patients (**Table 8**). In one case, after 6 months of therapy, disease symptoms flared up and led to a MAS episode which was promptly treated and cured. The patient continued CAN therapy after that episode, and treatment is still ongoing. Signs of arthritis were observed at baseline in 2/4 patients; improvement was noted after 3 months in only one patient who experienced a complete remission during the follow-up. In all three remaining cases, there was no evidence at 12 months of clinical disease signs (no fever, rash, arthritis, or lymphadenopathy).

The Laboratory Efficacy of CAN

Serum ferritin levels were increased at baseline in 2/4 patients and were normalized after 3 months in all four. Serum ferritin levels were increased after 6 months in one patient who experienced a MAS episode. As therapy was discontinued in one patient, laboratory parameters after 12 months were assessed only in 3. Ferritin levels in 2 of these appeared normal; in the third (the same patient who experienced MAS 6 months earlier) ferritin rose to 425 ng/ml. That same patient presented altered liver enzyme, ESR, and CRP values throughout the observation period. The transaminase levels were normal during follow-up in the other three cases. ESR was elevated at baseline and at the end of the third month in 3 of the 4 patients; it was reduced in one patient after 6 months, and it was reduced in another after 12 months. CRP was also higher at baseline and after 3 months in all of the patients, it was decreased in two patients at the 6 months time point, and in another at the 12-months time point.

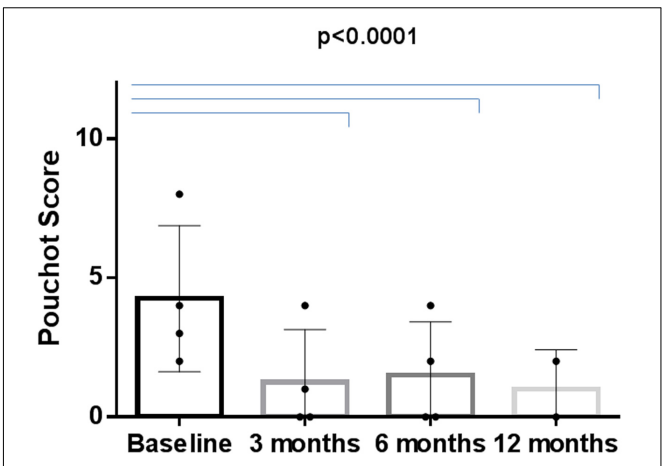

FIGURE 5 | Modifications in Pouchot's score changes during the 12-months study period in the CAN-treated patients.

The Impact of CAN on Concomitant Therapies during Follow Up

Although no patient discontinued steroids during the 12 months study period, the mean PDN dosage was significantly lower with

TABLE 8 | Clinical and laboratory features at baseline and during the 12-months study period in the CAN-treated patients.

Clinical and laboratory	Baseline ($n = 4$) (%)	3 months ($n = 4$) (%)	6 months ($n = 4$) (%)	12 months ($n = 3$) (%)
Fever	100	25	50	0
Rash	50	0	0	0
Pleuritis	25	0	0	0
Pneumonitis	0	0	0	0
Pericarditis	25	0%	0	0
Lymphadenopathy	50	25	0	0
Hepatomegaly or increased liver enzymes	25	25	25	33.3
Hyperferritinemia	50	0	25%	33.3
Leucocytosis	100	25	25%	0
Sore throat	25	0	0%	0
Milagia	100	25	25%	0
Arthritis	50	25	50%	0
Macrophage Activation Syndrome (MAS)	0	0	25%	0

TABLE 9 | Changes in concomitant therapy strategies during the 12-months study period in the CAN-treated patients.

Therapy	Baseline ($n = 4$)	3 months ($n = 4$)	6 months ($n = 4$)	12 months ($n = 3$)
Steroids	100%	100%	100%	100%
	143.7 ± 238.2 mg	8.2 ± 7.8 mg	16.2 ± 13 mg	10 ± 7 mg
DMARDs	50%	50%	50%	33%

respect to the baseline value (143.75 ± 238.23 mg) as early as at 3 months (8.2 ± 7.8 mg, $p < 0.0001$) and at 12 months (10 ± 7.07 mg, $p < 0.0001$) (**Table 9**). The concomitant use of DMARDs was similar at baseline (2/4 cases) and at the end of the 12 months follow-up period (1/3 cases) (**Table 9**). Information regarding the current therapy and the last follow-up are outlined in **Table 7**.

DISCUSSION

To our knowledge, this is the largest retrospective observational study evaluating the efficacy and safety of ANA and CAN in AOSD. Consistent with other studies, our data have confirmed the efficacy of IL-1-INH treatment in AOSD patients. It is well known that blocking IL-1, particularly IL-1β, represents standard therapy for a number of autoinflammatory conditions in which this cytokine plays a pivotal role. As far as IL-1α or IL-1β signal cascades are concerned, upon binding to the ligand-binding chain (IL-1RI), activation of the signaling pathway originates from the cytoplasmic Toll/IL-1 receptor (TIR) domain that associates with a TIR domain-containing adaptor, MyD88. Subsequent phosphorylation of several kinases leads to translocation of NF-κB to the nucleus and final expression of a large portfolio of inflammatory genes (Weber et al., 2010). The IL-1 family comprises 11 members (Dinarello, 2011), and some investigators (Colafrancesco et al., 2012; Priori et al., 2014) have shown that IL-1α/β and IL-18 are crucial in the pathogenesis of AOSD and are valid serological biomarkers of the disease (Colafrancesco et al., 2015).

First described as a treatment for AOSD in 2003, ANA, a recombinant version of the interleukin 1 receptor antagonist (IL1-RA), was the first IL-1 inhibitor used in clinical practice (Rudinskaya and Trock, 2003). Although the efficacy of ANA in AOSD has been described by several case-reports and case-series, due to the disease's rarity, large randomized control trials (RCT) are still lacking. A meta-analysis published in 2014 identified eight studies, including one RCT (Nordström et al., 2012) demonstrating that ANA seems to be effective in improving AOSD manifestations and in reducing mean steroid dosage over time in patients refractory to conventional therapies (Hong et al., 2014). That meta-analysis, which considered all studies published between 2010 and 2014 (sample size ranging from 6 to 28 patients for a total of 134 patients receiving ANA treatment (100 mg/day), showed an overall and complete remission rate of 81.66 and 66.75%, respectively, thus supporting the efficacy of ANA in more than half of the patients studied.

The largest study until now evaluating ANA efficacy, which analyzed 41 patients, was published in 2015. Approximately 68% of the patients were found to be responders; half achieved complete remission defined as the total disappearance of signs and symptoms of disease and normalization of laboratory parameters. As reported in previous reports summarized in **Table 10** (Lequerré et al., 2008; Laskari et al., 2011; Nordström et al., 2012; Giampietro et al., 2013; Gerfaud-Valentin et al., 2014b; Cavalli et al., 2015; Ortiz-Sanjuán et al., 2015; Rossi-Semerano et al., 2015), a complete remission was demonstrated in up to 80% of the patients studied.

In our cohort of 140 patients a prompt response to IL-1 inhibitors was demonstrated already after 3 months. Both clinical and laboratory parameters dramatically improved and remission was sustained over time and led to discontinuation of treatment in 28% of the cases during the follow-up. Most of the patients succeeded in achieving remission within 3 months of beginning

TABLE 10 | Studies on AOSD patients treated with interleukin-1 (IL-1) inhibitors published in the past.

	Lequerré et al., 2008	Laskari et al., 2011	Nordström et al., 2012	Giampietro et al., 2013	Gerfaud-Valentin et al., 2014b	Cavalli et al., 2015	Ortiz-Sanjuán et al., 2015	Rossi-Semerano et al., 2015
N° patients	15	25	12	28	6	20	41	35
N° patients *Complete response (Remission*)*	11 (73.3%)	20 (80%)	6 (50%)	15 (53.5%)	5 (83.3%)	14 (70%)	14 (34.1%)	16 (45.7%)
N° patients *Partial response****	2 (13.3%)	4 (16%)	N/A	4 (14.2%)	0	2 (10%)	14 (34.1%)	–
N° patients *Not effective*****	2 (13.3%)	1 (4%)	1	6 (21.4%)	1 (16.6%)	4 (20%)	7 (17.07%)	14 (40%)
Discontinued for reasons other than remission/AE	–	–	–	1 (3.5%) (pregnancy)	–	–	1 (2.4%) (pregnancy)	2 (5.7%) (at patient's request)
Steroid dosages reduced	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N/A
AE	2 (13.3%) Cutaneous reactions	3 (12%) Cutaneous reactions	11 (91.6%) Cutaneous reactions	2 (7.1%) Cutaneous reactions	0	2 (10%) Cutaneous reactions	8 (19.5%) Cutaneous reactions	3 (8.5%) Infections (pneumonia, 1 VZV reactivation, MAS and infection)
		7 (28%) Infections (2 RTI, 3 UTI, 1 GE, 1 tissue abscess)				2 (10%) VZV Reactivation	4 (9.7%) Infections (1 osteomyelitis, 1 RTI, 2 UTI, 1 VZV reactivation) 1 (2.4%) Leukopenia 1 (2.4%) myopahy	

*The times therapy efficacy were evaluated were different in the various studies. *Remission: no fever (<37°C), no increase in inflammatory markers (ESR, CRP or ferritin), no tender or swollen joints. **Partial response: clinical improvement without complete symptom improvement or complete normalization of inflammatory markers. ***Not effective: AOSD symptoms were unmodified during ANA treatment, including primary or secondary failure. RTI = respiratory tract infection; UTI, urinary tract infection; GE, gastroenteritis; VZV, varicella zoster virus; MAS, macrophage activation syndrome.*

therapy, a finding that is in agreement with reports in the literature describing the normalization of clinical, hematologic and biochemical parameters within hours to days after the first ANA injection. Pouchot's score revealed a dramatic improvement in patients' general clinical condition within 3 months time. Although the score lacks scientific validation, it is commonly used to evaluate patients' disease activity. Some investigators have proposed using it as a "severity" rather than "activity" score. Others have suggested giving it a prognostic value and of using the score of 7.0 as a cut-off at the time of diagnosis as predictive of a more severe outcome and an increased risk of mortality (Ruscitti et al., 2016).

Interestingly, response to therapy was achieved rapidly in the same way in the systemic and chronic articular patients studied as far as the different signs and symptoms of the disease were concerned.

The number of articular patients with arthritis symptoms as well as the mean DAS 28 value significantly ameliorated over the time. Although this aspect seems to be confirmed by other studies (Laskari et al., 2011; Gerfaud-Valentin et al., 2014a), the efficacy of ANA in improving articular manifestations continues to be controversial. Laskari et al. (2011) demonstrated an improvement in joint manifestations (evaluated by ACR50 and ACR70 response) in 93 and 87% of their patients, respectively. Although a very low number of treated patients (4 with a systemic polyciclic pattern and 2 with a chronic articular one), no difference in response to therapy was identified in the patient groups by the study conducted by Gerfaud-Valentin et al. We also reported on the apparent efficacy of ANA in 3 patients with AOSD who was refractory to conventional therapies and who showed marked improvement in joint manifestations after therapy was begun (Priori et al., 2008). According to a study by Cavalli et al. (2015), ANA proved to be more effective in patients with the systemic disease pattern with respect to the chronic articular one, especially in those cases experiencing severe complications such as MAS.

The increased efficacy of ANA in patients experiencing a prevalently systemic involvement is in line with a dichotomous view of AOSD. Indeed, many assume that the systemic form is mainly driven by IL-1 responsible for fever or increased serum inflammatory marker, while the articular pattern is more similar to rheumatoid arthritis and may be principally driven by TNF-α (Maria et al., 2014). Likewise, in a study carried out by Giampietro et al. (2013) the most impressive response was obtained in the systemic AOSD pattern; a slightly less dramatic effect was noted in the chronic articular phenotype. Ortiz-Sanjuán et al. (2015) likewise reported a persistence of joint involvement after one year of treatment in 41.5% of the patients studied. Definitive conclusions on the real effectiveness of ANA on joint involvement have yet to be drawn.

We did not observe any differences in the type of response in the two patterns of the disease in our study nor when the patients were stratified according to age, sex, and previous types of therapies. Interestingly, no differences were noted in the AOSD group receiving monotherapy with respect to those who were also receiving DMARDs. The fact that similar results were reported by Giampietro et al. (2013) seems to confirm the relevance of taking into consideration the cost-effectiveness of ANA in view of its efficacy as monotherapy and its steroid sparing effect. For the time being, there is no consensus on this issue. Although the differences were not significant in a study by Ortiz-Sanjuán et al. (2015) focusing on the use of ANA in combination with conventional immunosuppressive drugs, it did not appear to improve the systemic symptoms or joint manifestations more efficaciously than did the monotherapy. ANA seems to spare steroids with the non-negligible consequence of preventing complications attributable to the chronic intake of steroids.

In most published studies ANA was used at the standard dose of 100 mg per day. In our study, a few of the patients began with lower dosages (50 mg per day or 100 mg every other day). Those patients were for the most part the ones who later needed dosage upgrade to the conventional one. We also reported on one patient whose 100 mg per day dosage did not appear to be sufficiently effective; when dosage was later switched to 200 mg/day the patient showed a good response and tolerability. In the presence of a clinical improvement, ANA dose has been reduced in several cases of our cohort, more commonly than in other reports (Laskari et al., 2011; Gerfaud-Valentin et al., 2014b; Ortiz-Sanjuán et al., 2015).

The optimal duration of ANA treatment in AOSD has not yet been established. As we recently reported in another multicenter study, the off-label use of IL-1 inhibitors presents a wide variability. While they are more frequently employed at a dosage based on body weight in pediatric patients, in adults a standard dose of 100 mg is frequently used (Vitale et al., 2016). Contrary to our observations, Kötter et al. described a recurrence in disease activity after reducing ANA administration to alternate days (Kötter et al., 2007). During a previous study focusing on the use of IL-1 inhibitors in different conditions, we noted that adjusting the dosage by increasing the dose at each administration or decreasing the timing between injections proved to be successful in 66.7% of patients. With one exception, we did not report any relapse after therapy was discontinued because of remission. In agreement with other reports, tapering of dosage was well tolerated and there was no evidence of relapse (Laskari et al., 2011; Giampietro et al., 2013; Ortiz-Sanjuán et al., 2015; Vitale et al., 2016).

The current study was able to retrospectively evaluate not only the efficacy but also the safety of ANA. In agreement with our previous reports (Sfriso et al., 2016; Vitale et al., 2016) and those of others (outlined in **Table 10**), ANA appears to be a safe drug that does not pose risks of infections. In the current study, the frequency of AEs was higher (33.5%) then that previously reported (**Table 10**). The most relevant AEs noted were reactions at the injection site (28/140 cases, 20%) or diffuse allergic skin rashes (12/140 cases, 8.6%); in some cases, the AEs were so severe as to determine therapy discontinuation. Although with a lower frequency, cutaneous reactions were the most commonly reported AE even in other reports (**Table 10**). In the light of the current study (the largest cohort of patients carried out until now) and the findings of others, the risk of reactions must be taken into consideration when therapy is being decided. The short half-life of ANA and the need for daily subcutaneous injections are other not negligible disadvantages that also need to be considered.

Canakinumab is a fully human monoclonal antibody against IL-1β with a longer half-life (26 days), meaning that it can be administered every 8 weeks. It has been approved for periodic fevers such as Cryopyrin Associated Periodic Syndromes (CAPS) as well as for SJIA. Promising results have recently been reported in patients with AOSD refractory to ANA. The first report, which was published in 2012 (Kontzias and Efthimiou, 2012), was followed by others (Barsotti et al., 2014; Lo Gullo et al., 2014). Although a study by Rossi-Semerano et al. (2015) did not report any significant differences in AEs in the ANA and CAN therapy groups, no AEs occurred in our 4 patients who were treated with CAN. Indeed, no cutaneous reactions or infections of any kind were noted. CAN proved to be effective in 3/4 patients and perhaps responsible for therapy discontinuation due to complete remission in one. It should be underlined that the failure took place in a patient with a chronic articular pattern. Although a slight improvement in the patient's general condition was noted in that case, neither arthritis nor fever responded to therapy. In another case regarding a patient with a systemic pattern but also showing signs of arthritis, CAN proved to be efficacious. Definite conclusions concerning CAN efficacy in inflammatory joint involvement are not yet possible.

One patient also experienced MAS during therapy but completely recovered and subsequently continued therapy. Episodes of MAS also occurred during ANA treatment, in two cases with fatal outcomes. Although the percentage of patients experiencing recrudescence of the disease with severe life-threatening complications was quite low [6/140 (4.1%) = 5 cases during ANA treatment and 1 during CAN therapy], neither of the IL-1 inhibitors appeared to be capable of completely controlling these severe systemic manifestations.

Although the retrospective design and the open-label nature constitute potential limitations, the study represents the largest evaluation of IL-1 inhibition efficacy and safety in patients with AOSD. According to our findings, IL-1-INH seem to represent the best bDMARDs available and should be considered the first-line biologic treatment for patients not responding to conventional treatment. ANA can also be utilized as a monotherapy since it seems to have the same efficacy even when it is not associated to DMARDs. As ANA seems to be effective in improving all the clinical and laboratory features of AOSD, its use seems opportune regardless of the disease pattern. ANA also appears to be safe as far as the infectious risk is concerned. It should nevertheless be remember that development of skin reactions can at times be so invalidating as to induce therapy discontinuation.

Canakinumab also appears to be effective in AOSD, but in the current study it was possible to evaluate its efficacy only in patients who did not respond to ANA therapy or in whom ANA efficacy was lost. It cannot be excluded that those cases were more aggressive ones. It was also impossible to make any comparisons between ANA and CAN given the overwhelming differences in numbers.

CONCLUSION

Interleukin-1 inhibitors appear to be a highly effective treatment of AOSD. Although most data regarding the effect of IL-1-INH in AOSD concern ANA, case reports focusing on treatment with CAN suggest that it has a similar efficacy. Further studies and randomized controlled trials are of course warranted.

AUTHOR CONTRIBUTIONS

SC, RP, PG, and PS drafted the manuscript. SC and RP carried out the data analysis. All the authors contributed to the enrollment process, in monitoring the patients, and in data collection. The study was conceived and designed by LC and PS.

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REVIEW
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IL-1β Inhibition in Cardiovascular Complications Associated to Diabetes Mellitus

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Diabetes mellitus (DM) is a chronic disease that affects nowadays millions of people worldwide. In adults, type 2 diabetes mellitus (T2DM) accounts for the majority of all diagnosed cases of diabetes. The course of the T2DM is characterized by insulin resistance and a progressive loss of β-cell mass. DM is associated with a number of related complications, among which cardiovascular complications and atherosclerosis are the main cause of morbidity and mortality in patients suffering from the disease. DM is acknowledged as a low-grade chronic inflammatory state characterized by the over-secretion of pro-inflammatory cytokines, including interleukin (IL)-1β, which reinforce inflammatory signals thus contributing to the development of complications. In this context, the pharmacological approaches to treat diabetes should not only correct hyperglycaemia, but also attenuate inflammation and prevent the development of metabolic and cardiovascular complications. Over the last years, novel biological drugs have been developed to antagonize the pathophysiological actions of IL-1β. The drugs currently used in clinical practice are anakinra, a recombinant form of the naturally occurring IL-1 receptor antagonist, the soluble decoy receptor rilonacept and the monoclonal antibodies canakinumab and gevokizumab. This review will summarize the main experimental and clinical findings obtained with pharmacological IL-1β inhibitors in the context of the cardiovascular complications of DM, and discuss the perspectives of IL-1β inhibitors as novel therapeutic tools for treating these patients.

Keywords: diabetes, inflammation, cardiovascular complications, interleukin-1β, interleukin-1 inhibitors

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DIABETES, INFLAMMATION AND CARDIOVASCULAR COMPLICATIONS

Diabetes mellitus (DM) is a cardiometabolic disease that affects millions of people worldwide. Accordingly, the World Health Organization (WHO) has acknowledged the disease as epidemic. In adults, the prevalence of type 2 diabetes mellitus (T2DM) is markedly higher that that of type 1 diabetes mellitus (T1DM), since it accounts for at least 90% of all diagnosed cases of diabetes. The rapid increase in the prevalence of T2DM has emerged as a major global health problem, mostly due

to associated complications, medical costs and reduced life expectancy. The course of the T2DM is characterized by insulin resistance and a progressive loss of β-cell mass, together with the onset of vascular complications including coronary heart disease, stroke, peripheral vascular disease, and end stage renal disease, making it a leading cause of death worldwide.

Diabetes mellitus is nowadays acknowledged as a low-grade chronic inflammatory condition characterized by the over-secretion of pro-inflammatory cytokines. A growing body of evidence currently points at interleukin-1β (IL-1β), which is a major player in a wide array of auto-inflammatory diseases, to also act as key promoter of systemic and tissue inflammation in DM (Dinarello et al., 2010; Sumpter et al., 2011). Indeed, an enhanced expression of IL-1β in a high glucose milieu has been described in human monocytes and macrophages (Shashkin et al., 2006; Dasu et al., 2007), pancreatic islets (Maedler et al., 2004), myocardium (Niu et al., 2014), and aortic endothelium (Asakawa et al., 1997), while the upregulated IL-1β levels have been described in the heart and the retina and retinal vessels from diabetic rats, among other (Ares-Carrasco et al., 2009; Liu et al., 2012).

Many pieces of evidence, including studies in humans, suggest that IL-1β plays a role in insulin resistance, both in clinically overt T2DM and pre-diabetic states (van Asseldonk et al., 2011). Such conditions are characterized by an over-production of adipocytokines, including IL-1β, that are locally associated to the inflammation of the adipose tissue. Importantly, these adipokines can also be released to the circulation and impact on distant organs, including the heart or the vessels. In fact, increased systemic and vascular inflammation are considered key mechanisms underlying diabetic vasculopathy (Sowers, 2013). In this context, a key role of pro-inflammatory cytokines, such as IL-1β, in the development of cardiovascular complications of DM is being considered (Raines and Ferri, 2005; Goldberg, 2009; Sprague and Khalil, 2009; Frostegård, 2013; Krishnan et al., 2014).

Based on the above described autoinflammatory features of DM, the pharmacological approaches to treat diabetes should not only correct hyperglycaemia, but also target chronic inflammation in order to prevent the development of metabolic and cardiovascular complications. In this context, blocking IL-1β arises as a challenging therapeutic option to treat the progression of the disease and its complications.

IL-1β AND THE INFLAMMASOME

Interleukins are regulatory proteins with ability to accelerate or inhibit inflammatory processes, as well as other tissue responses. IL-1 belongs to the group of pro-inflammatory interleukins together with IL-2, IL-6, IL-7, IL-8, IL-15, IL-17, and IL-18. IL-1 may counterregulate anti-inflammatory cytokines such as IL-4, IL-10, IL-11, or other cytokines, such as IL-12 and IL-13 (Fisman et al., 2008). The IL-1 superfamily of cytokines comprises IL-1α, IL-1β, the endogenous regulator of IL-1 activity [a competitive IL-1 receptor antagonist (IL-1Ra)], IL-18, and the newly discovered IL-33, among other. The IL-1 superfamily is closely linked to both innate inflammation and immune responses. IL-1α is constitutively expressed in most cells of healthy subjects, although a role for this cytokine in disease is being validated in the context of sterile inflammation (Dinarello et al., 2012). In contrast, the expression of IL-1β is very limited in health, but it is markedly enhanced in blood monocytes, tissue macrophages and dendritic cells after stimuli like microbial products and other cytokines, such as tumor necrosis factor (TNF)α, IL-18, IL-1α, or IL-1β itself, as an autoinflammatory mechanism (Dinarello et al., 2012).

Interleukin-1β is produced as a precursor peptide (pro-IL-1β), which is N-terminal cleaved by caspase-1 or by the IL-1 converting enzyme (ICE) to form the active mature molecule. Caspase-1, an intracellular cysteine protease, needs firstly to be processed following the oligomerization of a complex of intracellular proteins termed the inflammasome. A key component of the inflammasome, NLRP3, plays a critical role in the secretion of IL-1β and in pyroptosis, which is an inherently inflammatory caspase 1-dependent mechanism of cell death triggered by various pathological stimuli, such as acute myocardial infarction (AMI) (Bergsbaken et al., 2009). Remarkably, a human autosomal mutation in NLRP3 results in enhanced caspase-1 activity and greater secretion of IL-1β (Franchi et al., 2009).

Interleukin-1β triggers intracellular signaling cascades through the activation of the interleukin-1 receptor, type I (IL-1R1), which also binds IL-1α. IL-1R1 is characterized by extracellular immunoglobulin-like domains and an intracellular Toll/interleukin-1R (TIR) domain, and it requires heterodimer formation with interleukin-1 receptor accessory protein (IL-1RAcP) to exert intracellular signaling (Palomo et al., 2015). IL-1R1 is expressed in all cells and its activation triggers multiple and sequential phosphorylation events that result in nuclear translocation of transcription factors. IL-1 activates JAK protein kinases that phosphorylate serine and threonine residues, which are the targets of the mitogen-activated protein kinase (MAPK) family. MAPKs then stimulate the translocation of nuclear factor (NF)-κB to the nucleus via the IRAK-TRAF6 pathway, and enhance nuclear binding of c-jun and c-fos, for activator protein (AP)-1 activation. Both NF-κB and AP-1 sites are present in the promoter regions of many inflammation-related IL-1-inducible genes that encode for diverse cytokines, adhesion molecules, chemokines or pro-inflammatory enzymes, among other (Palomo et al., 2015).

PHARMACOLOGICAL BLOCKADE OF IL-1β

In view of the growing pathophysiological relevance of IL-1β in a wide variety of diseases, novel biological drugs have been developed over the last years to antagonize the actions of the cytokine. The drugs targeting IL-1β that are currently approved for clinical use are anakinra, rilonacept, and canakinumab, while gevokizumab has received orphan drug designation. A brief description of these drugs follows below.

Anakinra is a short-acting recombinant non-glycosylated form of the naturally occurring IL-1Ra, which blocks the activity

of both IL-1α and IL-1β. Anakinra was first approved for the treatment of rheumatoid arthritis on a daily basis in 2001 (Dinarello et al., 2012). However, its clinical indications have been extended so far to other diseases such as the cryopyrin-associated periodic syndromes (CAPS), a group of rare inherited auto-inflammatory diseases associated to pathogenic variants in the IL-1-regulating genes NLRP3 and ILRN (Jesus and Goldbach-Mansky, 2014). Anakinra nowadays occupies a relevant position in IL-1 therapeutics as a result, of its excellent safety record even in long-term treatments, among other (Kullenberg et al., 2016).

The development of soluble decoy receptors was later introduced as a strategy to achieve IL-1 binding and neutralization. Rilonacept is a long-acting dimeric fusion protein that complexes the extracellular residues of the two IL1 receptor subunits, IL-1R1 and IL-1RAcP, to the Fc portion of IgG1. Rilonacept was approved in 2008 by the FDA for the treatment of CAPS. Like anakinra, rilonacept binds both IL-1α and IL-1β.

More recently, monoclonal antibodies that specifically target IL-1β have been developed. Canakinumab is a human IgG1k monoclonal antibody that binds and neutralizes soluble IL-1β, with no cross-reactivity with other interleukins, including IL-1α. Like rilonacept, canakinumab was approved for treating CAPS, other rare periodic fever syndromes, and juvenile arthritis (Dinarello et al., 2012). A second humanized IgG2 monoclonal antibody, gevokizumab, which also strongly binds IL-1β has been designed as orphan drug by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) for treating a series of rare conditions, including pyoderma gangrenosum, Schnitzler syndrome, chronic non-infectious uveitis or congenital hyperinsulinism. Another humanized IL-1 neutralizing antibody LY2189102 (Eli-Lilly and Company) is currently under study (Sloan-Lancaster et al., 2013).

Furthermore, a novel approach to block IL-1β has been developed by means of active vaccination against endogenous pro-inflammatory proteins. The vaccine hIL1bQb consists of full-length recombinant IL-1β coupled to virus-like particles, and it has been shown to produce endogenous anti-IL-1β antibodies in pre-clinical models. The vaccine is currently starting to being tested in patients (Cavelti-Weder et al., 2016).

Beyond their yet approved indications, these drugs are being currently used in both pre-clinical research and clinical trials to assess their applicability to other conditions in which IL-1β may play a key pathophysiological role. In the next sections, the main pre-clinical findings on the modulation of cardiovascular dysfunction by IL-1β pharmacological blockers will be summarized. Moreover, the clinical trials assessing the cardiovascular impact of IL-1β inhibition in the context of DM will be reviewed.

IL-1β BLOCKADE AND EXPERIMENTAL DIABETIC VASCULOPATHY

Endothelial Dysfunction

Endothelial dysfunction is a crucial and early manifestation of vascular diseases. It is characterized by the impairment of the relaxations induced by nitric oxide (NO) and other vasodilator compounds (like prostacyclin or the endothelium hyperpolarizing factor), while the endothelial vasoconstrictor factors are increased (Félétou and Vanhoutte, 2006). A pro-oxidant and pro-inflammatory vascular environment is another characteristic of endothelial dysfunction (Félétou and Vanhoutte, 2006). Endothelial dysfunction is linked to the early development of DM in both humans and experimental animal models, and associated to reactive oxygen species (ROS) over-production (Durante et al., 1988; Calver et al., 1992; Johnstone et al., 1993; Angulo et al., 1998; Rodríguez-Mañas et al., 2003a,b).

There is also increasing evidence suggesting that the development of an inflammatory environment in the vasculature by pro-inflammatory cytokines is followed by an impairment of endothelial function. Thus, acute systemic inflammation in response to *Salmonella typhi* vaccine produces a temporary but profound dysfunction of human arterial endothelium in both resistance and conduit vessels, which is related to cytokine production (Hingorani et al., 2000; Kharbanda et al., 2002). Moreover, it is now well established, either in experimental models and humans, that pro-inflammatory cytokines impair vascular reactivity in different vascular beds, including resistance vessels (Vila and Salaices, 2005).

Although the IL-1 pathway is considered at present a critical player in the pathophysiology of both T1DM and T2DM (Donath and Shoelson, 2011; Herder et al., 2015), the evidence on the impact of IL-1β on vascular function is still limited. In isolated resistance microvessels from non-diabetic animals, IL-1β produces endothelial dysfunction after different exposure times to the cytokine (Wimalasundera et al., 2003; Jiménez-Altayó et al., 2006; Vallejo et al., 2014). Interestingly, the impairment of endothelial function may occur even after a rather short incubation with IL-1β (30 to 120 min), when the possible pro-inflammatory responses triggered by the cytokine are not fully developed, as indicated by the lack of involvement of inducible pro-inflammatory enzymes that require *de novo* synthesis (Vallejo et al., 2014). This early endothelial dysfunction evoked by IL-1β is rather due to the IL-1 receptor-mediated activation of NADPH oxidase, which enhances superoxide anion ($O_2^{\bullet -}$) production (Vallejo et al., 2014). Interestingly, NADPH oxidase over-activation has been linked to excess ROS generation and the development of atherosclerosis in the context of diabetic vasculopathy (Olukman et al., 2010; Gray et al., 2013). The pharmacological blockade of IL-1 receptors by anakinra permits to attenuate both NADPH activation and endothelial dysfunction induced by IL-1β in microvessels from non-diabetic animals (Vallejo et al., 2014).

Interestingly, the intraperitoneal administration of anakinra can also partially recover the endothelial dysfunction observed in experimental diabetes after 15 days of diabetes induction (Vallejo et al., 2014). The animals receiving the drug exhibited a clear improvement of endothelium-dependent relaxations that was paralleled by the normalization of NADPH oxidase activity in the vascular wall (Vallejo et al., 2014). Intriguingly, the circulating levels of IL-1β were not found to be enhanced (Vallejo et al., 2014), as previously reported by others in

experimental models (Yazar et al., 2011) or in diabetic patients (Mooradian et al., 1991; Pereira et al., 2014). The local over-expression of IL-1β in the vascular wall may be responsible for a paracrine inflammatory response, similarly to that described in vitreous samples from patients with proliferative diabetic retinopathy (Patel et al., 2008). In addition, other long-lasting pro-inflammatory mechanisms induced by IL-1β can be involved in the endothelial dysfunction associated to experimental diabetes, since improved endothelium-dependent relaxation was observed after the pharmacological blockade of cyclooxygenase or the inducible form of nitric oxide synthase (iNOS) (Vallejo et al., 2014).

Vascular Inflammation and Atherosclerosis

Chronic vascular inflammation is at the basis of atherosclerosis, which in turn accounts for life-threatening complications of DM such as AMI or stroke. The pro-inflammatory cytokines IL-1β and IL-1α are widely expressed in human and experimental atherosclerotic lesions (Frostegård et al., 1999; Liu et al., 2014) and a large body of preclinical data reveals that IL-1β plays a major role in the progression and rupture of atherosclerotic plaques (Chi et al., 2004; Isoda et al., 2004; Merhi-Soussi et al., 2005; Chamberlain et al., 2006; Frostegård, 2013; Singla et al., 2016). Thus, a rationale is set for the use of pharmacological IL-1β blockers as tools to delay the onset and progression of atherosclerotic lesions.

One of the earliest steps of atherosclerosis is the recruitment of leukocytes by endothelial cells via the expression of adhesion molecules such as ICAM-1 and VCAM-1. The saturated fatty acid palmitate, which is linked to a higher risk of type 2 diabetes and cardiovascular disease, promotes the release of IL-1β by monocytes and their adhesion to human endothelial cells (Shikama et al., 2015). This effect can be blocked by anakinra, which prevents the induction of adhesion molecules elicited by IL-1β (Shikama et al., 2015).

An imbalanced trafficking and distribution of intracellular cholesterol has been associated with defective signaling and vascular cell dysfunction (Schreiber, 2002). In cultured vascular smooth muscle cells, serum amyloid A, which is expressed in atherosclerotic lesions, promotes cholesterol trafficking via the release of IL-1β (Pessolano et al., 2012). This effect can be inhibited by the recombinant form of IL-1Ra (Pessolano et al., 2012), and presumably, by IL-1β blocking drugs, although this point still needs to be addressed.

Moreover, the dysruption of the endoplasmic reticulum (ER) homeostasis by high glucose or other factors has been identified over the last years as an important mechanism that underlies many complications of type 2 such as endothelial dysfunction, vascular inflammation and atherosclerosis (Lenna et al., 2014). Chronic ER stress leads to the so-called unfolded protein response (UPR), which ultimately seeks to restore ER function but can also lead to cell death and apoptosis (Lenna et al., 2014). In a murine model of type 2 diabetes, the intraperitoneal injection of anakinra during 4 weeks diminished ER stress and macrophage infiltration, and improved ischemia-induced neovascularization and blood flow as compared with untreated animals (Amin et al., 2012). These effects were further accompanied by a normalization in the blood levels of cholesterol and adiponectin without affecting glycemia (Amin et al., 2012).

It is also worth noting that the pro-inflammatory signaling elicited by IL-1β in human vascular cells can be exacerbated by high glucose. In human endothelial cells, the expression of adhesion molecules such as ICAM-1 and VCAM-1 induced by IL-1β or TNF-α is augmented in the presence of extracellular high glucose (Azcutia et al., 2010). Functionally, the synergy between IL-1β and high glucose leads to an exaggerated leukocyte-endothelial adhesion *in vitro*, together with enhanced rolling flux and emigration of leukocytes in animal experimental models *in vivo* (Azcutia et al., 2010). Similarly, in human vascular smooth muscle cells IL-1β triggers the activation of pro-inflammatory pathways such as the ERK 1/2- NF-κB- inducible nitric oxide synthase (iNOS) axis (Lafuente et al., 2008). Such an effect of IL-1β is exaggerated proportionally to the concentration of extracellular glucose (Lafuente et al., 2008). As an explanation for this synergy, it has recently been shown that IL-1β permits the entry of extra glucose across the plasma membrane of human vascular smooth muscle cells (Peiró et al., 2016). Part of this excess intracellular glucose is then driven by the pentose phosphate pathway to produce NADPH that in turn fuels the pro-oxidant enzyme NADPH oxidase. The over-production of ROS seems ultimately to be responsible for the exaggerated NF-κB activation and iNOS induction triggered by IL-1β under high glucose conditions (Peiró et al., 2016). By using anakinra, not only the pro-inflammatory and pro-oxidant signaling elicited by IL-1β in vascular cells was blunted but also, and importantly, its exacerbation by extracellular high glucose (Peiró et al., 2016).

Despite the promising pre-clinical data available to date (**Figure 1**), the ability of anakinra or other IL-1β blocking drugs to prevent or retard diabetes-associated experimental atherosclerosis remains to be better established and requires additional research with *in vivo* models. The scarce data available on IL-1 blockade and atherosclerosis in patients suffering from T2DM will be presented later in this review.

Accelerated Vascular Aging

Type 2 diabetes, together with obesity, has been shown to accelerate aging processes and, particularly, vascular aging (Minamino and Komuro, 2007; Barton, 2010). Indeed, besides presenting endothelial dysfunction and a pro-inflammatory status, animal models of obesity or diabetes exhibit core hallmarks of vascular aging and cardiovascular pathologies, such as arterial stiffness, calcification, and endothelial cell senescence (Brodsky et al., 2004; Sloboda et al., 2012). In humans, different reports have evidenced that patients suffering from type 2 diabetes display a higher propensity to calcified arteries, especially in the vasculature of lower extremities and in the coronary artery (Chen and Moe, 2003; Zhu et al., 2012), while they also exhibit enhanced arterial stiffness (Laugesen et al., 2013). The presence of senescent

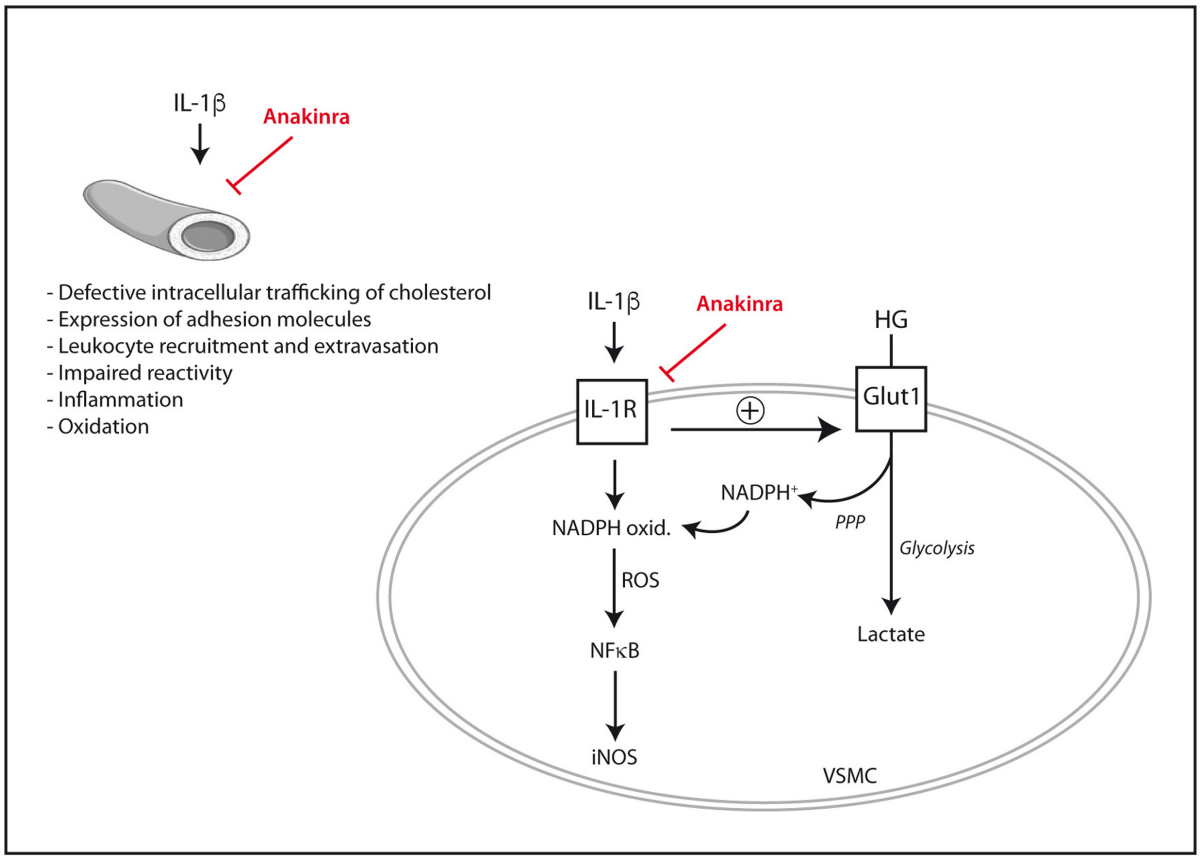

FIGURE 1 | Mechanisms by which IL-1β may directly promote vascular dysfunction and their inhibition by the IL-1Ra recombinant analog anakinra. In vascular smooth muscle cells (VSMC), IL-1β synergizes with extracellular high glucose (HG) to exacerbate pro-inflammatory signaling. iNOS, inducible nitric oxide synthase; Glut1, glucose transporter 1; IL-1R, interleukin-1 receptor; NADPH ox., NADPH oxidase; PPP, pentose phosphate pathway; ROS, reactive oxygen species.

endothelial cells has been demonstrated in atherosclerotic lesions from aortae and coronary arteries of rat models of diabetes (Chen et al., 2002; Minamino et al., 2002). Vascular senescence is associated to a series of morphological and metabolic disturbances that result in dysfunctional cell homeostasis (Erusalimsky, 2009) The senescence-associated secretory phenotype is characterized by enhanced cytokine secretion that in turn drives local inflammation and vascular dysfunction (Erusalimsky, 2009). Overall, the occurrence of vascular premature aging in patients suffering from obesity and/or T2DM favors the development of vascular disease, markedly enhances cardiovascular risk and worsens the life expectancy of these patients. To date, there are few studies available on the impact of IL-1β on accelerated vascular aging. It is known, however, that it can promote both alkaline phosphatase expression and mineralization in cultured vascular smooth muscle cells, as two mechanisms favoring vascular calcification (Lencel et al., 2011). We have also recently observed that IL-1β can induce the accumulation of senescence-associated β-galactosidase in cultured human endothelial cells, an effect that can be dampened by anakinra (Peiró et al., unpublished results).

IL-1β INHIBITION AND DIABETIC CARDIOMYOPATHY

Role of IL-1β in the Heart Failure

Heart failure (HF) is a complex clinical syndrome characterized by impaired cardiac function (left ventricular ejection fraction less than 40%), and enhanced inflammation which is associated with worsening outcomes in these patients. Although infection with microorganisms is not involved in the development of HF in most cases (sterile inflammation), inflammation has been implicated in the pathogenesis of HF. In this regard, IL-1β exerts crucial effects on most cell types involved in cardiac repair and injury, and beneficial and detrimental effects of IL-1β have been reported (Bujak and Frangogiannis, 2009). The pro-inflammatory and pro-fibrotic responses after myocardial injury serve to clear the wound and facilitate wound healing and scar formation, and an excessive inhibition of the inflammatory response can develop a defective scar (Frangogiannis et al., 2005). Also, in myocardial ischemia-reperfusion (I/R) injury, IL1β and IL1α pretreatment reduces ROS generation and a subsequent increase in glucose-6-phosphate dehydrogenase activity, which

provides cytoprotective effects against oxidation (Hedayat et al., 2010). However, excessive upregulation or chronic stimulation of IL1β and IL1α have been linked to deleterious responses.

IL-1β and Cardiac Inflammation

During an ischemic episode, myocardial contractile force diminishes, Ca^{+2} homeostasis is altered, O_2-derived ROS are generated, NO is released, and local production of cytokines, particularly TNF-α and IL-1β is increased. IL-1β was described as an early upregulated cytokine in cardiac inflammation that becomes chronically elevated after impaired myocardial function and LV hypertrophy following AMI (Toldo et al., 2014; Fang et al., 2015). These cytokines enhance the expression of cyclooxygenase-2, and phospholipase A2, as well as vascular adhesion molecules and several chemokines. Interactions between chemokines and cell adhesion molecules activate the innate and adaptive immune system on endothelial cells of leukocytes. Then, an immediate cytokine-mediated neutrophil and mononuclear cells recruitment and extravasation in the infarcted myocardium further damages heart muscle (Herskowitz et al., 1995). In addition, IL-1β signaling, together with Toll-like receptors activation (TLR), stimulate NF-κB to increase the expression of more cytokines, chemokines, and adhesion molecules (**Figure 2**). Remarkably, NF-κB and TLRs signaling upregulate also pro-IL-1β (Fang et al., 2015). Finally, IL-1β is able to activate spleen monocytopoiesis following AMI to stimulate further monocyte production. Thus, excessive IL-1β signaling and inflammation has been linked to increased incidence of arrhythmia and other AMI-related pathologies (Fang et al., 2015).

IL-1β, Cardiac Hypertrophy, and Contractile Dysfunction

After a cardiac insult, IL-1β is also involved in the myocardial hypertrophic growth response by direct gene expression to compensate for environmental stresses (**Figure 2**). The expression of IL-1β is increased in pressure and volume overload-induced cardiac hypertrophy (Dai et al., 2004; Higashikuni et al., 2013), and IL-1β induced growth of isolated cardiomyocytes (McTiernan et al., 1997). Transgenic mice with constitutively cardiac-specific overexpression of IL-1β produced myocyte hypertrophy and HF (Palmer et al., 1995). This hypertrophic response was NO-independent and lead to activation of the fetal gene program by upregulation of atrial natriuretic factor and β-myosin heavy chain. In addition, IL-1β, in synergism with TNF-α, have demonstrated an exacerbation of heart contractile dysfunction. IL-1β decreased myocardial contractility by overexpressing iNOS in cardiomyocytes. Then, NO was released to promote a cardiodepressant effect by directly blocking the mitochondrial activity and reducing energy depletion (Stein et al., 1996). This effect of IL-1β was enforced by amelioration of calcium regulatory genes such as sarcoplasmic reticulum Ca^{2+}-ATPase, phospholamban, and voltage dependent calcium channel, and subsequent decrease of basal and stimulated contractility (Oddis and Finkel, 1995; Tatsumi et al., 2000).

IL-1β, Cardiac Necrosis/Apoptosis, and Autophagy

The uncontrolled inflammation in the injured heart, as in AMI, induces cellular apoptosis and progressive cardiac dysfunction. The progression from cardiac injury to symptomatic HF is mainly due to a loss of functional cardiac myocytes through necrosis, autophagy/mitophagy, intrinsic and extrinsic apoptosis (Gordon et al., 2011). Autophagy and mitophagy have been recently characterized as essential cellular processes in the heart, but whether these functions as pro-death or pro-survival programs during disease conditions is still not completely understood. The link between autophagy, mitochondria, and inflammation in the heart has been established (Oka et al., 2012). In particular, in diabetes, mitochondrial DNA, a by-product of insufficient mitophagy, induced sterile inflammation and subsequent cardiac dysfunction through the TLR9 pathway, and increasing IL-1β, IL-6, and infiltration of $CD68^{+}$ macrophages.

Nevertheless, myocyte extrinsic apoptosis is the most significant mechanism of cell loss and the end point of pathological remodeling induced by cardiac injury, enhanced autonomic activity, and cytokine secretion (**Figure 2**). In AMI, the acute releasing of IL-1β and TNFα can regulate the survival or apoptosis of myocytes in infarcted zone. They also produce a negative inotropic effect as an adaptive response to delimit the injury and decrease myocardial energy demand (Schulz et al., 1995). *In vitro*, in cultured cardiac myocytes, IL-1β induced programmed cell death through a cGMP-independent NOS induction, and by generation of ROS, activation of caspases, and alteration of the cellular Bak/Bcl-xL ratio (Abbate et al., 2008). On the other hand, the presence of necrosis has been demonstrated in most cardiac injuries (Shinde and Frangogiannis, 2014). Necrotic cells release danger signals, activating innate immune pathways and triggering an intense inflammatory response. Stimulation of TLR signaling and complement activation prompted NF-κB and related upregulation of proinflammatory cytokines (i.e., IL-1β, IL-1α, and TNFα) and chemokines (i.e., MCP-1 and CCL2). Interestingly, some NF-κB target genes such as TNFα, and p53 directly induced also intrinsic apoptosis (Shakhov et al., 1990).

IL-1 and Cardiac Fibrosis

The adult mammalian myocardium contains abundant fibroblasts entrapped within the interstitial and perivascular extracellular matrix (ECM). After a massive sudden loss of cardiomyocytes following AMI, the limited regenerative capacity of the myocardium overcomes, resulting in the formation of a collagen-based scar (Prabhu and Frangogiannis, 2016). In the early stages of infarct healing, fibroblasts become pro-inflammatory cells, activating the inflammasome and producing cytokines, chemokines and proteases. NLRP3 inflammasome is predominantly upregulated in the cardiac fibroblasts of the ischemic myocardium in animal models with AMI (Fang et al., 2015). Then, pro-inflammatory cytokines, such as Interleukin-1, delay myofibroblast transformation, until the wound is cleared by infiltrated leukocytes from dead cells and matrix debris. Suppression of the inflammatory

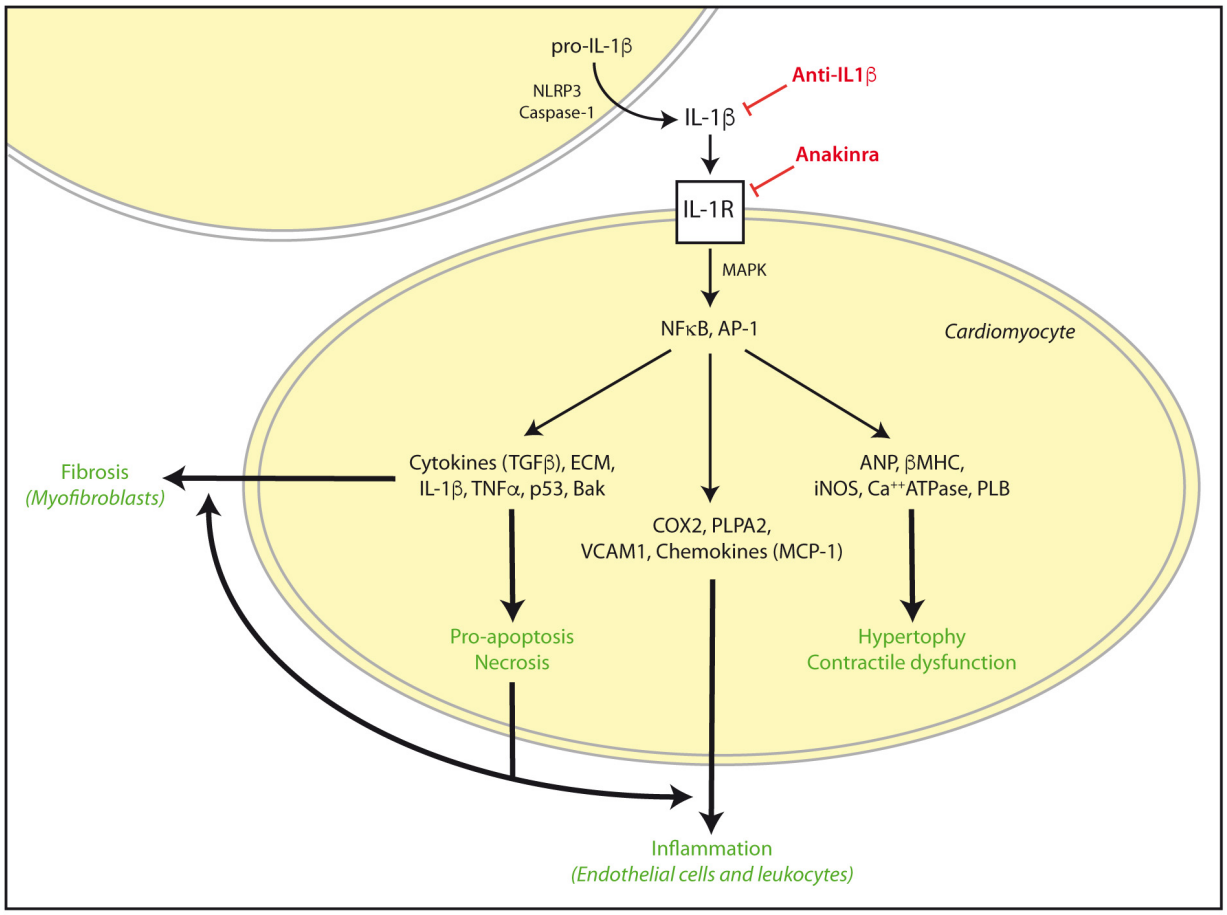

FIGURE 2 | IL-1β activation in cardiac damage. After cardiac injury such as AMI or I/R, exacerbated IL-1β signaling can induce NFκB and AP-1 activation and subsequent increase of pro-hypertrophic, contractile, inflammatory, apoptosis/necrosis, and fibrotic genes for autocrine and paracrine responses. These effects are reinforced in the diabetic myocardium, and can be stimulated by TNFα and TLRs signaling. However, some drugs may specifically target IL-1β actions by NLRP3, IL-1β, and IL-1R blockade. ECM, extra cellular matrix proteins.

response triggers activation of reparative cells. Thus, fibroblasts migrate, proliferate, undergo myofibroblast transdifferentiation, and deposit large amounts of ECM proteins to maintain the structural integrity of the infarcted myocardium. In particular, IL-1 signaling regulates reparative processes by modulating gene expression of growth factors and ECM proteins (i.e., procollagen α1-IV, α2-IV, and fibronectin) in fibroblasts and smooth muscle cells, and by altering the Matrix Metalloproteinase (MMP)/Tissue Inhibitor of Metalloproteinases (TIMP) balance (Prabhu and Frangogiannis, 2016) (**Figure 2**). IL-1 increased MMP-1, MMP-3, MMP-7, MMP-9, and MMP-13, and TIMP-1 and TIMP-2, via AP-1 and NF-κB activation (Siwik and Colucci, 2004). Again, the myocardial matrix also elicits TLRs and IL-1 signaling, which in turn stimulates NF-κB. The renin–angiotensin–aldosterone system and members of the TGF-β family play also a pivotal role in activation of myofibroblasts (Siwik et al., 2000). Formation of a mature cross-linked scar is associated with clearance of fibroblasts following inhibitory signals to restrain the fibrotic response (Shinde and Frangogiannis, 2014). However, excessive fibrosis has been linked to increased incidence of arrhythmia and other AMI-related pathologies (Xie et al., 2004). In this sense, significant upregulation of IL-1 can extend to non-infarcted areas and promote a second phase of elevated levels of cytokines, leading to interstitial fibrosis in the non-infarcted myocardium, and enhancing cardiac dysfunction (Shinde and Frangogiannis, 2014).

Blockade of IL-1β in Experimental HF

Several reports have demonstrated the benefit of blocking IL-1β in experimental models of HF. Early inhibition of IL-1β signaling is more likely to inhibit the inflammatory cascade, whereas late inhibition may predominantly abrogate the direct actions of IL-1β on fibroblasts (Bujak et al., 2008). In rodents, by using genetically engineered antibody to IL-1β, cardiac enlargement and dysfunction following AMI were reduced without affecting the infarct size (Abbate et al., 2010). By administration of a recombinant IL-1Ra in T1DM-induced diabetic cardiomyopathy, the IRAK2/CHOP-dependent apoptosis was attenuated, without affecting fasting blood glucose concentration (Liu et al., 2015). Interestingly, in T1DM-induced diabetic cardiomyopathy,

administration of an anti-TNF-α monoclonal antibody lessen cardiac TNF-α and IL-1β expression in correlation with cardiac collagen-I and -III content, and improvement of left-ventricle function (Westermann et al., 2007).

Other strategies to control IL-1β activity in the context of cardiomyopathy address the kallikrein–kinin system and the NLRP3/caspase-1/TLR pathway. Transgenic activation of kallikrein-1 ameliorated intramyocardial inflammation through reduction of adhesion molecules, IL-1β and TNF-α, and leukocyte infiltration, as well as endothelium dysfunction and oxidative stress in T1DM-induced diabetic cardiomyopathy (Tschöpe et al., 2005). Moreover, NLRP3-deficient mice subjected to I/R exhibited a marked improvement of cardiac function and reduction of hypoxic damage (Sandanger et al., 2013). NLRP3 gene silencing ameliorated pyroptosis and ROS release under high glucose in cardiomyocytes. ROS inhibition decreased also NF-κB and mature IL-1β (Luo et al., 2014a). Finally, NLRP3 and high-mobility group box-1 (HMGB1) knockdowns reduced cardiac hypertrophy and fibrosis, and restored cardiac function (Fuentes-Antras et al., 2014). Ablation of TLR4 successfully reverted architectural aberrations and restored cardiac dysfunction in T1DM mice. Thus, the role of HMGB1 as TLR4 ligand and upstream inducer of NF-κB and NLRP3 may shape a key pathogenic axis in diabetic cardiomyopathy, suggesting their potential as novel anti-inflammatory approaches. In this context, other ligands for TLR (TLR2 and TLR4) such as biglycan could also play a key role in amplifying fibrotic responses after cardiac injury (Beetz et al., 2016).

In this regard, statins as rosuvastatin have also exhibited beneficial proprieties against diabetic cardiomyopathy through inhibition of NLRP3 inflammasome and mature IL-1β, via suppression of the MAPKs activation (Luo et al., 2014b). Similar effects for pravastatin were observed also in obese T2DM rats with AMI (Li et al., 2006). Moreover, administration of the PDE-5 inhibitor tadalafil reduced circulating IL-1β and TNF-α, and associated chemokines RANTES, MIP-1β and MCP-1, after I/R induction in T2DM mice. In parallel, tadalafil upregulated the anti-inflammatory cytokine IL-10 and improved fasting glucose, whereas decreased infarct size (Varma et al., 2012). In addition, a glucocorticoid (methylprednisolone) treatment reduced expression of TLR4/NF-κB signaling and IL-1β, IL-6, TNF-α, and ICAM-1 on T1DM-induced diabetic cardiomyopathy with I/R injury (Hu et al., 2011). More importantly, the antiapoptotic property of anakinra has been demonstrated in models of I/R injury and AMI. This action was due to a decreased expression of pro-apoptotic mediators Bax, Bak, and caspase-1 and -3, which promote a reduction of infarct size and favorable ventricular remodeling (Abbate et al., 2008). Also, in T1DM mice, released IL-1β from cardiac macrophages stimulated with TLR2 and NLRP3 agonists, induced a decrease in potassium current and an increase in calcium sparks in cardiomyocytes, and subsequent cardiac arrhythmia. Interestingly, inhibition of IL-1β signaling by either anakinra or NLRP3 inhibitor (MCC-950) reduced these effects (Monnerat et al., 2016).

CLINICAL TRIALS ASSESSING IL-1β INHIBITION AND CARDIOVASCULAR COMPLICATIONS IN PATIENTS WITH DM

Although pharmacological inhibitors of the IL-1 pathway were first developed to control classical autoinflammatory diseases such as rheumatoid arthritis, the increasing evidence about a key role for this pathway in the pathophysiology of T1DM and T2DM (see reviews by Dinarello, 2014; Ballak et al., 2015; Herder et al., 2015; Pollack et al., 2016) provided a rationale for assessing their therapeutic value in the context of DM. Moreover, a growing bulk of pre-clinical data highlight the potential relevance of IL-1β in the development of atherosclerosis and cardiovascular dysfunction. Despite this, the number of studies conducted in humans to assess the benefit of IL-1β inhibition on cardiovascular outcomes in the context of DM still remains very limited today.

Interestingly, the success of the first clinical trial reported using a IL blocker, i.e., anakinra, in T2DM (Larsen et al., 2007; ClinicalTrials.gov identifier: NCT00303394) reinforced the proposal that the pharmacological control of the imbalance of the IL-1:IL-1Ra ratio could be a relevant approach for the treatment of this metabolic autoinflammatory disease. In such study, 70 adult patients were treated with a 100 mg daily dose during 13 weeks. An improvement of several markers of glucose metabolism, such as reduction in HbA1c and an increased C-peptide secretion were obtained, together with the reduction of systemic inflammatory markers, such as CRP and IL-6 levels (Larsen et al., 2007). Moreover, a 39-week follow-up of the same study after treatment withdrawal, showed not only a maintained reduction in inflammatory markers but also a persistent improvement in β-cell function, as expressed by the lower proinsulin/insulin ratio in the formerly anakinra-treated patients as compared with those in the placebo group (Larsen et al., 2009). However, this study did not assess any cardiovascular outcome in the patients enrolled.

Cardiovascular Outcomes

Although the results obtained with anakinra were encouraging, still there are characteristics that make the drug unsuitable for long-term treatment of T2DM, such as a short half-life (anakinra should be administered on a daily basis to maintain adequate suppression of IL-1β) or injection-site reactions, in addition to being an expensive option. It is also worth to note that an anti-IL-1 strategy that saves IL-1α activity could offer safety benefits. In fact, genome-wide association studies recently suggested that long-term dual IL-1α/β inhibition could increase cardiovascular risk mediated, at least in part, through an increase in proatherogenic lipid concentrations (The Interleukin 1 Genetics Consortium, 2015). Therefore, reducing specifically IL-1β activity with longer-lasting agents would provide a therapeutic improvement in terms of sustained inhibition of IL-1β. In this context, the monoclonal antibody canakinumab achieves IL-1β inhibition, while preserving IL-1α activity. Also, the significantly longer half-life of canakinumab (Chakraborty et al., 2012) makes the drug more suitable for assessing outcomes

that may require longer treatment times, such as cardiovascular ones.

In fact, canakinumab is currently being tested in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), which is the largest trial in progress for any anti-cytokine drug (ClinicalTrials.gov identifier: NCT01327846). The objective is to test whether canakinumab will reduce cardiovascular events in high-risk patients with T2DM who have high CRP levels (hsCRP > 2 mg/L) despite receiving optimal statin therapy. The trial is examining 17,200 individuals using three doses of the antibody in a placebo-controlled, randomized design in 146 centers. Although the primary outcome is based on the hypothesis that blocking IL-1β activity in these patients may also reduce the incidence in myocardial infarction and stroke, secondary end points include prevention and improvement of diabetes. CANTOS is an event driven trial due to be completed in 2017, when approximately 1400 cases of myocardial infarction, stroke, or cardiovascular death will have accrued (Ridker et al., 2011).

A preliminary proof-of-concept study has been already executed within the CANTOS trial; thus, 556 well-controlled type 2 diabetic subjects (baseline HbA1c of 7.4%) with high cardiovascular risk have been randomly allocated to receive monthly subcutaneous canakinumab at doses of 5, 15, 50, or 150 mg or placebo and followed over 4 months. Together with an important dose-dependent reduction in inflammatory markers (CRP and IL-6), there was a modest non-significant improvement in glucose control parameters (HbA1c, glycemia, and insulinemia), while lipoprotein levels were unchanged (Ridker et al., 2012). In terms of cardiovascular outcomes, there is great expectation for the results of the trial. If positive, CANTOS would reinforce the inflammatory hypothesis of atherothrombosis and support a cytokine-based therapy for the secondary prevention of cardiovascular disease in T2DM.

Apart from the expected results from CANTOS, there are very few studies available on vascular outcomes in patients with DM and most of them show no clear cardiovascular improvement upon IL-1β blockade. A smaller trial enrolled 189 patients with atherosclerosis in the carotid artery and/or the aorta and either T2DM or impaired glucose tolerance (ClinicalTrials.gov identifier: NCT00995930). The subcutaneous administration of canakinumab (150 mg) monthly for 12 months did not significantly affect vascular structure in terms of carotid intima thickness, or vascular function in terms of aortic distensibility measured by pulsed wave velocity (Choudhury et al., 2016). Canakinumab showed an anti-inflammatory effect by reducing the circulating levels of IL-6 and hs-CRP, but had no effect on metabolic indicators, like fasting glucose, HbA1c, or insulin sensitivity (Choudhury et al., 2016). Although no cardiovascular benefit was found, the results were not considered conclusive due to some limitations, including the short duration of the study to assess structural changes. Also, the lack of stratification of the patients enrolled based on their initial inflammatory status did not permit to identify those patients that would potentially benefit more from IL-1β inhibition. Finally, the dose administrated was sufficient to lower inflammatory markers but perhaps was not high enough to generate a maximal effect on atherosclerotic lesions.

Another prospective non-controlled pilot study was performed with only six patients with proliferative diabetic retinopathy, secondary to T1DM or T2DM, and receiving subcutaneous canakinumab (150 mg) every 8 weeks (ClinicalTrials.gov identifier: NCT01589029). No regression of retinal neovascularization was reported after 24 weeks of follow-up, although a positive effect on macular edema was observed (Stahel et al., 2016). Again, the dose of canakinumab may have been insufficient, with higher doses of 300 mg every 4 weeks being currently approved for uses such as systemic juvenile idiopathic arthritis.

A possible limitation for these studies, in addition to sample size or dosage, can be that the patients enrolled already exhibited advanced stage cardiovascular disease. Thus, parallel studies performed in diabetic patients without evidence of advanced vascular lesions would help shedding light on the capacity of canakinumab or other IL-1β blocking drugs to prevent or retard the onset of atherosclerosis or other vascular alterations in the context of DM.

Safety

Canakinumab was safe and well-tolerated in the above-mentioned cardiovascular trials, with no significant differences in adverse effects between treated and control groups (Choudhury et al., 2016; Stahel et al., 2016). The upcoming results of CANTOS will help shedding more light on the tolerability of long-term treatments with canakinumab.

In the context of T2DM, other studies not specifically designed to assess cardiovascular outcomes, have already provided information on the tolerability of canakinumab. A recent study pooled the data from trials that used different doses of the drug (Howard et al., 2014). These trials enrolled a total of 1026 patients with different routes of administration, treatment regimes and follow-up time (ClinicalTrials.gov identifiers: NCT00900146 and NCT00605475; Ridker et al., 2012; Rissanen et al., 2012; Hensen et al., 2013; Noe et al., 2014). The global analysis of the three trials demonstrated that canakinumab was safe and well tolerated over a treatment period up to 1.4 years at the four pooled doses evaluated, which was in agreement with safety findings reported in the individual studies (Howard et al., 2014). No significant differences were evidenced in terms of adverse effects, discontinuations or deaths between treatment and placebo (Howard et al., 2014). Overall, canakinumab seems safe and well tolerated by patients with T2DM. However, additional trials with longer follow-up times are still needed to assess whether the drug will be suitable for longer-term treatment of a chronic conditions such as T2DM.

CONCLUDING REMARKS

The growing body of evidence pointing at IL-1β as a key player in the development of DM and its cardiovascular complications provides nowadays a solid rationale for IL-1 blockade as a potential pharmacological approach to treat the disease.

While IL-1 inhibition seems to be rather promising in controlling the pro-inflammatory status and even in ameliorating glucose homeostasis in the context of DM, evidence is lacking on its potential benefits on the cardiovascular complications of the disease.

Most of the positive results obtained to date in terms of cardiovascular improvement arise from pre-clinical data mostly performed through *ex vivo* or *in vitro* approaches. Clearly, further experimental studies using *in vivo* experimental models are required to better understand the pharmacological effects and mechanisms of actions of IL-1β blockers on the cardiovascular system. This will help identifying which conditions may benefit more from IL-1β blockade and predicting potential adverse effects.

At present, the knowledge derived from studies in humans remains very limited. Despite promising results, additional studies need to be performed to better assess the tolerability, and, importantly, the long-term safety of the drugs that may achieve long-lasting IL-1β blockade. In terms of clinical efficacy, more trials are needed with an accurate patient selection based on the stage and complications of the disease. Studies such as the CANTOS trial are soon expected to shed light on the impact of long-lasting IL-1β inhibitors on the cardiovascular complications of DM. This and forthcoming studies, that may include novel pharmacological approaches such as therapeutic vaccination, will allow answering whether or not IL-1β is indeed a valuable therapeutic target to reduce the burden of cardiovascular diabetic complications.

AUTHOR CONTRIBUTIONS

CP, OL, RC, and CS-F designed, wrote, reviewed, and approved the manuscript.

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A Snapshot on the On-Label and Off-Label Use of the Interleukin-1 Inhibitors in Italy among Rheumatologists and Pediatric Rheumatologists: A Nationwide Multi-Center Retrospective Observational Study

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Background: Interleukin (IL)-1 inhibitors have been suggested as possible therapeutic options in a large number of old and new clinical entities characterized by an IL-1 driven pathogenesis.

Objectives: To perform a nationwide snapshot of the on-label and off-label use of anakinra (ANA) and canakinumab (CAN) for different conditions both in children and adults.

Methods: We retrospectively collected demographic, clinical, and therapeutic data from both adult and pediatric patients treated with IL-1 inhibitors from January 2008 to July 2016.

Results: Five hundred and twenty-six treatment courses given to 475 patients (195 males, 280 females; 111 children and 364 adults) were evaluated. ANA was administered in 421 (80.04%) courses, CAN in 105 (19.96%). Sixty-two (32.1%) patients had been treated with both agents. IL-1 inhibitors were employed in 38 different indications (37 with ANA, 16 with CAN). Off-label use was more frequent for ANA than CAN ($p < 0.0001$). ANA was employed as first-line biologic approach in 323 (76.7%) cases, while CAN in 37 cases (35.2%). IL-1 inhibitors were associated with corticosteroids in 285 (54.18%) courses and disease modifying anti-rheumatic drugs (DMARDs) in 156 (29.65%). ANA dosage ranged from 30 to 200 mg/day (or 1.0–2.0 mg/kg/day) among adults and 2–4 mg/kg/day among children; regarding CAN, the most frequently used posologies were 150 mg every 8 weeks, 150 mg every 4 weeks and 150 mg every 6 weeks. The frequency of failure was higher among patients treated with ANA at a dosage of 100 mg/day than those treated with 2 mg/kg/day ($p = 0.03$). Seventy-six patients (14.4%) reported an adverse event (AE) and 10 (1.9%) a severe AE. AEs occurred more frequently after the age of 65 compared to both children and patients aged between 16 and 65 ($p = 0.003$ and $p = 0.03$, respectively).

Conclusions: IL-1 inhibitors are mostly used off-label, especially ANA, during adulthood. The high frequency of good clinical responses suggests that IL-1 inhibitors are used with awareness of pathogenetic mechanisms; adult healthcare physicians generally employ standard dosages, while pediatricians are more prone in using a weight-based posology. Dose adjustments and switching between different agents showed to be effective treatment strategies. Our data confirm the good safety profile of IL-1 inhibitors.

Keywords: autoinflammatory disorders, treatment, interleukin (IL)-1, anakinra, canakinumab

INTRODUCTION

Inhibition of interleukin (IL)-1 was initially adopted for the treatment of rheumatoid arthritis (RA). To date, the receptor antagonist anakinra (ANA), the selective inhibitor of IL-1β canakinumab (CAN), the soluble decoy IL-1-receptor rilonacept, and the human-engineered monoclonal anti-IL-1β gevokizumab represent the four IL-1 inhibitors (IL-1-INH) available (Finch and Sleeman, 2015). However, only the first two agents, ANA and CAN, have been approved for clinical use in Europe.

Since its introduction in 2001 for RA, a number of other inflammatory pathologies have found to benefit from IL-1-INH, in particular monogenic autoinflammatory diseases (AIDs), including familial Mediterranean fever (FMF; Ben-Zvi and Livneh, 2014; Gül et al., 2015), tumor necrosis factor receptor-associated periodic syndrome (TRAPS; Brizi et al., 2012; La Torre et al., 2015; Lopalco et al., 2015b), mevalonate kinase deficiency/hyper-IgD syndrome (van der Hilst and Frenkel, 2010), and cryopyrin-associated periodic syndrome (CAPS; Cantarini et al., 2011; Caorsi et al., 2013; Scarpioni et al., 2015). However, a wide range of polygenic and multifactorial autoinflammatory conditions characterized by at least a partial deregulation of IL-1 have recently been described as responsive to IL-1-INH (Cantarini et al., 2012a,b, 2015c; So et al., 2013; Cavalli and Dinarello, 2015; Lopalco et al., 2015a). Among others, adult-onset Still's disease (AOSD; Naumann et al., 2010; Nordström et al., 2012), systemic juvenile idiopathic arthritis (SOJA; Hedrich et al., 2012; Ruperto et al., 2012a,b), Behçet's disease (BD; Cantarini et al., 2012a,b, 2015b; Vitale et al., 2014; Emmi et al., 2016) and crystal-induced arthritis (So et al., 2007; Schlesinger et al., 2012) are prime examples of multifactorial AIDs showing a good response to IL-1-INH. As a whole, an increasing number of disorders have proven to be characterized by molecular modifications resembling those found in monogenic AIDs. Therefore, the good clinical response shown by monogenic AIDs to IL-1-INH induced clinicians to try

the path of IL-1 inhibition in an increasing number of disorders previously labeled as possible polygenic AIDs on the basis of laboratory findings.

Nowadays, in Italy ANA is indicated for the treatment of RA, in association with methotrexate, and CAPS, while CAN is indicated for CAPS, SOJA, and gout. Consequently, the use of IL-1-INH is often done with off-label modality, therefore without the possibility of real and effective monitoring strategies on long-term effectiveness and safety. For this reason, we conducted a multicenter observational study to perform a nationwide evaluation about the use of IL-1-INH for different conditions with both on-label and off-label modalities in order to provide a description of IL-1-INH use in real life and deduce practical implications as reference points for physicians requiring to use IL-1-targeted inhibition.

PATIENTS AND METHODS

We retrospectively collected demographic, clinical, and therapeutic data from both adult and pediatric patients treated with IL-1-INH from January 2008 to July 2016 in 23 Italian reference Centers for pediatric and adult patients. Collected data included patients' age, gender, disease, disease duration, age at disease onset, response to IL-1-INH, previous and concomitant treatments, dosages employed, and modifications of dosages or frequency of administration, duration of treatment, and causes for discontinuation, including adverse events (AEs) and severe AEs (SAEs).

The primary aims of our study were: (i) to identify the frequency of ANA and CAN prescription as approach in on-label and off-label use; (ii) to describe the percentage of patients needing to switch from ANA to CAN and vice versa focusing the reasons for switching due to AEs, loss of efficacy, primary inefficacy; (iii) to identify the clinical outcome after switching from one to another agent; (iv) to investigate whether dosage adjustments can contribute to the achievement of a secondary response to treatment; (v) to describe the IL-1-INH safety profile and identify any correlation between the age of patients and the occurrence of AEs based to their severity; (vi) to highlight reasons for discontinuation.

The secondary aims were to describe: (i) different dosages employed for ANA and CAN, distinguishing between pediatric and adult subjects; (ii) the number of biologic agents administered before starting IL-1 inhibition; (iii) previous and concomitant use of corticosteroids and disease modifying anti-rheumatic drugs (DMARDs) differentiating by age; (iv) different therapeutic indications differentiating by age.

Describing the frequency of complete response, partial response, and failure to IL-1-INH represented an ancillary end-point.

Response to IL-1-INH was graded as complete, partial, or failing. The evaluation of response was not standardized, however the normalization of inflammatory markers (erythrocyte sedimentation rate, ESR, <15 mm/h; C-reactive protein, CRP, level <0.5 mg/dl) and the disappearance of all previously identified signs and symptoms were considered as criteria for a complete response. Partial response was retained for patients with clinical improvement, but not fulfilling the criteria for complete response. Finally, a treatment was labeled as failing when neither clinical nor laboratory improvements were observed.

For comparisons between adults and children, patients were classified as pediatric when aged <16 years.

The study was approved and reviewed by the local Ethical Committee (AOUS, Azienda Ospedaliera Universitaria Senese) and was conducted according to the declaration of Helsinki.

Statistical Analysis

Descriptive statistics was evaluated for sample size, mean, and standard deviation for quantitative variables. For quantitative data, pair wise comparisons were performed by means of unpaired *t*-test for parametric data and Mann-Whitney *U*-test for non-parametric data after assessing data normality by using Anderson–Darling test. For qualitative data comparisons were performed by means of Chi-square or Fisher's exact test when required. Significance was defined as $p < 0.05$.

RESULTS

We evaluated 526 treatment courses administered to 475 patients (195 males; 280 females) who underwent IL-1-INH between January 2008 and July 2016. The mean ± *SD* age of patients was of 36.36 ± 22.18 years, the mean ± *SD* age at symptom onset and at diagnosis were of 24.47 ± 19.99 and 29.31 ± 20.18 years, respectively. Patients aged <16 years were 111 (23.4%), corresponding to 135 (25.7%) treatment courses, 93 of which with ANA (68.9%) and 42 (31.1%) with CAN. The mean ± *SD* age of pediatric patients was 10.2 ± 3.8 years (range 1.75–16.0 years). Patients aged more than 16 years were 364. The mean ± *SD* age of adults was 44.8 ± 18.7 (range 16.75–89.0 years). **Table 1** shows demographic and clinical data of all patients enrolled, also distinguishing by different therapeutic indications.

Overall, ANA was administered in 421 (80.04%) courses and CAN in 105 (19.96%) courses. Sixty-two (32.1%) patients had been treated with both IL-1-INH. ANA was prescribed on-label in 60 (14.3%) cases, while CAN was prescribed on-label in 46 (43.8%) cases. Off-label prescribing was significantly more frequent for ANA than CAN ($p < 0.0001$). **Figure 1** graphically describes differences in the on-label use of ANA and CAN.

IL-1-INH were associated with corticosteroids in 285 (54.18%) courses (276 patients, 52.5%) and DMARDs in 156 (29.65%) courses (151 patients, 31.8%). Distinguishing by age, IL-1-INH *plus* corticosteroids were administered to 46 (41.4%) pediatric patients, corresponding to 62 (45.2%) pediatric treatment courses (46 with ANA and 16 with CAN), and 214 (51.6%) adult patients, corresponding to 223 (57.03%) treatment courses (198 with ANA and 25 with CAN). Concomitant corticosteroids were significantly more frequently used among adults than children ($p = 0.002$). DMARDs *plus* IL-1-INH were administered to 27 pediatric patients, corresponding to 32 (23.7%) pediatric treatment courses (23 with ANA and 9 with CAN), and 124 (31.7%) adults, corresponding to 124 (23.6%) treatment courses (115 with ANA and 9 with CAN).

TABLE 1 | Demographic and clinical data of all patients enrolled in the study.

	N° patients	*M* (%)	Pediatric patients (%)	Mean age ± *SD* (years)	Age at onset mean ± *SD* (years)	Age at diagnosis mean ± *SD* (years)	Diagnostic delay mean ± *SD* (years)
All	475	195 (41.05%)	111 (23.36%)	36.46 ± 22.13	24.44 ± 20.05	29.36 ± 20.19	4.89 ± 9.60
AOSD	78	27 (34.61%)	0 (0%)	47.33 ± 16.03	39.95 ± 16.15	41.82 ± 15.93	1.87 ± 5.38
SOJA	72	34 (47.22%)	53 (73.61%)	11.98 ± 5.29	6.71 ± 4.62	7.07 ± 4.62	0.21 ± 0.43
BD	46	17 (36.95%)	1 (2.17%)	39.54 ± 13.32	27.41 ± 12.60	31.24 ± 12.41	3.83 ± 6.03
RA	42	9 (21.42%)	0 (0%)	67.55 ± 12.51	44.90 ± 12.05	45.95 ± 11.83	1.23 ± 2.21
FMF	34	11 (32.35%)	4 (11.76%)	41.79 ± 19.77	18.96 ± 15.54	36.07 ± 19.31	17.11 ± 15.40
USAID	32	13 (40.62%)	19 (59.37%)	19.42 ± 15.12	8.17 ± 12.87	15.00 ± 14.86	6.83 ± 9.87
CAPS	30	19 (63.33%)	9 (30%)	33.86 ± 21.44	19.22 ± 20.56	28.38 ± 21.77	8.84 ± 13.20
TRAPS	29	12 (41.37%)	3 (10.34%)	39.18 ± 17.25	20.05 ± 15.82	33.68 ± 17.01	13.56 ± 15.23
IRAP	23	13 (56.52%)	4 (17.39%)	40.17 ± 21.43	34.65 ± 22.25	36.17 ± 21.96	1.52 ± 3.98
CRMO	11	3 (27.27%)	6 (54.54%)	13.91 ± 5.01	9.48 ± 5.03	10.59 ± 5.17	1.11 ± 1.75
OTHERS	78	37 (47.43%)	12 (15.38%)	36.46 ± 22.13	24.44 ± 20.05	29.36 ± 20.19	4.73 ± 9.60

AOSD, Adult Onset Still's Disease; BD, Behçet's Disease; CAPS, Cryopyrin-Associated Periodic Syndrome; CRMO, Chronic Recurrent Multifocal Osteomyelitis; FMF, Familial Mediterranean Fever; IRAP, Idiopathic Recurrent Acute Pericarditis; RA, Rheumatoid Arthritis; SOJA, Systemic Onset Juvenile Idiopathic Arthritis; TRAPS, Tumor Necrosis Factor Receptor-Associated Periodic Syndrome; USAID, Undifferentiated Systemic AutoInflammatory Disease; M, Male; SD, Standard Deviation.

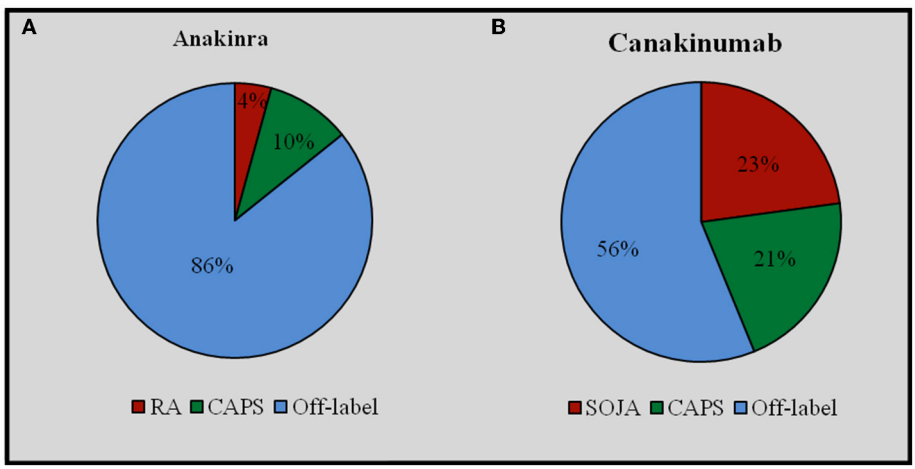


FIGURE 1 | On-label and off-label use of Anakinra (A) and Canakinumab (B). CAPS, Cryopyrin-Associated Periodic Syndrome; RA, rheumatoid arthritis; SOJA, Systemic Onset Juvenile Idiopathic Arthritis.

Concomitant DMARDs were more frequently administered in adults, without reaching statistical significance ($p = 0.06$). **Figure 2** *plus* **Figure 3** and **Table 2** better specify concomitant therapies during IL-1 inhibition.

ANA dosage ranged from 30 mg/day subcutaneously to 200 mg/day (or 1.0–2.0 mg/kg/day) among adults and 2–4 mg/kg/day among pediatric patients; however, 18 out of 28 (64.3%) patients aged from 13 to 16 years were administered the standard dose of 100 mg/day, as more frequently described for adults. One 15-year-old female patient diagnosed with undifferentiated connective tissue disease and suffering from macrophage activation syndrome was treated with ANA at a dosage of 200 mg/day for 8 months. Specifically, the most frequently employed ANA dosages were as follows: 100 mg/day in 322 out of 421 cases (76.5%) as standard posology, 2 mg/kg/day in 64 patients (15.2%), 1–2 mg/kg/day in 18 (4.3%) subjects based on the patient's body weight. Regarding CAN, the most frequently employed posologies were as follows: 150 mg every 8 weeks in 41 out of 105 (39.04%) cases; 150 mg every 4 weeks in 19 patients (18.1%); 150 mg every 6 weeks in 10 patients (9.5%); 4–5 mg/kg every 4 weeks in 11 (10.5%) patients; other dosages (1–4 mg/kg every 4 weeks, 2 mg/kg every 8 weeks, 300 mg every 4 weeks) were employed in 4 (3.8%) subjects. Finally, a patient with CAPS and a second patient with mevalonate kinase deficiency were treated with CAN at a dosage of 300 mg every 8 weeks and a 2-year-old patient diagnosed with CAPS was treated with CAN at the dosage of 4 mg/kg every 8 weeks. **Figure 4** summarizes the frequency of administration of different dosages employed for ANA and CAN.

Regarding response, ANA showed complete effectiveness in 256/421 (60.8%) treatment courses and partial effectiveness in 116 (27.6%) subjects, while ANA led to no response in

49 (11.6%). Distinguishing by age, among pediatric patients ANA was completely effective in 66/93 (71%) cases, partially effective in 23/93 (24.7%) cases, and led to no response in 4 (4.3%) cases. In adults, ANA was completely effective in 190/328 (57.9%) treatment courses and partially effective in 93 (28.4%), while 45 (13.7%) patients proved no response and 2 (0.6%) were lost at follow-up. Regarding CAN, among children a complete response was achieved in 26/42 (61.9%) patients, while a partial response was obtained in 14 (33.3%) subjects; failure was identified in 2 (4.8%) patients. Among adults, CAN led to complete response in 38/63 (60.3%) patients and partial response in 19 (30.2%), while failure was observed in 6 (9.5%) subjects. **Table 3** summarizes information about complete or partial response, and treatment failure in adults and children treated with ANA and CAN, distinguished by the pertinent diagnosis.

The frequency of failure was significantly higher among patients treated at a dosage of 100 mg/day than patients treated with 2 mg/kg/day ($p = 0.03$). This finding was not maintained when differentiating by the different treatment indications. Regarding CAN, no statistical differences were identified in complete response, partial response and failure according to the different dosages administered ($p = 0.43$).

The mean ± *SD* duration of treatment was 24.4 ± 27 months for both IL-1-INH, corresponding to 24.34 ± 27.03 months for ANA and 24.52 ± 27.06 months for CAN, as well as 26.6 ± 28.6 months for pediatric patients and 24.39 ± 27.04 months for adults. No significant differences were identified between adults and children regarding treatment duration ($p = 0.51$).

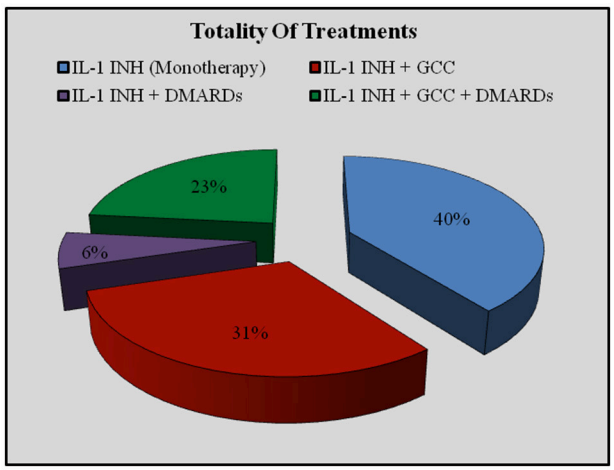
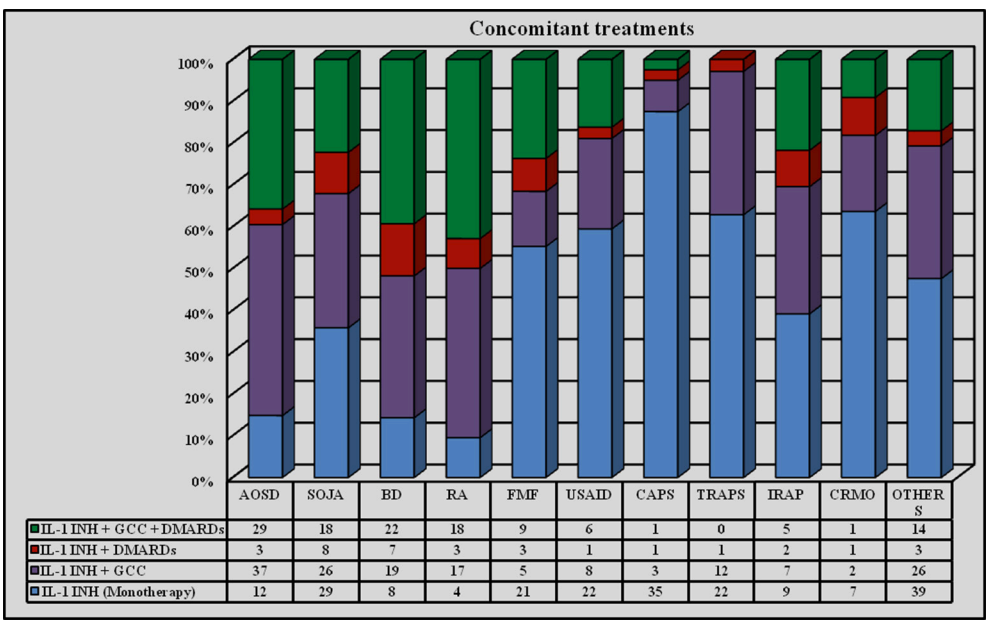


FIGURE 2 | Use of concomitant therapies during IL-1 inhibition on the whole of treatment courses. DMARDs, disease modifying antirheumatic drugs; GCC, glucocorticosteroids; IL-1-INH, IL-1 inhibitors.

Therapeutic Indications

As pointed-up in **Table 4**, IL-1-INH were administered due to 38 different indications, 37 for ANA and 16 for CAN. In pediatric patients IL-1-INH were administered for 16 different indications, 15 for ANA and 10 for CAN; in adults the therapeutic indications were 36, 30 of which for ANA and 15 for CAN. Among patients with complete response the different indications were 25, 23



	AOSD	SOJA	BD	RA	FMF	USAID	CAPS	TRAPS	IRAP	CRMO	OTHERS
IL-1 INH + GCC + DMARDs	29	18	22	18	9	6	1	0	5	1	14
IL-1 INH + DMARDs	3	8	7	3	3	1	1	1	2	1	3
IL-1 INH + GCC	37	26	19	17	5	8	3	12	7	2	26
IL-1 INH (Monotherapy)	12	29	8	4	21	22	35	22	9	7	39

FIGURE 3 | Use of concomitant therapies during IL-1 inhibition distinguishing by different indications. AOSD, Adult Onset Still's Disease; BD, Behçet's Disease; CAPS, Cryopyrin-Associated Periodic Syndrome; CRMO, Chronic Recurrent Multifocal Osteomyelitis; FMF, Familial Mediterranean Fever; IRAP, Idiopathic Recurrent Acute Pericarditis; RA, Rheumatoid Arthritis; SOJA, Systemic Onset Juvenile Idiopathic Arthritis; TRAPS, Tumor Necrosis Factor Receptor-Associated Periodic Syndrome; USAID, Undifferentiated Systemic AutoInflammatory Disease.

TABLE 2 | Previous and concomitant treatments administered to all patients.

	Previous treatments			Concomitant treatments	
	Corticosteroids (%)	DMARDs (%)	Biologics (%)	Corticosteroids (%)	DMARDs (%)
ALL	427/526 (81.17%)	310/526 (58.93%)	165/526 (31.36%)	285/526 (54.18%)	156/526 (29.65%)
AOSD	77/81 (95.06%)	60/81 (74.07%)	17/81 (20.98%)	66/81 (81.48%)	32/81 (39.50%)
SOJA	58/81 (71.60%)	38/81 (46.91%)	22/81 (27.16%)	44/81 (54.32%)	26/81 (32.09%)
BD	44/56 (78.57%)	43/56 (76.78%)	33/56 (58.92%)	41/56 (73.21%)	29/56 (51.78%)
RA	42/42 (100%)	42/42 (100%)	22/42 (52.38%)	35/42 (83.33%)	21/42 (50%)
FMF	30/38 (78.37%)	23/38 (59.45%)	13/38 (35.13%)	14/38 (36.84%)	12/38 (31.57%)
USAID	27/37 (72.97%)	23/37 (62.16%)	10/37 (27.02%)	14/37 (37.83%)	7/37 (18.91%)
CAPS	26/40 (65%)	16/40 (72.97%)	14/40 (35%)	4/40 (10%)	2/40 (5%)
TRAPS	27/35 (77.14%)	3/35 (8.57%)	9/35 (25.71%)	12/35 (34.28%)	1/35 (2.85%)
IRAP	21/23 (91.30%)	11/23 (47.82%)	0/23 (0.00%)	12/23 (52.17%)	7/23 (30.42%)
CRMO	7/11 (63.63%)	3/11 (27.27%)	1/11 (9.09%)	3/11 (27.27%)	2/11 (18.18%)
OTHERS	69/82 (81.36%)	48/82 (58.93%)	24/82 (31.36%)	40/82 (48.78%)	17/82 (20.73%)

AOSD, Adult Onset Still's Disease; BD, Behçet's Disease; CAPS, Cryopyrin-Associated Periodic Syndrome; CRMO, Chronic Recurrent Multifocal Osteomyelitis; FMF, Familial Mediterranean Fever; IRAP, Idiopathic Recurrent Acute Pericarditis; RA, Rheumatoid Arthritis; SOJA, Systemic Onset Juvenile Idiopathic Arthritis; TRAPS, Tumor Necrosis Factor Receptor-Associated Periodic Syndrome; USAID, Undifferentiated Systemic AutoInflammatory Disease; DMARDs, Disease Modifying Antirheumatic Drugs.

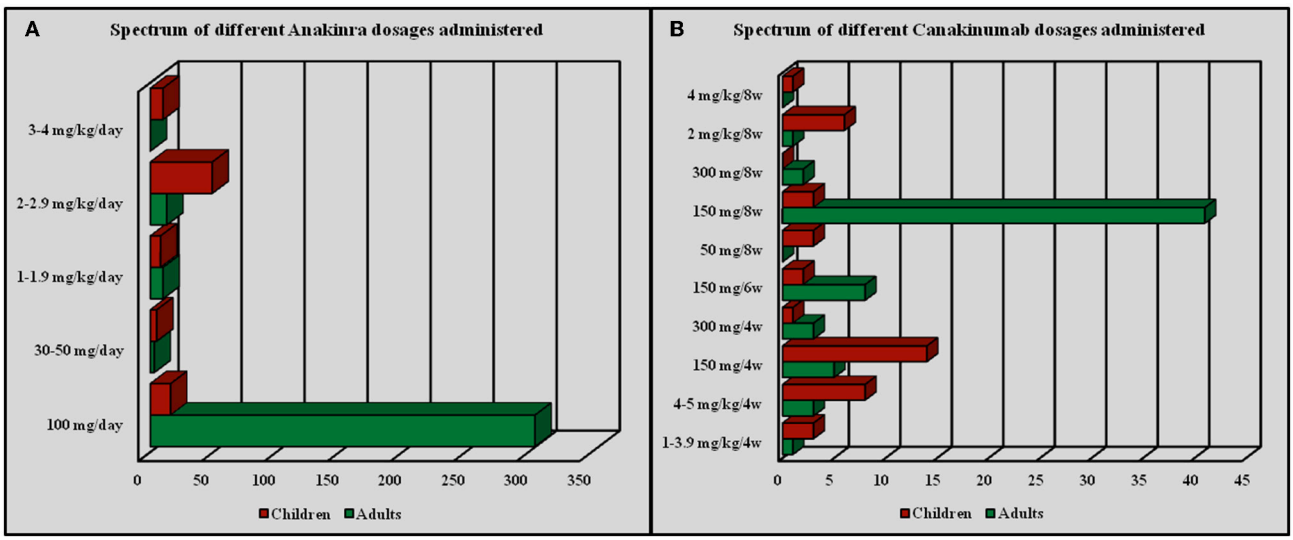


FIGURE 4 | Frequency of administration for different dosages employed with Anakinra (A) and Canakinumab (B).

of which for ANA and 12 for CAN. For patients with partial response the indications were 25, 24 of which for ANA and 12 for CAN. Among patients with failing response the number of indications was 22, 21 of which for ANA and 6 for CAN. The number of indications for IL-1-INH was significantly higher among adults than in pediatric subjects ($p < 0.0001$), but there were no significant differences in the number of indications when ANA and CAN were analyzed separately ($p = 0.41$ and $p = 0.23$, respectively). There were no statistical differences in the number of indications for patients with complete, partial, and failing response neither on the total number of IL-1-INH ($p = 0.71$), nor for ANA ($p = 0.80$) and CAN ($p = 0.25$). When ANA represented the first anti-IL-1 approach, the number of indications stood at 37, while for CAN as the first anti-IL-1 agent the number of indications amounted to 13. Consequently, the number of indications was significantly higher for patients undergoing ANA as first IL-1-INH ($p < 0.0001$); **Figure 5** represents the first-line employment of ANA and CAN for the different indications.

Previous Treatments

ANA was employed as first line biologic approach in 323 (76.7%) cases, while CAN was employed as first biologic in 37 cases (35.2%). Consequently, the frequency of ANA administration as first line biologic approach was significantly higher compared to CAN ($p < 0.0001$). ANA and CAN were employed as second biologic line in 52 (12.4%) and 42 (40%) cases, respectively; third

TABLE 3 | Response to IL-1 inhibition among adult and child patients.

	Anakinra						Canakinumab					
	Children (93)			Adults (328)			Children (42)			Adults (63)		
	CR	PR	F	CR	PR	F	CR	PR	F	CR	PR	F
ToT	66/93 (70.96%)	23/93 (24.74%)	4/93 (4.3%)	190/328 (57.92%)	93/328 (28.35%)	45/328 (13.71%)	26/42 (61.90%)	14/42 (33.33%)	2/42 (4.76%)	38/63 (60.31%)	19/63 (30.16%)	6/62 (9.53%)
AOSD	0/0 (0%)	0/0 (0%)	0/0 (0%)	61/78 (78.20%)	10/78 (12.82%)	7/78 (8.97%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	2/3 (66.66%)	1/3 (33.33%)	0/3 (0%)
SOJA	38/44 (86.36%)	4/44 (9.09%)	2/44 (4.54%)	12/13 (92.3%)	1/13 (7.4%)	0/13 (0%)	12/20 (60%)	7/20 (35%)	1/20 (5%)	3/4 (75%)	1/4 (25%)	0/4 (0%)
BD	0/1 (0%)	1/1 (100%)	0/1 (0%)	15/40 (37.5%)	19/40 (47.5%)	6/40 (15%)	0/1 (0%)	1/1 (100%)	0/1 (0%)	7/14 (50%)	6/14 (42.85%)	1/14 (7.14%)
RA	0/0 (0%)	0/0 (0%)	0/0 (0%)	12/42 (28.57%)	24/42 (57.14%)	6/42 (14.28%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
FMF	1/3 (33.33%)	2/3 (66.66%)	0/3 (0%)	13/29 (44.82%)	9/29 (31.03%)	7/29 (24.13%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	3/4 (75%)	1/4 (25%)	0/4 (0%)
USAID	13/19 (68.42%)	6/19 (31.57%)	0/19 (0%)	6/12 (50%)	3/12 (25%)	3/12 (25%)	3/4 (75%)	1/4 (25%)	0/4 (0%)	1/2 (50%)	1/2 (50%)	0/2 (0%)
TRAPS	1/1 (100%)	0/1 (0%)	0/1 (0%)	14/20 (70%)	5/20 (25%)	1/20 (5%)	3/3 (100%)	0/3 (0%)	0/3 (0%)	8/11 (72.72%)	3/11 (27.27%)	0/11 (0%)
CAPS	5/6 (83.33%)	1/6 (16.66%)	0/6 (0%)	8/12 (66.66%)	4/12 (33.33%)	0/12 (0%)	5/7 (71.43%)	2/7 (28.57%)	0/7 (0%)	11/15 (73.33%)	1/15 (6.66%)	3/15 (20%)
IRAP	3/4 (75%)	1/4 (25%)	0/4 (0%)	15/19 (78.94%)	3/19 (15.78%)	1/19 (5.26%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
CRMO	1/6 (16.66)	3/6 (50%)	2/6 (33.33%)	3/5 (60%)	1/5 (20%)	1/5 (20%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
OTHERS	4/9 (44.44%)	5/9 (55.55%)	0/9 (0%)	31/58 (53.44%)	14/58 (24.13%)	13/58 (22.41%)	3/5 (60%)	2/5 (40%)	0/5 (0%)	3/10 (30%)	5/10 (50%)	2/10 (20%)

AOSD, Adult Onset Still's Disease; BD, Behçet's Disease; CAPS, Cryopyrin-Associated Periodic Syndrome; CRMO, Chronic Recurrent Multifocal Osteomyelitis; FMF, Familial Mediterranean Fever; IRAP, Idiopathic Recurrent Acute Pericarditis; RA, Rheumatoid Arthritis; SOJA, Systemic Onset Juvenile Idiopathic Arthritis; ToT, totality of treatments; TRAPS, Tumor Necrosis Factor Receptor-Associated Periodic Syndrome; USAID, Undifferentiated Systemic Autoinflammatory Disease. CR, complete response; PR, partial response; F, failure.

TABLE 4 | List of indications for which IL-1 inhibitors were administered.

List of indications	N° of treatments
ANAKINRA (421/526)	
Adult onset Still's disease	78/421(18.52%)
Ankylosing spondylitis	1/421(0.23%)
Autoinflammatory syndrome induced by adjuvants (ASIA) syndrome	1/421(0.23%)
Behçet's disease	41/421(9.73%)
Blau syndrome	1/421(0.23%)
Chondrocalcinosis	3/421(0.71%)
Chronic recurrent multifocal osteomyelitis	11/421(2.61%)
Cryopyrin-associated periodic syndromes	18/421(4.27%)
Familial Mediterranean fever	32/421(7.6%)
Gout	5/421(1.18%)
Histiocytic panniculitis	1/421(0.23%)
Hyper-IgD syndrome	5/421(1.18%)
Idiopathic recurrent acute pericarditis	23/421(5.46%)
Idiopathic uveitis	2/421(0.47%)
Juvenile idiopathic arthritis	4/421(0.95%)
Mevalonic aciduria	1/421(0.23%)
NLRP12-associated familial cold autoinflammatory disease	2/421(0.47%)
Osteoarthritis	1/421(0.23%)
Periodic fever	5/421(1.18%)
Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) syndrome	1/421(0.23%)
Polychondritis	1/421(0.23%)
Polyserositis	1/421(0.23%)
Psoriatic arthritis	1/421(0.23%)
PSTPIP1-associated myeloid-related-proteinaemia inflammatory syndrome	1/421(0.23%)
Pyoderma gangrenosum	1/421(0.23%)
Pyogenic arthritis, pyoderma gangrenosum, acne (PAPA) syndrome	2/421(0.47%)
Rheumatoid arthritis	42/421(9.97%)
SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) syndrome	3/421(0.71%)
Sarcoidosis	1/421(0.23%)
Schnitzler's syndrome	7/421(1.66%)
Sweet's syndrome	2/421(0.47%)
Systemic-onset juvenile idiopathic arthritis	57/421(13.53%)
Tumor necrosis factor receptor-associated periodic syndrome	21/421(4.98%)
Vasculitic urticaria	1/421(0.23%)
Undifferentiated connective tissue disease	4/421(0.95%)
Undifferentiated spondyloarthritis	1/421(0.23%)
Undifferentiated systemic autoinflammatory disease	31/421(7.36%)
CANAKINUMAB (105/526)	
Adult onset Still's disease	3/105(2.85%)
Behçet's disease	15/105(14.28%)
Cryopyrin-associated periodic syndromes	22/105(20.95%)
Epidermolysis bullosa	1/105(0.95%)
Familial Mediterranean fever	6/105(5.71%)
Hyper-IgD syndrome	4/105(3.8%)
Idiopathic uveitis	2/105(1.90%)

(Continued)

TABLE 4 | Continued

List of indications	N° of treatments
Juvenile idiopathic arthritis	2/105(1.90%)
Mevalonic aciduria	1/105(0.95%)
NLRP12-associated familial cold autoinflammatory disease	2/105(1.90%)
Periodic fever	1/105(0.95%)
Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) syndrome	1/105(0.95%)
Systemic-onset juvenile idiopathic arthritis	24/105(22.85%)
Tumor necrosis factor receptor-associated periodic syndrome	14/105(13.33%)
Vasculitic urticaria	1/105(0.95%)
Undifferentiated systemic auto-inflammatory disease	6/105(5.71%)

line in 28 (6.7%) and 12 (11.4%) patients, respectively; fourth line in 8 cases for ANA (1.9%) and 9 for CAN (8.6%); fifth line in 5 (1.2%) and 4 (3.8%) cases; more than fifth line in 5 (1.2%) and 1 (0.9%) cases. **Table 5** displays the clinical outcome for ANA and CAN used for different treatment lines.

Before starting IL-1-INH, corticosteroids had been already employed in 428 out of 526 (81.4%) cases, 95 (22.2%) of which were pediatric ones; DMARDs had preceded IL-1 inhibition in 309 cases (58.7%), 57 (18.4%) of which were pediatric ones. In addition, 35 pediatric (21.2%) and 130 adult subjects (78.8%) had been previously treated with at least one biologic agent different from IL-1-INH. **Table 2** shows details about previous therapeutic approaches, while **Figure 6** shows the amount of the specific DMARDs and biologics previously administered.

Switching from a First to a Second Anti-Il-1 Agent

The number of patients switched from ANA to CAN was significantly higher than patients switched from CAN to ANA ($p < 0.0001$). Specifically, the number of patients firstly treated with ANA and then switched to CAN amounted at 60 (57.1%); conversely, although with a complete response, 2 (0.5%) patients (diagnosed with SOJA and CAPS) needed to be switched from CAN to ANA because of mild leukopenia and loss of efficacy, respectively.

Reasons for switching from ANA to CAN were as follows: loss of efficacy ($n = 29$, 48.3%) after a mean $\pm$ *SD* treatment period of 25.97 $\pm$ 24.47 months, lack of compliance ($n = 7$, 11.7%), and lack of efficacy ($n = 6$, 10%). However, no data were available about the reason for ANA discontinuation in 18 (30%) patients despite a treatment duration ranging from 3 to 132 months.

Regarding clinical outcome, complete response was achieved in 40 (66.7%) cases switched from ANA to CAN, partial response in 17 (28.3%) and failure in 3 (0.5%). Conversely, both patients switched from CAN to ANA proved to be completely responsive to the second anti-IL-1 agent.

Concomitant Treatments

IL-1-INH were associated with corticosteroids in 285 (54.2%) cases, 244 patients being treated with ANA and 41 with CAN. Concomitant DMARDs were employed in 156 (29.66%) cases, of which 138 (88.46%) were using ANA and 18 (11.54%) CAN. Methotrexate was employed in 67 (42.9%) patients, colchicine in 32 (20.5%), cyclosporine A in 25 (16.03%), hydroxychloroquine in 12 (7.7%), salazopyrine in 7 (4.5%), leflunomide in 6 (3.8%), azathioprine in 6 (3.8%), mycophenolate mofetil in 1 (0.6%) case.

In six cases more than one DMARD was associated with IL-1 inhibition: colchicine *plus* hydroxychloroquine, colchicine *plus* methotrexate, methotrexate *plus* leflunomide, methotrexate *plus* cyclosporine A, and salazopyrine *plus* leflunomide each in one case, respectively. Patients requiring two DMARDs were diagnosed with BD ($n = 3$), SOJA ($n = 1$), polyarticular juvenile idiopathic arthritis ($n = 1$), and RA ($n = 1$); these patients were treated with ANA in all cases. **Figure 7** graphically shows the frequency of concomitant DMARDs and the distinction according to ANA and CAN use.

Adverse Events

Seventy-six (14.4%) patients reported AEs and 10 (1.9%) SAEs. Specifically, AEs included skin reactions (28 injection site reactions, 5.3%; 29 generalized skin involvement, 5.5%), disorders of hematopoiesis ($n = 6$, 1.15%), infections ($n = 4$, 0.7%), gastrointestinal affections ($n = 2$, 0.3%), flu-like symptoms ($n = 2$, 0.4%), increase of aminotransferases ($n = 1$, 0.2%), thrombophlebitis ($n = 1$, 0.2%), unspecified temporary breathing problems ($n = 1$, 0.2%), unspecified problems ($n = 2$, 0.4%). Seventy-two (94.7%) patients were on ANA and 4 on CAN treatment.

AEs were significantly more frequent among patients with concomitant therapy compared to patients with either concomitant treatments ($p = 0.01$), or previous DMARDs treatment ($p = 0.04$).

Distinguishing among different types of AEs, no significant differences were found on the basis of previous and concomitant therapies; in particular, no difference was found for skin reaction among patients with or without previous DMARDs and/or biologic treatment and patients with or without co-administered DMARDs ($p = 0.94$).

SAEs recorded were as follows: pneumonia in three adult patients with AOSD; trophic ulcers of lower limbs in one patient with AOSD; herpetic keratitis in one patient with RA; and anaphylaxis in four (0.6%). Finally, one patient with BD was diagnosed with pleural mesothelioma after 3 months of ANA treatment. Death occurred in five patients, all treated with ANA. Nine out of 10 patients with SAEs were under ANA treatment. **Table 6** summarizes clinical characteristics of patients with SAEs and in which death occurred.

Figure 8 shows the frequency of AEs in patients undergoing ANA treatment; as regards CAN, skin rash ($n = 3$) and gastrointestinal symptoms (diarrhea, abdominal pain; $n = 2$) represented the most frequently recorded AEs; flu-like symptoms ($n = 1$) and asthenia ($n = 1$) were also reported after CAN administration.

When patients were subdivided into three age groups (<16 years, 16–65 years, and >65 years), AEs proved to be significantly more frequent after the age of 65 compared to both pediatric patients and subjects aged between 16 and 65 ($p = 0.003$ and p

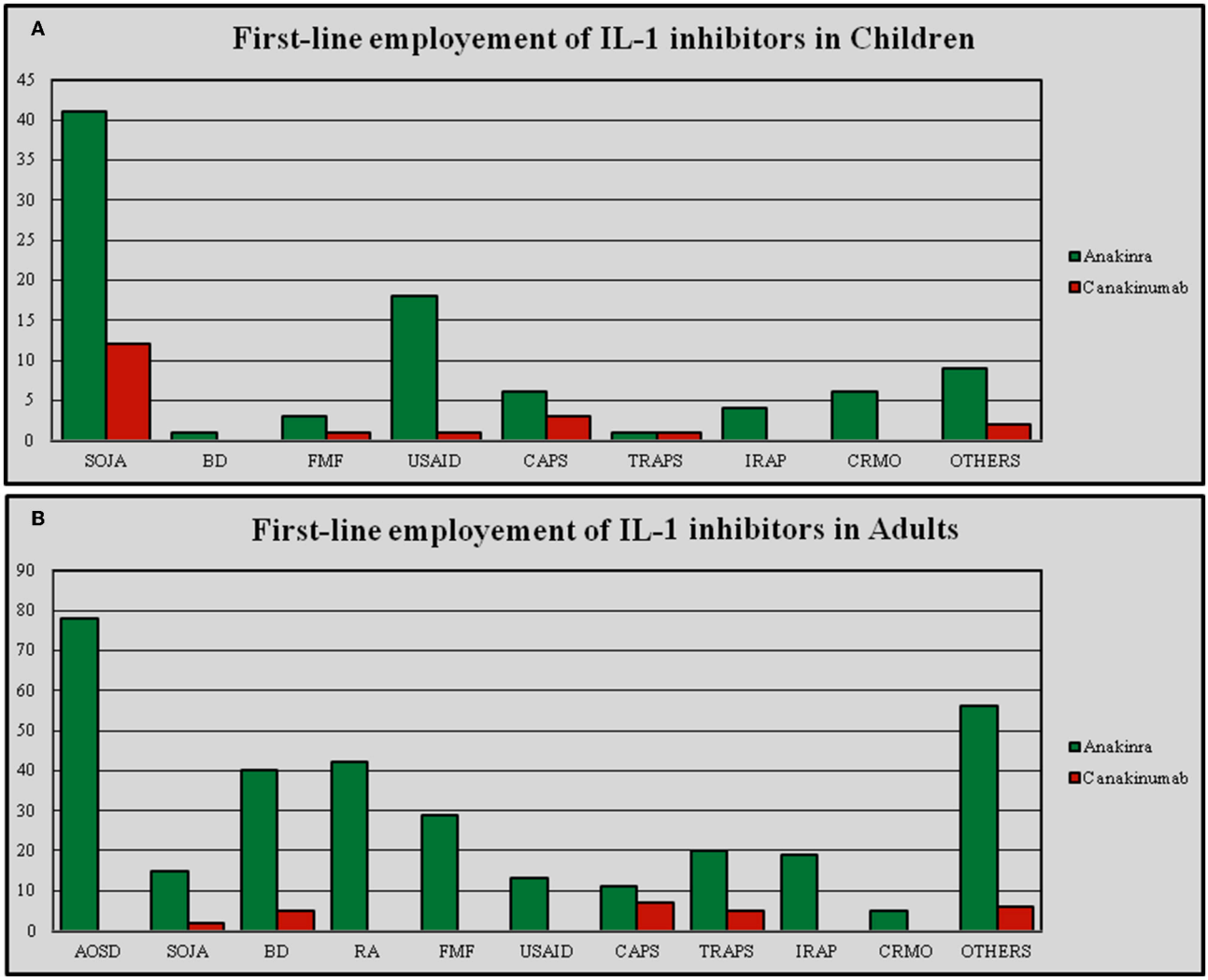


FIGURE 5 | First-line employment of Anakinra and Canakinumab in different indications differentiating between pediatric (A) and adult patients (B). AOSD, Adult Onset Still's Disease; BD, Behçet's Disease; CAPS, Cryopyrin-Associated Periodic Syndrome; CRMO, Chronic Recurrent Multifocal Osteomyelitis; FMF, Familial Mediterranean Fever; IRAP, Idiopathic Recurrent Acute Pericarditis; RA, Rheumatoid Arthritis; SOJA, Systemic Onset Juvenile Idiopathic Arthritis; TRAPS, Tumor Necrosis Factor Receptor-Associated Periodic Syndrome; USAID, Undifferentiated Systemic AutoInflammatory Disease.

= 0.03, respectively). **Table 7** describes the frequency of different AEs according to the age of patients.

In 61 (71.8%) cases AEs led to treatment discontinuation, in 59 cases treated with ANA and in two cases treated with CAN. Regarding patients remaining under ANA treatment despite AEs, most were characterized by injection site reactions ($n = 13$); others were interested by infections (otitis, $n = 1$; infection of the upper respiratory tract, $n = 1$; bronchitis, $n = 1$; pneumonia, $n = 1$), transient leukopenia ($n = 1$), thrombophlebitis ($n = 1$), widespread skin rash with eosinophilia ($n = 1$). Patients continuing CAN despite AEs had presented localized cutaneous erythema ($n = 2$) and flu-like symptoms ($n = 1$).

Dose Adjustments

Dose adjustments were performed for 117 treatment cycles (22.4%), 88 (75.2%) for ANA and 29 (24.8%) for CAN; no statistical differences were identified in the number of dose adjustments between ANA and CAN ($p = 0.29$).

Regarding subjects treated with ANA, an increase of the dose was performed in 12 cases, bringing about a recovery of effectiveness in 7 (58.3%) cases. Conversely, a decrease of the dosage was attempted in 76 patients, leading to maintenance of therapeutic efficacy in 89.4% ($n = 68$) of cases. Among patients treated with CAN, an increase of dosage was performed in 15.2% ($n = 16$) of patients, obtaining a recovery of efficacy in 10 (62.5%). On the contrary, a reduction of dosage was attempted in 12 (11.4%) subjects, leading to maintenance of efficacy in 11 cases out of 12 (91.6%). One patient (0.9%) with TRAPS underwent an increase of the interval between CAN administrations without changing the dosage and without losing efficacy. **Figure 9** shows the number of patients undergoing an increase/decrease of IL-1 INH dosage with related clinical outcome.

TABLE 5 | Percentages of complete response, partial response, and failure to IL-1 inhibition, based on previous biologic therapies.

Totality of treatments (526)	Type of response	0 previous biologics (362/526)	1 previous biologic (92/526)	2 previous biologics (40/526)	3 previous biologics (17/526)	4 previous biologics (9/256)	≥5 previous biologics (6/256)
	Complete	243/362 (67.12%)[ab]	53/92 (57.60%)[cd]	14/40 (35%)[ac]	3/17 (17.64%)[bd]	4/9 (44.44%)	2/6 (33.33%)
	Partial	83/362 (22.92%)[ef]	32/92 (34.78%)	19/40 (47.5%)[e]	10/17 (58.82%)[f]	3/9 (33.33%)	2/6 (33.33%)
	Failure	36/362 (9.94%)	7/92 (7.60%)	7/40 (17.5%)	4/17 (23.52%)	2/9 (22.22%)	2/6 (33.33%)
Totality of treatments (Anakinra) (421)	**Type of response**	**0 previous biologics (323/421)**	**1 previous biologic (52/421)**	**2 previous biologics (28/421)**	**3 previous biologics (8/421)**	**4 previous biologics (5/421)**	**≥5 previous biologics (5/421)**
	Complete	219/323 (67.80%)[ghi]	21/52 (40.38%)[g]	10/28 (35.71%)[h]	2/8 (25%)[i]	2/5 (40%)	2/5 (40%)
	Partial	72/323 (22.29%)[lmn]	25/52 (48.07%)[l]	13/28 (46.42%)[m]	4/8 (50%)[n]	1/5 (20%)	1/5 (20%)
	Failure	32/323 (9.90%)	6/52 (11.53%)	5/28 (17.85%)	2/8 (25%)	2/5 (40%)[o]	2/5 (40%)
Totality of treatments (Canakinumab) (105)	**Type of response**	**0 previous biologics (37/105)**	**1 previous biologic (42/105)**	**2 previous biologics (12/105)**	**3 previous biologics (9/105)**	**4 previous biologics (4/105)**	**≥5 previous biologics (1/105)**
	Complete	23/37 (62.16%)	33/42 (78.57%)[op]	4/12 (33.33%)[o]	2/9 (22.22%)	2/4 (50%)	0/1 (0%)
	Partial	10/37 (27.02%)[q]	8/42 (19.04%)[rs]	6/12 (50%)[r]	6/9 (66.66%)[qs]	2/4 (50%)	1/1 (100%)
	Failure	4/37 (10.81%)	1/42 (2.38%)	2/12 (16.66%)	1/9 (11.11%)	0/4 (0%)	0/1 (0%)

[a]$p = 0.0001$, [b]$p < 0.0001$, [c]$p = 0.02$, [d]$p = 0.0031$, [e]$p = 0.001$, [f]$p = 0.002$, [g]$p = 0.0003$, [h]$p = 0.001$, [i]$p = 0.0181$, [l]$p = 0.0002$, [m]$p = 0.009$, [n]$p = 0.0850$, [o]$p = 0.005$, [p]$p = 0.002$, [q]$p = 0.04$, [r]$p = 0.0573$, [s]$p = 0.008$.

Reasons for Discontinuation

Discontinuation of anti-IL-1 regimen was performed in 246 (46.6%) cases, 225 (91.5%) while on ANA and 21 (8.5%) on CAN. The reasons for discontinuation were as follows: loss of efficacy ($n = 75$, 30.4%), lack of efficacy ($n = 57$, 23.2%), disease remission ($n = 38$, 15.4%), occurrence of AEs ($n = 33$, 13.4%) and SAEs ($n = 7$, 2.9%), poor compliance ($n = 14$, 5.6%), death ($n = 5$, 2%), pregnancy ($n = 2$, 0.8%). Eight cases (3.2%) were lost at follow-up. **Figure 10** shows the reasons for discontinuation by distinguishing between ANA and CAN. As this figure shows, the percentage of poor compliance is higher in patients treated with CAN, though no statistical significance was reached ($p = 0.49$).

DISCUSSION

In this study, we aimed at evaluating how IL-1-INH are currently used in a group of Italian rheumatological and pediatric Centers, principally in terms of therapeutic indications, dosages employed, clinical management, and safety issues, thus identifying differences between adults and patients aged <16 years. In particular, by virtue of the large number of patients enrolled, we would provide useful real-life-related data for the physician needing to resort to IL-1 inhibition.

To date, therapeutic indications for ANA include only RA and CAPS, while on-label use of CAN refers to CAPS, SOJA, and gout. Nevertheless, thanks to vivid basic and clinical research efforts, innate immunity has recently proven to have an important role in a wide number of disorders beyond monogenic AIDs (Banerjee and Saxena, 2012; Dickie et al., 2012; Vitale et al., 2012; Caso et al., 2013; Sheedy and Moore, 2013; Van Tassell et al., 2013; Baskar et al., 2016). Sure enough, inflammatory pathways of innate immunity have been found to affect a lot of pathologies previously classified as exclusively belonging to the field of adaptive immune disorders or degenerative diseases (Martinon et al., 2006; Rigante et al., 2011; Kotas et al., 2013; Ruscitti et al., 2015; Thueringer et al., 2015). Consequently, in the last years a large number of old and new clinical entities have been suggested as possible further therapeutic indications for IL-1-INH (Cantarini et al., 2010, 2015b; Caso et al., 2014; Finetti et al., 2014; D'Elia et al., 2015; Imazio et al., 2016). Therefore, whilst now it is absolutely clear that tumor necrosis factor (TNF)-inhibitors represent a therapeutic option of first order compared to IL-1-INH in RA, a resurgence of interest has occurred for these agents to treat a wide number of diseases located somewhere along a continuity of aberrant innate or adaptive immune responses (Larsen et al., 2007; McGonagle et al., 2007; Shin et al., 2009; Tanzi et al., 2011; Abbate et al., 2013; de Koning et al., 2013; Howard et al., 2014; Néel et al., 2014; Van Tassell et al., 2014; Lopalco et al., 2015b, 2016; Vitale et al., 2015; Annicchiarico et al., 2016).

In this context, Rossi-Semerano et al. (2015) have recently published an interesting national cross-sectional observational study on 189 patients from 38 France Centers. As for our results, they also observed that AOSD and SOJA represented the clinical conditions more frequently requiring IL-1-INH; also, ANA was more commonly employed than CAN with off-label modality.

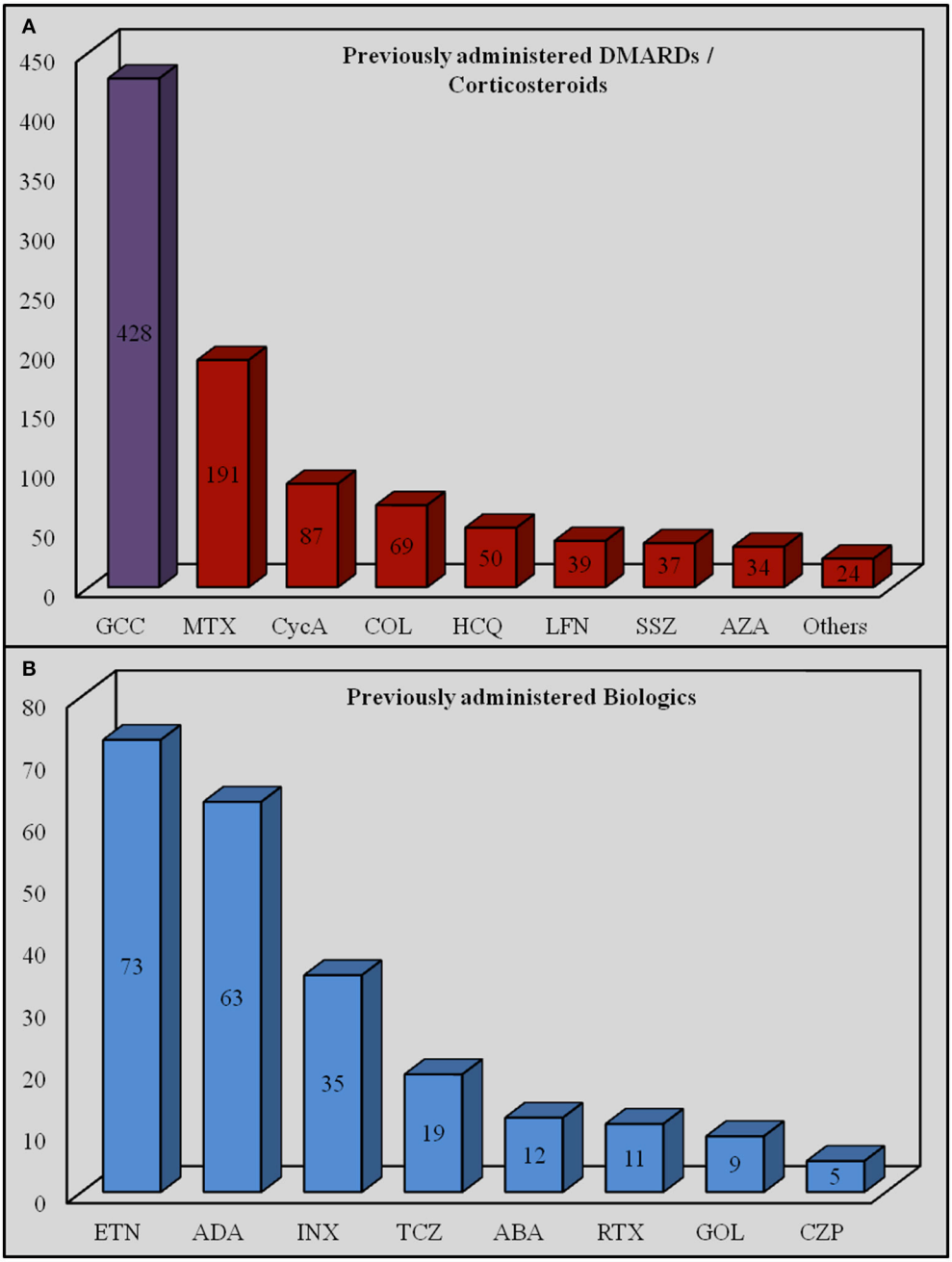


FIGURE 6 | Amount of specific disease modifying antirheumatic drugs/corticosteroids (A) and biologic agents (B) previously administered. ABA, abatacept; ADA, adalimumab; AZA, azathioprine; COL, colchicine; CycA, cyclosporine A; CZP, certolizumab pegol; INX, infliximab; ETN, etanercept; GCC, glucocorticoids; GOL, golimumab; HCQ, hydroxychloroquine; LFN, leflunomide; MTX, methotrexate; RTX, rituximab; SSZ, sulfasalazine; TCZ, tocilizumab.

Regarding clinical outcome, they found a higher response rate among patients suffering from Schnitzler's syndrome, gout, CAPS, and AOSD. Both ANA and CAN appeared safe as for our patients: most of AEs were classified as minor, while most of the time SAEs were represented by severe infections. However, while Rossi-Semerano et al. (2015) observed a number of patients with liver abnormalities and weight gain, we identified only one case of liver toxicity and did not observe any case of weight increase.

Coming back to our study, in our experience most of treatment courses were due to off-label indications, ranging from different monogenic AIDs to a wide variety of polygenic and multifactorial disorders, as summarized in **Table 4**. This is evident for both IL-1-INH, but as for Rossi-Semerano et al.

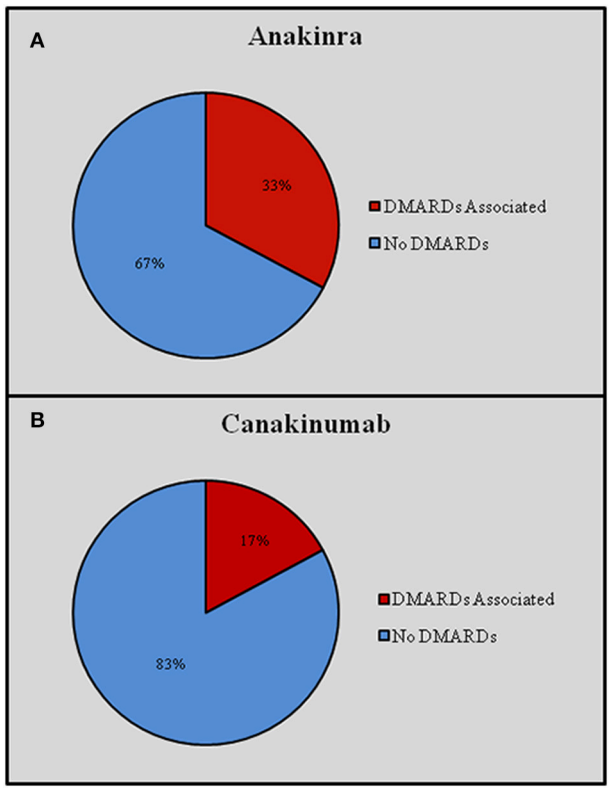


FIGURE 7 | Frequency of disease modifying antirheumatic drugs (DMARDs) concomitantly administered with Anakinra (A) and Canakinumab (B). DMARDs, disease modifying antirheumatic drugs.

(2015), the percentage of off-label use was significantly higher among patients treated with ANA. We think that this is probably related to the high manageability of ANA and to its shorter half-life (Church and McDermott, 2009), but also to the longer experience gained with ANA, and perhaps to cost-related implications. As a whole, the number of different indications for IL-1-INH was significantly higher in adults than in pediatric patients, suggesting that the off-label use of ANA and CAN is more frequently advised by adult health care physicians. However, we did not find statistical differences in the number of indications among patients presenting with complete, partial and failing response, concluding that IL-1-INH are used with awareness of pathogenetic mechanisms also when administered with off-label modality.

In addition to the large number of therapeutic indications, the off-label use of IL-1-INH also manifests with a wide variability of dosages administered both among children and adults. Noteworthy, dosages were more frequently employed based on the body weight among pediatric patients, while adults were more frequently treated with standard dosages. On the contrary, teenage patients (aged <16 years) were frequently treated with a standard dose, as occurring in adults, as a result of the transition from pediatric to adult health care. However, since the body weight affects the pharmacokinetics of drugs (Urien et al., 2013), the two different modalities of IL-1-INH administration may influence the degree of effectiveness. In this regard, we found that the frequency of failure was significantly higher among patients treated with ANA at the dosage of 100 mg/day compared to dosage of 2 mg/kg/day. Conversely, no similar results were identified for CAN, probably due to a lower impact of the body weight related to the much longer half-life (Church and McDermott, 2009), but also to the tiny sample sizes obtained after dividing our CAN population for different dosages.

As expected, we observed that the number of patients switched from ANA to CAN was significantly higher than vice versa, as a likely result of the higher costs of CAN and, here again, of the easy handling of ANA. In fact, the little experience to date available in the off-label context and the relatively scarce supporting literature set physicians advising ANA for safety reasons. Nevertheless, data available on CAN safety (Alten et al., 2011; Ruperto et al., 2012a,b; Imagawa et al., 2013; Howard et al., 2014; Gül et al., 2015), also supported by the present study, make switching from CAN to ANA a reasonable medical choice. In addition, our results demonstrate that patients requiring being moved-on from CAN to ANA showed a complete response after the change of therapy. However, switching between the two IL-1-INH represents a concrete and effective therapeutic opportunity in both directions. Indeed, only 0.5% of patients needing to be switched from ANA to CAN showed a failure after change of therapy, and two-thirds of subjects converted from ANA to CAN even showed a complete response. Consequently, these results confirm previously reported data on the concrete and effective role of switching from a first to a second IL-1-targeted inhibitor (Brizi et al., 2012; Galeotti et al., 2012; Cantarini et al., 2015b; Lopalco et al., 2015b; Emmi et al., 2016).

Similarly, adjusting IL-1-INH dosages by increasing the dose at each administration or decreasing the timing between injections have proved to be successful choices in 66.7% of patients treated with ANA or CAN. These findings suggest that increasing the dosage of IL-1-INH in patients with unsatisfactory results can be a feasible therapeutic strategy. On the other hand, decreasing the dosage in patients with sustained drug-induced quiescence seems to represent another possible way to go when the clinical condition is suitable. Indeed, 92.9% of patients treated experienced maintenance of complete response despite the dose tapering.

Regarding which agent physicians chose as a first option, ANA was employed as first line biologic agent in a greater number of patients, probably due to the higher use of this drug in off-label prescription in reference to the aforementioned explanations. Similarly, the number of DMARDs administered before and during IL-1-INH reflects the frequency with which ANA and CAN were prescribed for complex and multifactorial indications. Actually, the frequency of concomitant and previous DMARDs was higher in adults than pediatric patients and in the population treated with ANA. These findings confirms that IL-1-INH are more likely aimed at treating multifactorial disorders in such cases, whose pathogenesis involves different cell and cytokine pathways and often requires a combination therapy rather than monotherapy. Read backward, these data also suggest that during childhood IL-1-INH (especially CAN) are more frequently used in monogenic AIDs, generally not requiring DMARDs.

TABLE 6 | General features of patients displaying severe adverse effects (SAEs) and in whom death occurred.

N°	Gender	Age	Diagnosis	Age at disease onset	Number of previous DMARDs	Concomitant DMARDs	Number of previous biologics	Previous GCC	Concomitant GCC	IL-1 inhibitor	Treatment duration (months)	SAEs
GENERAL FEATURES OF PATIENTS WITH OCCURRENCE OF SEVERE ADVERSE EFFECTS												
1	M	52	AOSD	49	1	NO	0	YES	NO	Anakinra	17	Pneumonia
2	M	65	AOSD	52	2	YES	1	YES	YES	Anakinra	110	Lower limbs ulcers
3	M	67	AOSD	64	1	YES	1	YES	YES	Anakinra	9	Pneumonia
4	F	15	CAPS	2	1	NO	0	YES	NO	Anakinra	2	Anaphylaxis
5	M	52	FMF	49	1	YES	0	YES	YES	Anakinra	3	Pleural mesothelioma
6	F	76	RA	49	7	NO	2	YES	YES	Anakinra	48	Herpetic keratitis
7	F	64	SAPHO	33	2	YES	4	YES	NO	Anakinra	13	Pneumonia
8	M	15	USAID	0.2	0	NO	0	YES	NO	Anakinra	1	Anaphylaxis
9	F	23	USAID	17	0	NO	0	NO	NO	Anakinra	23	Anaphylaxis
10	M	12.6	SOJA	2	3	NO	0	YES	NO	Canakinumab	80	Anaphylaxis
N°	**Gender**	**Age**	**Diagnosis**	**Age at disease onset**	**Number of previous DMARDs**	**Concomitant DMARDs**	**Number of previous biologics**	**Previous GCC**	**Concomitant GCC**	**IL-1 inhibitor**	**Treatment duration (months)**	**Cause of death**
GENERAL FEATURES OF PATIENTS FOR WHOM DEATH OCCURRED												
11	F	20	AOSD	17	1	NO	0	YES	YES	Anakinra	n.k.	MAS
12	F	32	AOSD	32	0	NO	0	YES	YES	Anakinra	0.5	Myocarditis
13	F	59	AOSD	49	4	YES	1	YES	YES	Anakinra	120	Dilated cardiomyopathy

Patients n°3 and n°7 accounted here have to be reported also among patients in whom death occurred. AOSD, Adult Onset Still's Disease; CAPS, Cryopyrin-Associated Periodic Syndrome; FMF, Familial Mediterranean Fever; IRAP, Idiopathic Recurrent Acute Pericarditis; MAS, macrophage activation syndrome; n.k., not known; RA, Rheumatoid Arthritis; SAPHO, Synovitis Acne Pustolosis Hyperostosis and Osteitis syndrome; SOJA, Systemic Onset Juvenile Arthritis; TRAPS, Tumor Necrosis Factor Receptor-Associated Periodic Syndrome; USAID, Undifferentiated Systemic AutoInflammatory Disease; DMARDs, disease-modifying antirheumatic drugs; GCC, glucocorticoids; M, male; F, female.

As a whole, AEs and SAEs interested one-sixth and 1.7% of patients, thus confirming the good safety profile of IL-1-INH. Interestingly, patients with previous or concomitant use of DMARDs showed a higher frequency of AEs, probably due to the intrinsic safety issues related to the additional employment of DMARDs as well as to the wide number of clinically complex diseases requiring additional immunosuppressive therapies. However, we did not observe any impact of DMARDs on the occurrence of skin reactions. This is an interesting finding, as our results are the opposite of that presented by Rossi-Semerano et al. (2015).

In any case, according to the previous experience with ANA, skin reactions did not lead to treatment discontinuation, confirming that this kind of AEs are usually transient and improves after the first weeks of treatment, with no need for ANA withdrawal (Lequerré et al., 2008).

As we look at SAEs, 40% of them were represented by anaphylactic reactions occurring in three patients treated with ANA and one patient treated with CAN. The other SAEs were severe bacterial infections in three cases, one case of severe herpetic keratitis, and one case of pleural mesothelioma. Death occurred in five cases, two of which as a consequence of infectious SAEs (pneumonia in both cases). However, these infections appeared more likely related to the existing comorbidities and to the globally poor clinical condition than to an actual compromising effect of ANA. In particular, two cases of severe infections had been diagnosed with AOSD and one case with SAPHO syndrome; moreover, AOSD represented the indication requiring IL-1-INH in four out of five cases in whom death occurred. In this regard, the mortality rate in AOSD is reported in up to 10% of patients and overwhelming infections (as also the macrophage activation syndrome and the myocarditis reported in two of our dead patients) represent a major cause of death. Pneumonia itself has been identified as a frequent cause of mortality and an unfavorable prognostic factor in AOSD (Zeng et al., 2009; Kim et al., 2012). Conversely, the dynamics of death of the patient with SAPHO were not completely clear, and we cannot explain in which context pneumonia led to death. Finally, the case of pleural mesothelioma was diagnosed after only 3 months of ANA administration, so we do not ascribe any cancerogenetic effect to IL-1 inhibition.

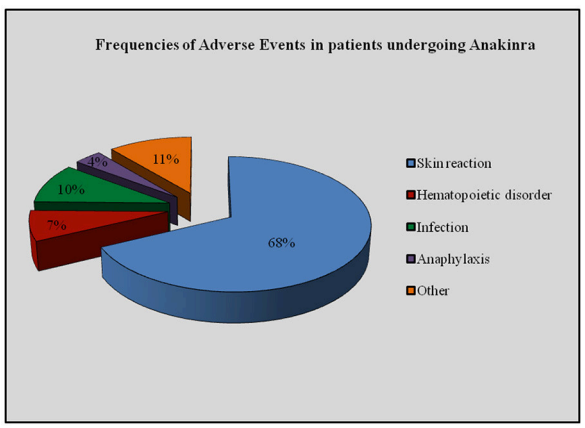


FIGURE 8 | Frequency of adverse events in patients undergoing Anakinra.

An intriguing finding of the present study was represented by the significant higher occurrence of AEs in subjects aged more than 65 years than in others, children included. This result conflicts with the evidence reported by Rossi-Semerano et al. (2015), identifying a higher incidence of AEs among children than in adults. Since no notable differences exist between the two studies in terms of percentages of pediatric and adult patients, this difference could be explained by the higher number of indications observed in our population. Consequently, while a careful monitoring should be guaranteed for all patients, elderly subjects could deserve a closer follow-up, especially for poorly investigated indications.

Noteworthy, none of our patients experienced tuberculosis infection or reactivation, corroborating our previous results on the same matter (Cantarini et al., 2015a; Lopalco et al., 2016) and suggesting that use of IL-1-INH is relatively safe compared

TABLE 7 | Frequency of different adverse events and severe adverse events according to the age of patients.

Age (years)	N° treatments	Total reactions	Generalized skin reaction	Injection site reaction	Hematopoiesis disorders	Infection	Gastrointestinal involvement	Anaphylaxis	Other
ADVERSE EVENTS AND SEVERE ADVERSE EVENTS (DIVIDED BY AGE)									
Total number of AEs: 76 out of 526 treatments									
All Ages	526	76 (14.44%)	29	28	6	4	2	–	7
0–15.99	130	11 (8.46%)	1	7	–	–	1	–	2
16–64.99	331	50 (15.1%)	22	16	3	4	1	–	4
≥65	65	15 (23.07%)	6	5	3	–	–	–	1
Total number of SAEs: 10 out of 526 treatments									
All Ages	526	10 (1.9%)	–	–	–	4	–	4	2
0–15.99	130	3 (2.3%)	–	–	–	–	–	3	–
16–64.99	331	4 (1.2%)	–	–	–	2	–	1	1
≥65	65	3 (4.61%)	–	–	–	2	–	–	1

AEs, Adverse Events; SAEs, Severe Adverse Events.

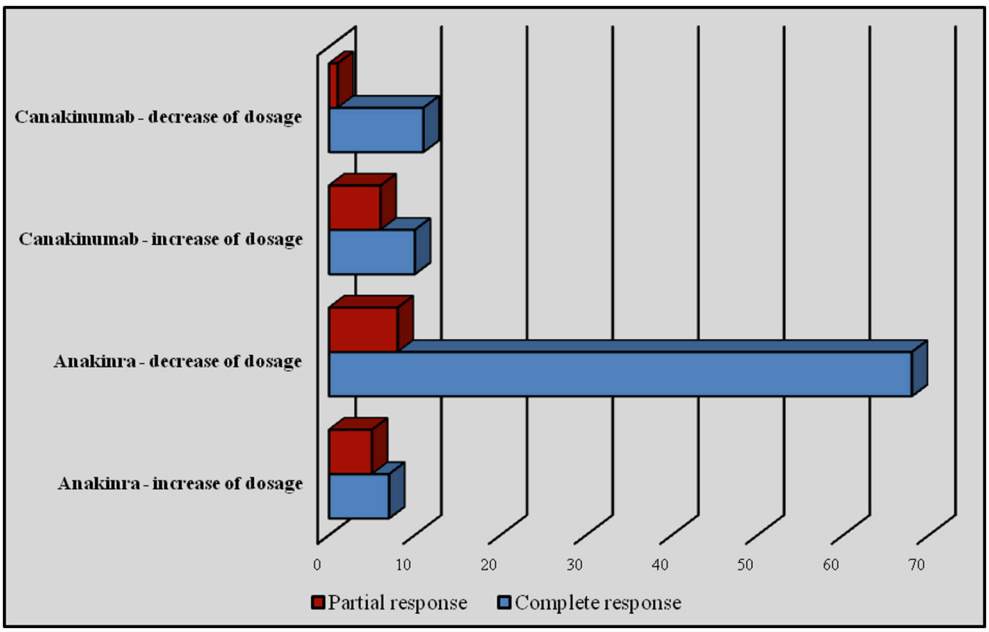


FIGURE 9 | Number of patients undergoing an increase or decrease of IL-1 INH dosage with related clinical outcome.

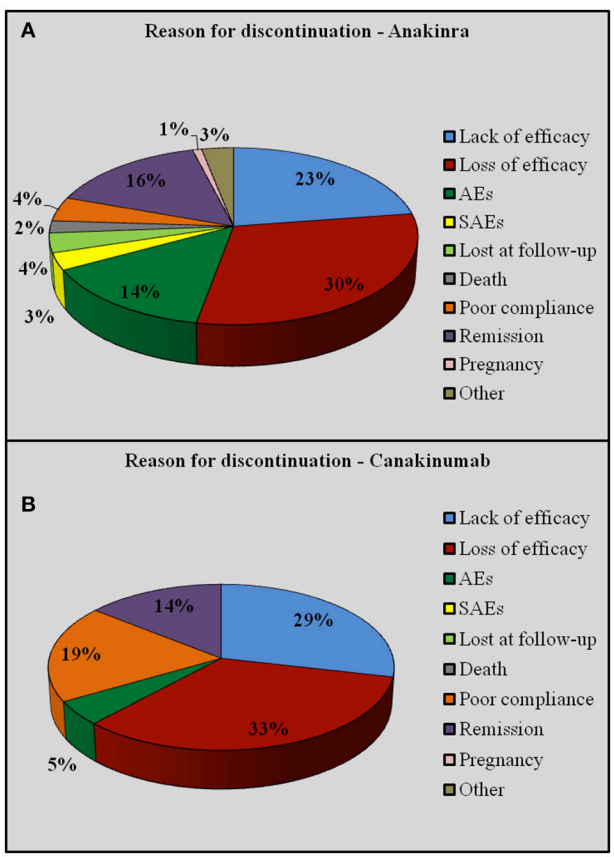


FIGURE 10 | Reasons for discontinuation distinguishing between Anakinra (A) and Canakinumab (B).

to other therapeutic tools in those geographical areas where tuberculosis is an endemic issue.

Because of the retrospective design, this study has some limitations: firstly, although data collection was quite exhaustive, some information was lacking due to the inability to collect all clinical data. This was especially true about the reasons for discontinuation, which remained without explanation in 18 cases treated with ANA. Secondly, a limitation of the causality assessment is highly probable in a retrospective study. In addition, the huge number of therapeutic indications made impossible a sharp statistical analysis on the response to IL-1-INH. Indeed, fragmentation of cases among 38 different indications and three possible outcomes (complete response, partial response, and failure) led to small sample sizes and convincing conclusions could not be drawn. As a result, we performed an overall description on the response and placed the issue of clinical response among the ancillary end-points. However, this is the first study showing how and when IL-1-INH are prescribed in Italy, highlighting different therapeutic choices in terms of starting dosages, dose adjustments, and switching from one to another IL-1-INH as well as the assessment of safety profile on a large number of patients.

In conclusion, our data show that treatment with IL-1-INH is mostly used in off-label regimen. Nevertheless, the high amount of complete and partial clinical response obtained suggests that IL-1-INH are administered in clinical conditions mostly characterized by the pathogenetic involvement of IL-1 cytokine network. Accordingly, most of the patients were concomitantly treated with DMARDs and had been previously administered with other biologic agents different from IL-1-INH, especially anti-TNF drugs. The off-label use of IL-1-INH has been more frequent for ANA and for adult

patients. The wide spectrum of dosages administered for IL-1-INH is a further interesting information emerging from our data: while adult health care physicians generally employ standard dosages of IL-1-INH, pediatricians are more frequently inclined to use a weight-based posology, which seems to be a more adequate therapeutic strategy because of pharmacokinetic implications about drug-tissue concentrations. Furthermore, switching from a first to a second IL-1-INH and increasing dosages appear to be useful in order to obtain a more successful clinical response. According to our findings, switching from CAN to ANA is a less common therapeutic choice than the reverse. However, patients undergoing this procedure showed complete response, and consequently we think that this therapeutic option should be kept into much greater account. The present study confirms the good safety profile of IL-1-INH in terms of low risk of tuberculosis. In addition, the majority of AEs were mild or moderate and did not require treatment discontinuation. On the other hand, SAEs and deaths reported were mostly connected to the underlying disease or other comorbidities. Finally, our data show that a slightly closer follow-up may be useful in patients over 65 years of age.

AUTHOR CONTRIBUTIONS

AV, DR, and Luca Cantarini wrote the manuscript. LCa designed the study and finally revised the manuscript. AV and LCa: data analysis. AI, PS, GLo, GE, MC, RMan, RC, RP, RT, SG, GD, MF, RG, AS, MA, DC, MCM, RMar, FL, CF, SC, FR, PG, OV, EV, MP, LCe, EC, AO, GP, GVi, AM, ES, CSt, GVa, MM, SD, AT, GLa, BF, FD, FI, LP, CSa, MG: patients enrollment, follow-up of the patients and data collection.

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Peri- and Postoperative Treatment with the Interleukin-1 Receptor Antagonist Anakinra Is Safe in Patients Undergoing Renal Transplantation: Case Series and Review of the Literature

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In patients undergoing solid organ transplantation, the presence of an interleukin-1 (IL-1) driven disease may require the addition of IL-1 inhibiting drugs to the standard immunosuppressive regimen to protect against inflammation and negative graft outcome. Three patients undergoing renal transplantation were treated perioperatively with the interleukin-1 receptor antagonist anakinra. Kidney function increased rapidly in all three and the only complications seen were minor infections. *In vitro* studies report associations between serum and urinary levels of IL-1β and IL-1 receptor antagonist and negative graft outcome, and studies in animals and two small human trials illustrate a possible protective effect of anti-IL-1 therapy after solid organ transplantation. Peri- and postoperative use of anakinra is safe and effective in patients undergoing renal transplantation.

Keywords: anakinra, interleukin 1, organ transplantation, renal transplantation, immunosuppressive drugs

INTRODUCTION

Patients undergoing solid organ transplantation depend on life-long immunosuppressive therapy with T-cel inhibiting drugs to prevent allograft rejection. In patients suffering from an interleukin-1 (IL-1) driven inflammatory disease, continuation of IL-1 inhibition is important because inflammation in these diseases is not controlled by the standard immunosuppressive drugs used after renal transplantation, and when anti-IL-1 directed therapy is stopped these patients are at high risk of recurrence of inflammation, associated with severe symptoms, such as arthritis, peritonitis, meningitis and many others.

IL-1 is a potent proinflammatory cytokine, which actions are mitigated by the circulating IL-1 receptor antagonist (IL-1RA). The recombinant IL-1RA anakinra is a very effective treatment for IL-1 driven diseases, such as autoinflammatory diseases and rheumatoid arthritis.

Little is known on the effects of perioperative IL-1 inhibition during renal transplantation. Hypothetically, the additional immunosuppressive effect of anti-IL-1 treatment on top of the standard immunosuppressive regimen could increase the risk of complications, especially

infections. On the other hand, cessation of anti-IL-1 therapy in IL-1 driven diseases may lead to recurrence of systemic inflammation, which may negatively influence short- and long-term transplant function and in some diseases may increases the risk of amyloidosis in the allograft.

We have recently performed renal transplantation in three patients with IL-1 driven diseases while continuing anti-IL-1 therapy with anakinra before, during and after the transplantation[1].

CASE SERIES

Case 1

A now 70-year old man developed recurrent fever episodes 12 years ago. One year after the first fever attack he developed end-stage renal disease. Renal biopsy at that time showed thrombotic microangiopathy (TMA). He was temporarily treated with plasmapheresis and was started on hemodialysis, which was later switched to peritoneal dialysis. After multiple episodes of fever and polyarthritis he was diagnosed with adult onset Still's disease (AOSD) and treatment with anakinra 100 mg on alternating days was started, which was later increased to the standard dose of 100 mg daily when renal function spontaneously increased. Four years after anakinra was started, this patient received a renal transplantation from a living related donor. He was started on immunosuppressive therapy with mycophenolate mofetil (MMF), tacrolimus and prednisone. Anakinra 100 mg daily was continued without interruption, only a single dose was skipped at the day of transplantation. After transplantation, kidney function improved rapidly and he could be discharged from the hospital 7 days postoperative. In the first year after the transplantation he experienced only two minor infections: an abscess located at an old drain entry site was drained 2 months after the transplantation and he was admitted for 5 days 6 months after the transplantation because of an upper respiratory tract infection. During these episodes anakinra and other immunosuppressive drugs were continued. 14 months after the transplantation this patient has been admitted for 34 days, and again 7 months later for 11 days, including several days in the intensive care unit, because of fever, encephalopathy and renal failure. These episodes were caused by exacerbations of the underlying AOSD. Two consecutive kidney biopsies showed endo- and extracapillary glomerulonephritis without signs of rejection or TMA. Treatment with high-dose steroids led to recovery of kidney function. Because of these severe exacerbations and apparent anakinra failure, anakinra was switched to the anti-interleukin-6 receptor antibody tocilizumab. The transplantation is now 2.5 years ago and this patient is doing well.

Case 2

A now 22-year old woman was diagnosed with mutation negative cryopyrin-associated periodic syndrome (CAPS)/chronic infantile neurologic, cutaneous, articular (CINCA) syndrome at the age of 10. She had been suffering from rash, chronic meningitis, bone dysplasia and growth retardation since birth.

From the first year of life recurrent episodes of pyelonephritis had been present, associated with vesicourethral reflux, leading to impaired renal function and proteinuria. These had been progressive since the age of 17.

Directly after she was diagnosed with CAPS, she was started on anakinra. 8 years later she switched to the selective IL-1β antibody canakinumab. Two years later she developed end stage renal disease due to a combination of recurrent pyelonephritis, use of NSAIDs and hypertension. Kidney biopsy was contraindicated because of small kidney size, but there were no other signs of AA amyloidosis. A pre-emptive renal transplantation was planned and 2 months before, canakinumab was switched back to anakinra 100 mg on alternating days; the shorter half life of the latter makes it more easy to stop in case of complications. This patient received a renal transplantation of a living related donor almost 2 years ago. She was started on immunosuppressive therapy with MMF, tacrolimus and prednisone, while anakinra was continued. Kidney function increased rapidly and she could be discharged 6 days after the transplantation. Because of incomplete control of inflammation 4 months after the transplantation the dose of anakinra was increased stepwise to 100 mg each 36 h and later to 100 mg daily.

She is now doing well. She has been admitted three times since the transplantation: 3 months post-transplantation because of influenza and primo-Epstein Barr virus (EBV) infection (2 days) and both 8 and 11 months after the transplantation because of diarrhea, with positive norovirus PCR during the last episode. This may be related to her job at a children's day care center. MMF was switched to azathioprine because of diarrhea. She still uses anakinra 100 mg each day.

Case 3

A 29-year old Turkish man was known with familial Mediterranean fever (FMF) since the age of 6 and slowly progressive nephrotic syndrome due to AA amyloidosis, which had been proven in a rectal biopsy at the age of 17. He had been on colchicine treatment since diagnosis, but without complete compliance and without proper medical guidance. He was only referred to a tertiary treatment center 11 years after the diagnosis of amyloidosis because of further deterioration of renal function after a pneumonia associated with vomiting and diarrhea with persistent use of ACE-inhibitors and NSAIDs. Hemodialysis had been initiated 1 month before referral. Renal biopsy at the time of referral showed AA amyloidosis. Because of unsatisfactory inflammatory control on colchicine, anakinra 100 mg on alternating days was started. This resulted in rapid control of inflammation. 15 months later he received a renal transplant from a living related donor. While continuing anakinra, he was started on standard immunosuppressive therapy with MMF, tacrolimus and prednisone. Renal function increased rapidly, but 4 days after the transplantation he developed a typical FMF-attack with fever, headache and malaise. The dose of anakinra was increased to 100 mg daily, because of suspected decreased plasma levels of anakinra due to increased renal clearance. This resulted in prompt resolution of symptoms. He was discharged

[1] All three patients gave their consent for anonymous publication of their data.

9 days after the transplantation. He is now 10 months after the transplantation. He has been admitted only once for 2 days 3 months after the transplantation because of pneumonia.

Six months after the transplantation, MMF has been switched to azathioprine on patient's request to simplify the drug regimen. He is doing well, has normal kidney function and still uses anakinra 100 mg daily.

DISCUSSION AND LITERATURE REVIEW

IL-1 Inhibition during and after Organ Transplantation in Patients with IL-1 Driven Inflammatory Diseases

In three patients who underwent renal transplantation, the use of the IL-1RA anakinra in combination with MMF/azathioprine, tacrolimus and prednisone was safe and well tolerated. Few complications were seen; all were minor infections, which are common in renal transplant patients. Kidney function increased rapidly in all three patients and no drug-drug interactions were observed.

The only other patients undergoing renal transplantation with perioperative anakinra treatment reported in literature are two FMF patients described in a case report (Moser et al., 2009) and a case series (Ozcakar et al., 2016). Both had end stage renal failure due to AA amyloidosis. No complications were observed in either patient. Ozcakar et al. continued anakinra perioperatively, while it is unclear from the report of Moser et al. how they handled the drug in the perioperative period. In our three cases, we only skipped a single dose of anakinra on the day of transplantation. These scarce cases on continuing anakinra perioperatively in renal transplantation patients give an indication that it can be safely combined with immunosuppressants used to prevent allograft rejection.

There are several reports on the safety of anakinra when initiated after renal transplantation and other solid organ transplantations in patients with IL-1 driven diseases. A number of case reports and two case series describe 5 adults and adolescents with FMF and one adult with CAPS that were started on anakinra in combination with azathioprine or tacrolimus and prednisone 1–3 years after renal transplantation. All report no complications and good graft function (Leslie et al., 2006; Alpay et al., 2012; Celebi et al., 2014; Ozcakar et al., 2016). A seventh renal transplant patient treated with tacrolimus developed neutropenia 2 months after anakinra was started because of gout. The start of anakinra was also associated with decreased allograft function. It could be debated whether this really can be attributed to anakinra as this patient was 20 years post-transplantation and already known with chronic graft dysfunction (Direz et al., 2012).

All our patients received an allograft from a living related donor. Of the other reported patients that underwent renal transplantation during or after the initiation of anakinra, donor type was reported in only two: one patient that started anakinra after renal transplantation received a kidney from a living related donor (Leslie et al., 2006), while the other received an allograft from a deceased donor 5 months after the initiation of anakinra (Moser et al., 2009). In this latter patient, the immunsuppressive drug regimen post-transplantation was the same as in the cases described here (tacrolimus, MMF and prednisone) and there was immediate graft function (Moser et al., 2009).

Anakinra was also reported to be safe after liver transplantation and hematopoietic stem cell transplantation in one patient each (Petropoulou et al., 2010; Yilmaz et al., 2014). In all patients anakinra effectively reduced inflammation, reflected by rapid resolution of symptoms and decrease of serum inflammatory markers.

Our third case and a patient reported by Moser et al. (2009) illustrate that, equal to the need for dose adjustment in patients with impaired renal function, it is important to increase the dose of anakinra when renal function increases after renal transplantation to avoid recurrence of inflammation. Increased renal clearance of anakinra results in decreased anti-inflammatory effect. When decreased renal function was present, the patients reported here were treated with anakinra 100 mg every 36–48 h, depending on the severity of renal function loss, instead of the standard dose of 100 mg daily.

IL-1 Inhibition after Organ Transplantation in Humans or Animals in the Absence of an Inflammatory Disease

In pancreatic islet transplantation, treatment with IL-1RA in apes (Danobeitia et al., 2015) or mice (Sahraoui et al., 2014) increased engraftment and efficacy of treatment. In mice undergoing pancreatic islet transplantation, treatment with IL-1RA gene therapy also led to better engraftment(Hsu et al., 2009) In humans, two trials including a total of 14 patients with type 1 diabetes mellitus, showed that treatment with anakinra during the first 1–2 weeks after the transplantation in combination with anti-thyomocyte globulin (ATG) and prednisone or etanercept/tacrolimus/MMF led to better engraftment and did not lead to increased risk of infections (Takita et al., 2012; Maffi et al., 2014).

In mice and rats undergoing high risk cornea transplantation, topical treatment with IL-1RA (Dana et al., 1997; Yamada et al., 1998; Dekaris et al., 1999; Jie et al., 2004a,b; Dana, 2007) or IL-1RA gene therapy (Yuan et al., 2012) reduced the incidence of graft dysfunction.

The Role of IL-1 in Renal Transplantation

In case of renal transplantation in patients with IL-1 related inflammatory disorders, inhibition of the increased IL-1 signaling is necessary to suppress the symptoms due to the inflammatory disease. It could be hypothesized that suppression of inflammation could also benefit the renal allograft, as the risk of possible impaired graft function or allograft rejection due to increased IL-1 serum levels may be important after transplantation. Studies on this subject show an association between increased IL-1 signaling and negative graft outcome.

In rats, during acute renal allograft rejection IL-1 mRNA is upregulated in kidney and spleen (Nagano et al., 1997) and IL-1 expression is upregulated in mononuclear, mesangial and endothelial cells in the graft (Tilney et al., 1993).

In humans, polymorphonuclear cells (PBMCs) (De Serres et al., 2011, 2012; Batal et al., 2014) and monocytes (Weimer et al., 2003) of patients with chronic rejection, glomerulonephritis or tubulopathy secrete more IL-1β than cells derived from non-rejecting patients or patients with normal renal graft biopsies. In patients with chronic renal graft dysfunction, the expression of IL-1β mRNA in the arterial wall of the renal arteries is upregulated (Zegarska et al., 2002).

Patients with acute renal allograft rejection have increased urinary excretion of IL-1β (Teppo et al., 1998). A single study showed higher serum IL-1β levels in patients with decreased graft function after transplantation (Caban et al., 2009) compared to patients with immediate graft function, although it has to be kept in mind that the concentration of circulating IL-1β and IL-1RA does not directly reflect the level of inflammation *in vivo*.

Polymorphisms in the genes encoding IL-1α, -β, and IL-1RA in renal transplant recipients are presumed to be associated with acute rejection (Manchanda et al., 2006; Manchanda and Mittal, 2008), short-term (Manchanda et al., 2006), and long-term graft function (Haldar et al., 1999), but this has not been reproduced in other studies (Marshall et al., 2000; Lee et al., 2004; Uboldi de Capei et al., 2004; Seyhun et al., 2012). This is most likely due to too small cohort sizes, differences in patient selection and case definition. Donor genotype does not influence graft outcome (Marshall et al., 2001).

Studies on the role of IL-1RA after renal transplantation show a protective effect. Low serum IL-1RA after transplantation is associated with delayed graft function (Sadeghi et al., 2003). Right before and during acute rejection, urinary excretion of IL-1RA was found to be decreased in some (Teppo et al., 1998, 2001; Xu et al., 2013), but not all studies (Sadeghi et al., 2003; Reinhold et al., 2012). These contradictory results may arise due to differences in patient selection, living or postmortal donors, immunosuppresive drug regimens, definition of allograft rejection, interval between transplant and rejection, or the cytokine measurement methods used. Higher urinary IL-1RA excretion is associated with better 1-year renal allograft function (Pereira et al., 2012) and the production of IL-1RA in renal biopsies taken before and during rejection is decreased compared to biopsies of non-rejecting kidneys (Oliveira et al., 1997; de Oliveira et al., 2002).

Renal transplantation is accompanied by ischemia-reperfusion injury, which may lead to delayed and impaired graft function. IL-1 is released during early reperfusion and induces apoptosis and an inflammatory response. There are few studies on the protective effects of IL-1RA in renal ischemia-reperfusion injury. During experimental ischemic clamping of murine native kidneys, one study showed that intraperitoneal treatment with a very high dose of IL-1RA (80 mg/kg) leads to significant reduction of tubular necrosis 24 h–5 days after ischemia-reperfusion and less apoptosis after 5 days (Rusai et al., 2008), but this has not been reproduced (Daemen et al., 2001), probably because of differences in the IL-1RA dose used and the methods used to detect ischemia-reperfusion injury. In a cardiac ischemia-reperfusion model in rabbits, intraventricular injection of 10–40 mg/kg IL-1RA after experimental myocardial ischemia decreased myocardial apoptosis (Li et al., 2004).

There are no studies on the effects of IL-RA treatment on reperfusion-ischemia injury in humans, and as the Il-1RA doses used in mice are high (approximately 4–300 mg/kg, compared to 2 mg/kg in children and a total of 100 mg in patients >40 kg in humans) these results cannot be directly extrapolated to a possible protective effect in humans. There are no reports on worse graft outcome after organ transplantation in patients with IL-1 driven inflammatory diseases, but as the number of patients with these kinds of diseases is limited, this might not be clinically evident. All three patients reported here had immediate graft function and no signs of rejection.

Besides possible worse graft outcome due to rejection or reperfusion-ischemia injury, the occurrence of secondary AA amyloidosis with consequent long-term graft loss due to prolonged uncontrolled inflammation in some IL-1 driven diseases is an additional reason why continuation of IL-1 inhibition after renal transplantation is important in patients with these diseases. Adequate inflammatory control protects against the development of AA amyloidosis.

CONCLUSION

In our three patients that used IL-1 inhibitors before, during and after renal transplantation, continuing the IL-1 receptor antagonist anakinra on top of the standard immunosuppressive drug regimen with MMF, tacrolimus and prednisone was safe and well tolerated. Before and after transplantation the dose of anakinra should be adjusted to the renal function, as this influences clearance.

AUTHOR CONTRIBUTIONS

CM wrote manuscript, performed literature search and review. MB provided case, corrected manuscript. FM provided case, corrected manuscript. AS provided case, corrected manuscript

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OPINION
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IL-1 Vaccination Is Suitable for Treating Inflammatory Diseases

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RELEVANCE OF VACCINATION

Anti-cytokine therapy associated with immunosuppressive drugs has demonstrated high efficiency in treatment of several autoimmune diseases, including rheumatoid arthritis (RA). Current treatments approved for use in patients are targeting inflammatory cytokines TNF-α, IL-1, or IL-6 and more drugs are undergoing clinical evaluation (Semerano et al., 2016a).

Three types of cytokine inhibitors had been developed in RA. The great majority of them is constituted by monoclonal antibodies (mAbs) directed against the cytokine or its receptor. A second category is based on recombinant proteins combining soluble receptors of cytokine stabilized by the Fc domain of IgG1 antibodies. A third type of anti-cytokine drugs is constituted by recombinant receptor antagonist that binds to receptor without induction of signaling.

One common feature of these inhibitors is their high interaction with the cytokine or its receptor that could induce excessive inhibition and some drawback for development. A common side effect is the production, after treatment, of anti-drug antibodies that result in frequent primary or secondary resistance to the treatment. In addition all these drugs have limited effect in the majority of cases; for instance in rheumatoid arthritis, targeted treatments induce 6-month remission in 25–30% patients, and a response different of remission in 50% cases. Partial resistance represents, in the first 6 months, 70% of patients. Furthermore, available anti-cytokine drugs are expensive and need frequent long-term administration.

In this context, a novel type of anti-cytokine drugs based on vaccination is emerging (Semerano et al., 2012). In this case, therapeutic antibodies are produced by the individual itself. This approach has the advantage to generate antibodies that are well-tolerated because of the absence of xenogenic epitopes.

The saga of anti-TNF-α vaccination recapitulates the different steps of such strategy. Several vaccination approaches were conducted in parallel to develop a vaccine against TNF-α. In order to induce a B cell response, DNA vaccination, introduction of a foreign Th cell epitope and coupling TNF-α (or peptides of TNF-α) with carrier proteins were developed in animal models of RA.

A vaccine (TNF-K) constituted by coupling human TNF-α to the carrier protein KLH (keyhole limpet hemocyanin) was studied extensively in animal models of arthritis and in several clinical trials. TNF-K vaccine induced the generation of anti-human TNF-α antibodies that didn't cross react with mouse TNF-α. Transgenic mice expressing human TNF-α (TTG mice) were a suitable model of arthritis to evaluate the efficacy of TNF-K vaccine. In this model, TNF-K vaccine resulted in the production of anti-TNF-α neutralizing antibodies that protected mice against established arthritis (Le Buanec et al., 2006). Anti-TNF-α antibody production was reversible and could not be stimulated by TNF-α administration, as this approach did not induce any cellular-mediated immunity against TNF-α. Therapeutic antibody production was not altered by methotrexate co-administration and the protective effect was correlated with the levels of anti-TNF-α antibody

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production in sera of vaccinated mice. TNF-K efficacy was compared to infliximab in TTG mice and showed a delay before arthritis inhibition linked to the induction of therapeutic antibodies. This delay did not induce significant alterations of the paws versus infliximab-treated mice, and could not be reduced by simultaneous administration of infliximab in mice during TNF-K vaccination. Indeed, co-administration of TNF-K and infliximab led to a lower response to the vaccine, probably due to epitope masking: TNF-K being constituted with the full human cytokine, infliximab could recognize TNF-α, and increase the clearance of the vaccine. Experiments in animal models of *Listeria monocytogenes* and *Mycobacterium tuberculosis* recently shown that mice vaccinated with a TNF conjugate did not develop a hypersensitivity to these infections (Assier et al., 2016). A first clinical trial (phase IIa) was conducted with RA patients who previously experienced secondary failure of TNF-α inhibitors. Several doses of TNF-K and different schedules of administration were used in order to give rise to therapeutic anti-TNF-α antibodies (Durez et al., 2014). TNF-K vaccine was well tolerated and this first trial held promising clinical improvements. However, a second clinical trial (phase IIB/II) conducted with a larger panel of RA patients did not reach significant therapeutic benefits. This failure could be due to the absence of detection of neutralizing anti-TNF-α antibodies in sera of patients.

Similarly to anti-TNF studies, several anti-cytokine vaccination approaches were developed in autoimmune models against different cytokines including IL-1β (**Table 1**). These vaccines were composed either with entire cytokine or peptide of cytokine, linked to various carrier proteins (KLH, VLP, Ova, DTT). The use of the full cytokine to compose a vaccine presents some limitations. The cost of recombinant purified cytokines is high and their use induces a polyclonal antibody response against all exposed epitopes. Among these antibodies, only a variable proportion will exert neutralizing capacities and there is a potential risk of unwanted reaction against a shared epitope.

The use of peptide of cytokine allows limiting antibody generation to selected epitopes. In return, the conformation and the peptidic sequence of the cytokine that interact with the receptor have to be determined. Peptides are often selected in interacting zone of cytokine with its receptor in order to block this interaction. Beyond this highest potential selectivity, the cost of peptides is lower and could lead to similar protections than its entire cytokine counterpart. In this sense, VEGF neutralization by both vaccinal approaches was studied in the collagen-induced arthritis (CIA) model. Vaccines were either constituted by the full length mouse VEGF-A or a peptide (Vpep1) selected in the sequence of VEGF-A, linked to the carrier protein KLH. Vpep1 was chosen for its potential interaction with the VEGF-A co-receptor Neuropilin-1, implied in pathologic angiogenesis. Both types of VEGF vaccines led to the production of anti-VEGF polyclonal neutralizing antibodies. Clinical and histological scores of inflammation and destruction were reduced as well as synovial vascularization (Semerano et al., 2016b). Thus, restraining antibody response to a single peptide sequence with a peptide vaccine could protect immunized mice from arthritis.

The use of peptides of cytokine could be also an asset when cytokine are composed of two chains that could be shared with other cytokines. A vaccinal approach against IL-23 was conducted with peptides chosen in the IL-23p19 subunit. IL-23 is important for the generation of Th17 lymphocytes that are implied in autoimmune diseases. IL-23 is constituted by the IL-12p40 subunit, shared with IL-12, and the specific IL-23p19 subunit. Because IL-12 and IL-23 could have opposite effects in autoimmune models, it was of great importance to inhibit selectively IL-23. In this context, two different teams have conducted experiments in CIA and colitis models. The first vaccine constituted by IL-23p19 peptides linked to KLH was protective in CIA model, whereas the second constituted by IL-23p19 peptides fused to hepatitis B core antigen was protective in colitis.

TARGETING IL-1 IN CHRONIC DISEASES

IL-1 is a major inflammatory cytokine that act at the systemic and local levels (Cavalli and Dinarello, 2015). IL-1 is encoded by two distinct genes giving rise to two related but functionally distinct proteins: IL-1α and IL-1β that interact with two IL-1 receptors. IL-1R2, as a decoy receptor, binds to IL-1 but does not transmit a signal. Signaling is assumed by IL-1R1 that is expressed on the surface of most cell types. IL-1α and IL-1β are primarily expressed as precursors. IL-1α precursor could be release from necrotic cells in a fully active form. Thus, IL-1α acts as an alarmin and could induce sterile inflammation. By contrast, IL-1β precursor is inactive and need to be cleaved to become active. Several enzymes are implied, as proteinase-3 and elastase from neutrophils or caspase-1 from hematopoietic cells. In other cells, caspase-1 exists as a pro-enzyme that needs to be cleaved by a macromolecular complex named the inflammasome. NLRP3 (also known as cryopyrin) is one of the major component of inflammasome. Mutations leading to a gain of function of NLRP3 protein are associated with high amounts of IL-1β secretion and autoinflammatory diseases.

In brief, IL-1α is expressed locally, whereas IL-1β is expressed at the systemic level and in inflamed sites as synovial fluids in rheumatoid arthritis or gouty arthritis (McInnes and Schett, 2007; Richette and Bardin, 2010). IL-1α and IL-1β binding could be limited by the naturally occurring inhibitor IL-1 receptor antagonist (IL-1Ra). IL-1Ra can bind to IL-1 receptor with greater affinity than either IL-1α or IL-1β. A recombinant version of IL-1Ra (anakinra) was developed, but its therapeutic use is limited by its short-lived effect leading to frequent injections to the patient. Two other IL-1 blockers were developed and present lower clearance rates. Rilonacept is a soluble decoy receptor that inhibits primarily IL-1β, but also IL-1α, and canakinumab is a monoclonal antibody that neutralizes specifically IL-1β.

It was suggested that specific targeting of IL-1β, may be beneficial over systemic IL-1 blockade. As an example, canakinumab was effective for the treatment of acute gouty arthritis, whereas rilonacept was not. In animal models, several studies targeting IL-1α and IL-1β by a vaccinal approach reinforce this hypothesis. Vaccines were obtained by linking mouse or human IL-1 cytokines to Virus Like Particles (VLPs).

TABLE 1 | Applications of anti-cytokine vaccinations in autoimmune diseases.

Cytokine target	Product	Diseases	Animal species	References	Clinical trial
mIL-1β	Mouse IL-1β peptides/KLH	Arthritis	Mouse	Bertin-Maghit et al., 2005	Pre-clinical
mIL-1 (α and β)	Mouse IL-1α/IL-1β/VLP	Arthritis	Mouse	Spohn et al., 2008	Pre-clinical
hIL-1β and mIL-1β	Human and murine IL-1β/VLP	Type 2 Diabetes	Mouse	Spohn et al., 2014	Pre-clinical
hIL-1β	Human IL-1β/VLP	Type 2 Diabetes	Human	Cavelti-Weder et al., 2016	Phase I
mIL-6	Modified mouse IL-6	Arthritis, MS	Mouse	Galle et al., 2007	Pre-clinical
	Mouse IL-6 peptide/KLH	SSc	Mouse	Desallais et al., 2014	Pre-clinical
	Mouse IL-6 peptide/KLH	DTH	Monkey	Desallais et al., 2016	Pre-clinical
hIL-15	Modified human IL-15	Arthritis	Monkey	Rodríguez-Álvarez et al., 2016	Pre-clinical
mIL-17A	Mouse IL-17A/Ova	MS	Mouse	Uyttenhove and Van Snick, 2006	Pre-clinical
	Mouse IL-17A/VLP	Arthritis, MS	Mouse	Röhn et al., 2006	Pre-clinical
	Mouse IL-17A/VLP	Autoimmune Myocarditis	Mouse	Sonderegger et al., 2006	Pre-clinical
mIL-18	Mouse IL-18 plasmid	SLE	Mouse	Bossù et al., 2003	Pre-clinical
mIL-23	Mouse IL-23p19 peptide/KLH	Arthritis	Mouse	Ratsimandresey et al., 2011	Pre-clinical
	Mouse IL-23p19 peptide/HBc Ag	Chronic Colitis	Mouse	Guan et al., 2013	Pre-clinical
hIFN-α	Human IFN-α/KLH	SLE	Hum. Transg. Mouse	Zagury et al., 2009	Pre-clinical
	Human IFN-α/KLH	SLE	Hum. Transg. Mouse	Mathian et al., 2011	Pre-clinical
	Human IFN-α/KLH	SLE	Human	Lauwerys et al., 2013	Phase I/II
	Human IFN-α/KLH	SLE	Human	Ducreux et al., 2016	Phase I/II
hTNF-α	Human TNF-α/KLH	Arthritis	Hum. Transg. Mouse	Le Buanec et al., 2006	Pre-clinical
		Arthritis	Hum. Transg. Mouse	Delavallée et al., 2008	Pre-clinical
		Arthritis	Hum. Transg. Mouse	Biton et al., 2011	Pre-clinical
		Arthritis	Mouse	Assier et al., 2012	Pre-clinical
		Arthritis	Hum. Transg. Mouse	Semerano et al., 2013	Pre-clinical
		Arthritis	Human	Durez et al., 2014	Phase IIa
mTNF-α	Modified mouse TNF-α	Arthritis, Cachexia	Mouse	Dalum et al., 1999	Pre-clinical
	Human TNF-α plasmid	Arthritis	Mouse	Shen et al., 2007	Pre-clinical
	Mouse TNF-α peptide/VLP	Arthritis	Mouse	Chackerian et al., 2001	Pre-clinical
	Mouse TNF-α/TNF-α peptide/VLP	Arthritis, Infections	Mouse	Spohn et al., 2007	Pre-clinical
	Mouse TNF-α peptide/KLH	Septic shock	Mouse	Capini et al., 2004	Pre-clinical
	Mouse TNF-α peptide/KLH	Arthritis	Mouse	Sun et al., 2016	Pre-clinical
	Mouse TNF-α peptide/DTT	Arthritis	Mouse	Zhang et al., 2016	Pre-clinical
rTNF-α	Rat TNF-α plasmid	Arthritis	Rat	Wildbaum et al., 2000	Pre-clinical
mVEGF-A	Mouse VEGF/VEGF peptide/KLH	Arthritis	Mouse	Semerano et al., 2016b	Pre-clinical

DTH, delayed-type hypersensitivity; DTT, transmembrane domain of diphtheria toxin; MS, multiple sclerosis; HBc Ag, hepatitis B core antigen; KLH, keyhole limpet hemocyanin; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; VLP, virus-like particles.

These compounds led to high production of anti-IL-1α or anti-IL-1β neutralizing antibodies by vaccinated mice. In the CIA model, both type of vaccines conduct to a higher protection against arthritis than daily administration of mouse IL-1Ra. In a second model of arthritis, the Collagen Antibody-Induced Arthritis model (CAIA), immunization with the IL-1β vaccine protects strongly against arthritis, whereas anti-IL-1α vaccine has no effect (Spohn et al., 2008). IL-1β vaccine was shown to be also effective in a type 2 diabetes model, and its human counterpart was well tolerated in a phase I clinical trial in patients with type 2 diabetes (Cavelti-Weder et al., 2016). In another study, IL-1β was specifically targeted by vaccines constituted

by peptides of IL-1β linked to KLH. Peptide sequences were chosen in regions interacting with the receptor and led to the generation of neutralizing anti-IL-1β antibodies. One IL-1β vaccine showed a protective effect in the CIA model on clinical and histological signs of inflammation (Bertin-Maghit et al., 2005). Thus, selective inhibition of IL-1β gave promising results and could be achieved with a peptide-based vaccine. However, targeting IL-1 or its receptor remains an unresolved issue, with contrasting animal and *in vitro* data arguing in favor of either specific cytokine or receptor blockade, but no conclusion that is relevant to the clinical use of these agents.

CANDIDATES FOR A VACCINE TARGETING IL-1

The best candidates are chronic diseases with flares, IL-1 dependent, and showing some improvement in previous clinical trials with currently used anti-IL-1 treatments. The low cost of development could allow treatments of patients in Southern countries, where access to healthcare is a concern for most of people. Research on vaccination targeting IL-1β is still ongoing. Preclinical studies with a peptide-based vaccine targeting IL-1β are currently focused on carrier protein and adjuvant, and clinical studies are planned. The use of vaccination targeting IL-1 extends now from initial rheumatic diseases, such as RA and crystal-induced arthritis (such as gout or acute chondrocalcinosis), to autoinflammatory diseases, such as systemic juvenile idiopathic arthritis (JIA), cryopyrin-associated periodic syndromes (CAPS), and familial Mediterranean fever (FMF). IL-1 inhibitors are also promising in two highly prevalent inflammatory diseases, encountered as co-morbidities in patients with rheumatic diseases, namely arteriosclerosis and type 2 diabetes.

AUTHOR CONTRIBUTIONS

EA and MB draft the manuscript. NB and JZ revised the manuscript.

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REVIEW
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The P2X7 Receptor-Interleukin-1 Liaison

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Interleukin-1β (IL-1β) plays a central role in stimulation of innate immune system and inflammation and in several chronic inflammatory diseases. These include rare hereditary conditions, e.g., auto-inflammatory syndromes, as well as common pathologies, such as type II diabetes, gout and atherosclerosis. A better understanding of IL-1β synthesis and release is particularly relevant for the design of novel anti-inflammatory drugs. One of the molecules mainly involved in IL-1β maturation is the P2X7 receptor (P2X7R), an ATP-gated ion channel that chiefly acts through the recruitment of the NLRP3 inflammasome-caspase-1 complex. In this review, we will summarize evidence supporting the key role of the P2X7R in IL-1β production, with special emphasis on the mechanism of release, a process that is still a matter of controversy. Four different models have been proposed: (i) exocytosis via secretory lysosomes, (ii) microvesicles shedding from plasma membrane, (iii) release of exosomes, and (iv) passive efflux across a leaky plasma membrane during pyroptotic cell death. All these models involve the P2X7R.

Keywords: interleukin-1β, P2X7 receptor, NLRP3 inflammasome, caspase-1, inflammation

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THE INFLAMMATORY PROCESS

Inflammation has been the object of countless studies and experimental observations since its definition more than 2000 years ago (Celso, De Medicina, 47 CE). Nevertheless, many aspects of this process are not fully understood, and therefore inflammation is still nowadays a field of extensive investigation, especially in view of its crucial role in the pathogenesis of many acute and chronic diseases. Accordingly, inflammation is a fertile ground of research for the development of novel drugs. Diverse chemical mediators with pro- or anti-inflammatory activity have been identified over the years. These range from histamine to bioactive lipids, e.g., prostaglandins and leukotriens, from free radicals, e.g., reactive oxygen species (ROS) and nitric oxide (NO), to cytokines, e.g., interleukins (ILs) and tumour necrosis factor (TNF). Among all these mediators, interleukin-1β (IL-1β) is recognized as one of the earliest and most potent pro-inflammatory agents synthesized and released in response to infectious agents and injuries, and therefore central to both septic and sterile inflammation (Gabay et al., 2010; Dinarello, 2011).

AN OVERVIEW ON INTERLEUKIN-1 (IL-1)

The term Interleukin-1 (IL-1), also known as leukocyte endogenous mediator, hematopoietin 1, endogenous pyrogen, catabolin and osteoclast activating factor, was used in the past to indicate a factor mediating many different pro-inflammatory and catabolic effects.

The history of IL-1 begins with studies on the endogenous factor produced by activated leukocytes that causes fever. As such, IL-1 was originally described in Menkin (1943), who reported the isolation of a pyrogenic euglobulin from inflammatory exudate named "pyrexin" or "endogenous pyrogen."

These initial studies were followed by the groundbreaking contributions of Beeson (1948) who confirmed Menkin's observation and further reported that an endotoxin-free, protein-containing material, released from rabbit peritoneal leukocytes, caused the rapid onset of fever after injection into rabbits. This was the first time in which the mechanism behind fever, in the absence of infection, was described. After Beeson's paper, there was a surge of studies on the links between infection/inflammation and fever, that culminated in the demonstration by Bodel and Atkins (1967) that human blood monocytes produced a pyrogen, similar to that released by rabbit neutrophils, by *de novo* synthesis.

Gery and Waksman (1972) described the effect on lymphocyte proliferation of soluble factors released in response to antigenic or mitogenic stimuli, and a few years later Dinarello and Bernheim (1981) purified the human leukocytic pyrogen from peripheral blood mononuclear cells (PBMCs) *in vitro* stimulated with heat-killed *Staphylococcus epidermidis*. Leukocytic pyrogen was also shown to enhance T cells responses to antigens and to promote synthesis of acute phase proteins (Kampschmidt et al., 1973).

Initially, the vast number of biological activities attached to a single molecule generated some confusion in the scientific community, however, with the cloning of IL-1 by Lomedico et al. (1984), the use of recombinant IL-1 established that IL-1 was indeed a pleiotropic cytokine mediating a great variety of inflammatory, as well as immunological, responses. Thanks to the seminal work of Dinarello, we now know that IL-1 is the founding member of a family of cytokines.

The IL-1 cytokine family consists of 11 members with different roles in inflammation. Seven of them, i.e., IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ, own well-demonstrated pro-inflammatory properties, whereas four members, IL-1Ra, IL-36Ra, IL-37 and IL-38, are anti-inflammatory (Garlanda et al., 2013; Borthwick, 2016). Cytokines of the IL-1 family ligate and activate specific plasma membrane receptors, the IL-1 receptor family, comprised of 10 members, named IL-1R1, IL-1R2, IL-1R3 (IL-1RAcP), IL-R4 (ST2), IL-1R5 (IL-18Rα), IL-1R6 (IL-R36), IL-1R7 (IL-18Rβ), IL-1R8 (SIGIRR: single Ig IL-1R-related molecule or TIR8: three Ig domain-containing IL-1R related), IL-1R9 (IL-33R), IL-1R10 (TIGIRR-1) (Garlanda et al., 2013).

Interleukin-1β (IL-1β), a crucial factor of host defense in response to infections and injuries, is the best characterized and most extensively studied member of the IL-1 family (Dinarello, 1996). In the last decade, IL-1β has also emerged as a causative agent and a therapeutic target for an expanding number of systemic and local inflammatory conditions named "auto-inflammatory diseases." The auto-inflammatory diseases include rare hereditary conditions as well as common pathologies. Recently, increasing evidence shows that the same pathogenetic mechanisms responsible for the activation of innate immunity in inherited auto-inflammatory diseases may also play a key role in sustaining inflammation in several frequent multifactorial pathologies, such as type II diabetes, gout, pseudogout, and atherosclerosis (Ginaldi et al., 2005).

Il-1β, usually not expressed by healthy resting cells, is mainly produced by activated inflammatory cells of the myeloid lineage. Production of IL-1β is a multistep process involving synthesis of immature pro-IL-1β, proteolytic cleavage to mature IL-β and, finally, release into the extracellular environment. Synthesis of the immature full-length pro-IL-1β is started with the recognition via Toll-like receptors (TLRs) of molecules derived from invading micro-organisms [pathogen-associated molecular patterns (PAMPs)] (Janeway, 2001). Once synthesized, the 31 kD pro-IL-1β undergoes a proteolytic cleavage catalyzed by caspase-1 (casp-1) which removes 116 N-terminal aminoacids to generate the 17 kD bioactive form, now ready to be secreted. If conversion to the 17 kD form does not occur, pro-IL-1β is polyubiquitinated and targeted for proteasomal degradation (Ainscough et al., 2014). Activation of casp-1, in turn, depends on assembly and activation of inflammasomes, multisubunit organelles that convert pro-casp-1 to active casp-1 (Thornberry et al., 1992; Martinon et al., 2002; Ogura et al., 2006).

The NLRP3 inflammasome has been investigated in depth and recognized as a very, likely the most, efficient machinery for pro-IL-1β maturation, and the biology of this cytokine has been intimately intertwined with that of the inflammasomes and of inflammasome-activating agents (Martinon et al., 2002; Di Virgilio, 2013). Inflammasomes are high molecular weight protein complexes assembled in the cytosolic compartment in response to a variety of stimuli, either of exogenous (PAMPs) or endogenous [danger/damage associated molecular patterns (DAMPs)] origin. PAMPs include bacteria- as well as virus derived components, whereas DAMPs encompass different classes of molecules normally segregated inside the cells (Venereau et al., 2015). DAMPs are released in response to invasion by micro-organisms (septic inflammation) as wells as to physical, chemical, metabolic non-infectious agents (sterile inflammation) (Gallucci et al., 1999). DAMPs released in the extracellular milieu fulfill the task of alerting surrounding cells, especially of immune lineages, of an incumbent danger or a damage (Venereau et al., 2015; Nie et al., 2016). Among DAMPs, extracellular ATP and other nucleotides play an undisputed role.

Nucleotide signaling is central in IL-1β maturation and release, as well as in other immune responses, such as neutrophil and macrophage chemotaxis, intracellular microbe killing, NADPH-oxidase activation, T lymphocyte proliferation and differentiation (Di Virgilio, 1995; Bours et al., 2006; Ferrari et al., 2006; Junger, 2011; Eltzschig et al., 2012; Idzko et al., 2014; Cekic and Linden, 2016). Extracellular ATP acts at plasma membrane purinergic P2 receptors, chiefly the P2X7 receptor (P2X7R) subtype, to drive NLRP3 inflammasome activation and IL-1β processing and release (Ferrari et al., 1997). ATP is released into extracellular environment during inflammation, ischemia, hypoxia, or other harmful events, via lytic (e.g., cell necrosis) or non-lytic (e.g., exocytosis, plasma membrane channels or pores) pathways. Pathways for non-lytic ATP release include pannexins (Dahl, 2015), connexins (Evans et al., 2006), ABC transporters (Cantiello, 2001), secretory vesicles (Sneddon and

Westfall, 1984; Wang et al., 2013), and the P2X7R (Pellegatti et al., 2005; Suadicani et al., 2006).

THE P2X7R

Several reports underscore the pivotal role of ATP-mediated P2X7R activation in IL-1β release from activated immune cells (monocytes, macrophages, and microglia) (Di Virgilio et al., 1998; Pelegrin et al., 2008; Sanz et al., 2009). Macrophages from genetically modified mice lacking the P2X7R, ASC or NLRP3, do not release IL-1β in response to ATP (Solle et al., 2001; Mariathasan et al., 2004, 2006). Moreover, oxidized ATP, an irreversible blocker of the P2X7R (Murgia et al., 1993) abrogates ATP-induced IL-1β release from immune cells (Ferrari et al., 1997). P2X7R stimulation also induces fast release into the cytosol of oxidized mitochondrial DNA (mitoDNA) that promotes NLRP3 inflammasome assembly by direct interaction (Nakahira et al., 2011; Shimada et al., 2012).

The P2X7R is a bi-functional ATP-gated plasma membrane ion channel that upon sustained stimulation undergoes a transition that generates a non-selective pore permeable to aqueous solutes of MW up to 900 Da (Di Virgilio, 2000). The P2X7R is widely distributed in human tissues, the highest expression being in cells of the immune and inflammatory systems, especially of the myeloid lineage (Di Virgilio, 1995, 2015; Karmakar et al., 2016). The P2X7R is the seventh, and latest to be cloned, member of the P2X receptor (P2XR) subfamily activated by an agonist concentration about 100 fold higher than the other members of the family. P2XRs are ATP-gated channels permeable to monovalent (Na^+, K^+) and divalent (Ca^{2+}) cations formed by the assembly of the same (homo) or different (hetero) P2X subunits. Six homomeric (P2X1R-P2X5R and P2X7R) and six heteromeric (P2X1/2R, P2X1/4R, P2X1/5R, P2X2/3R, P2X2/6R, and P2X4/6R) functional P2XRs have been described so far (Dubyak, 2007; North, 2016). Among P2X subunits, the P2X7 is generally thought not to assemble with the others, and thus forming P2X7 only homomeric channels. High sequence homology of P2X7R with the P2X4R (41% identity, 71% similarity), suggests a common origin by gene duplication. Therefore, the solved crystal structure for zebrafish P2X4R (Kawate et al., 2009; Hattori and Gouaux, 2012) has been used to model the 3D conformation of the P2X7R (Jiang et al., 2013). Useful insights as to ATP binding pocket, ion permeation pathway, site of antagonist binding and interaction with allosteric modulators are also derived from the crystal structure of the panda P2X7R (Karasawa and Kawate, 2016). Further information are ensued by recent 3D resolution of the human P2X3R (Mansoor et al., 2016).

The P2X subunits are characterized by a large extracellular loop, which includes agonist- and antagonist-binding sites, two short transmembrane domains, and intracellular N- and C-termini. The P2X7R with an extended C-terminal tail of 239 aa and an overall length of 595 aa, is the largest in the P2XR family. Transmembrane domains are responsible for the interactions among subunits and the formation of the ion-permeation pathway (Hattori and Gouaux, 2012; Grimes and Young, 2015). The intracellular C-tail interacts with different intracellular molecules such as heat shock proteins (HSP), cytoskeletal components, kinases and possibly also with membrane proteins. Among these latter, pannexin-1 and connexin-43 hemichannels have been variably implicated in the formation of the P2X7R-associated large-conductance pore and therefore in P2X7R-dependent IL-1β secretion, and in the release of extracellular ATP (Pelegrin and Surprenant, 2007; Baroja-Mazo et al., 2013). P2X7R has also been found to interact directly with components of inflammasomes, such as NLRP2, ASC (apoptosis-associated speck-like protein containing a CARD) and NLRP3 (Minkiewicz et al., 2013; Franceschini et al., 2015; Salaro et al., 2016). P2X7R activation by ATP is one of the most potent stimuli for NLRP3 inflammasome activation (Mariathasan et al., 2006; Munoz-Planillo et al., 2013).

THE NLRP3 INFLAMMASOME

Inflammasomes are cellular organelles with a fundamental role in inflammation and cell death (Martinon et al., 2002; Guo et al., 2015; Rathinam and Fitzgerald, 2016). The basic scaffold subunit is a nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) that contains a C-terminal leucine-rich repeat (LRR) domain, a central NACHT nucleotide-binding domain (NOD) and an N-terminal pyrin domain (a CARD domain in the NLRC4 inflammasome). The pyrin domain of the NLR scaffold subunit interacts with the pyrin domain of an adaptor molecule named ASC. NLR-driven ASC recruitment drives pro-casp-1 activation via CARD domains present on both ASC and pro-casp-1, thus resulting in pro-casp-1 cleavage and casp-1 activation. Casp-1 then cleaves pro-IL-1β and pro-IL-18 to produce the mature forms of both cytokines (Benko et al., 2008; Schroder and Tschopp, 2010; Broz and Dixit, 2016; Prochnicki et al., 2016). Inflammasomes play a cardinal role in innate immunity thanks to their ability to sense PAMPs and DAMPs (He et al., 2016a; Kim et al., 2016). Within the subfamily of NLRP inflammasomes (i.e., inflammasomes based on NLR scaffold molecules with an N-terminal pyrin domain) NLRP3 is currently enjoying the widest popularity as crucial sensor for a large number of danger signals and as the main platform for IL-1β processing. Activating stimuli for the NLRP3 inflammasome include bacterial toxins, flagellin, muramyl dipeptide, viral nucleic acids and fungal products, as well as endogenous components such as ATP, cholesterol crystals, monosodium urate, glucose and amyloid β, environmental pollutants, such as silica, asbestos or physical agents such as UV radiations (Kim et al., 2016).

The identity of the activating stimulus of the NLRP3 inflammasome has been a hot issue ever since its discovery. Nowadays there is basically general consensus on the key role played by K^+ efflux, which seems to be the final common pathway for many different agents (Munoz-Planillo et al., 2013). Most efficient NLRP3 activators include extracellular ATP, K^+ ionophores, and several extracellular crystals, all known to decrease the cytosolic K^+ level. The mechanism whereby these different agents lower K^+ is not entirely clear, but many converge

on P2X7R activation (Alves et al., 2014; Prochnicki et al., 2016). In fact, while P2X7R opening or nigericin, a carboxylic K^+ ionophore, directly allow K^+ efflux along its concentration gradient, the mechanism by which crystals, such as monosodium urate, deplete intracellular K^+ is obscure. To support the contribution of K^+ depletion, drugs inhibiting the Na^+/K^+-ATPase also trigger NLRP3 inflammasome activation (Walev et al., 1995; Munoz-Planillo et al., 2013). Albeit inhibition of Na^+/K^+-ATPase also causes plasma membrane depolarization, there is no evidence that depolarization itself may trigger P2X7R pore opening and/or IL-1β release (Di Virgilio, 2013). The central role of intracellular K^+ is further supported by the finding that a K^+ drop is also necessary to allow recruitment of the Nima-related kinase (NEK)7 protein to the NLRP3 inflammasome (He et al., 2016b). On the other hand, the mechanism by which the drop in the K^+ concentration drives NEK7 recruitment, NLRP3 inflammasome assembly and activation is utterly unknown.

The NLRP3 inflammasome can be also activated by a non-canonical pathway involving casp-11. Casp-11, and its human orthologs casp-4 and -5, function as cytosolic LPS sensors (Shi et al., 2014). Once activated by LPS, casp-11 induces cleavage of the plasma membrane channel pannexin-1 (Yang et al., 2015) producing two events consisting of K^+ efflux, that activates NLRP3, and release of ATP that acts as a P2X7R agonist to promote further NLRP3 activation and cell death (Yang et al., 2015). The casp-11/pannexin-1/NLRP3 inflammasome axis is proposed to promote IL-1β/IL-18 production (Yang et al., 2015). In addition, active casp-11 triggers pyroptosis via cleavage of Gasdermin D (GSDMD) leading to accumulation of free active N-terminal domains of this protein which disrupt cellular functions by forming plasma membrane pores (He et al., 2015; Vince and Silke, 2016). Casp-11-mediated cell death, like casp-1-induced pyroptosis, requires cleavage of the GSDMD pyroptotic factor (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). Casp-11 mediated cell death is indeed abrogated in GSDMD deficient cells and, although it is not clear if GSDMD is the terminal pyroptotic factor, its N-terminal domain released following casp-11-dependent cleavage is sufficient to cause pyroptosis (Kayagaki et al., 2015; Shi et al., 2015). It has been proposed that, since casp-1 is required for both pyroptosis and IL-1β cleavage, IL-1β is passively released alongside DAMPs following plasma membrane rupture (Vince and Silke, 2016). The finding that in macrophages lack of GSDMD has no effect on NLRP3-stimulated IL-1β processing by casp-1 but prevents IL-1β secretion (He et al., 2015; Shi et al., 2015), suggests that casp-1 is necessary for IL-1β cleavage whereas GSDMD is indispensable for its release. Recent findings have revealed that in human monocytes stimulated with LPS casp-4 and -5 act as key determinants in one-step non-canonical NLRP3 inflammasome activation culminating with IL-1β release (Vigano et al., 2015). This one-step pathway has been suggested to require Syk activity and Ca^{2+} influx due to CD14/TLR4-mediated LPS internalization (Vigano et al., 2015). NLRP3 activation and IL-1β release can also be driven by K^+ independent mechanisms involving ROS generation or RIPK1/FADD/casp-8 recruitment (Zhou et al., 2011; Heid et al., 2013; He et al., 2016a; Sanman et al., 2016). Converging experimental findings seem to rule out a role for cytosolic Ca^{2+} increases (Brough et al., 2003; Rada et al., 2014; Katsnelson et al., 2015). In some non-immune cells, e.g., astrocytes, IL-1β maturation has been reported to be due to P2X7-dependent NLRP2 stimulation via a process involving direct NLRP2, P2X7R, pannexin-1 interaction (Minkiewicz et al., 2013). Finally, IL-1β can also be processed independently of inflammasome/casp-1 activation, as shown in casp-1 deficient mice, where pro-IL-1β to IL-1β extracellular conversion is catalyzed by various neutrophil proteases such as elastase, proteinase-3, granzyme A and cathepsine G (Fantuzzi et al., 1997; Joosten et al., 2009).

P2X7R stimulation by itself has no or little effect on pro-IL-1β cytoplasmic accumulation, therefore cells need priming by agents that promote IL-1β gene transcription, which mainly occur via NFκB activation. Typical priming agents are bacterial lipopolysaccharide, zymosan and poly(I:C) (Ferrari et al., 1996; Facci et al., 2014).

IL-1β RELEASE

The canonical pathway for the export of cellular proteins into the extracellular space involves the ER and the Golgi apparatus that together form the endo-membrane system through which the vast majority of proteins are either targeted to the extracellular space or to specialized sub-cellular compartments. At variance with other cytokines, IL-1β lacks the conventional leader/signal peptide and therefore is not targeted to the conventional ER-Golgi secretory pathway (Rubartelli et al., 1990). This leads to IL-1β accumulation into the cytosol after translation on free ribosomes. Moreover, conversion of pro-IL-1 β to the mature form by inflammasomes also takes place in the cytosol. Therefore, release of mature IL-1 β requires non-classical mechanisms of export from the cytosolic compartment (Rubartelli et al., 1990; Wewers, 2004; Eder, 2009). A number of different possible mechanisms have been proposed (Dubyak, 2012) and summarized in **Figure 1**. They include exocytosis via secretory lysosomes (Andrei et al., 1999; Andrei et al., 2004), microvesicle shedding from plasma membrane (MacKenzie et al., 2001; Bianco et al., 2005; Pizzirani et al., 2007), release of exosomes (Qu et al., 2007), and, lastly, passive efflux across a leaky plasma membrane during pyroptotic cell death (Bergsbaken et al., 2009; Martin-Sanchez et al., 2016). The P2X7R has been implicated in all these processes.

EXOCYTOSIS OF IL-1β-CONTAINING SECRETORY LYSOSOMES

Rubartelli et al. (1990) presented the first evidence for a non-classical secretory pathway for IL-1β release. Blockade of protein transport and secretion through the ER-Golgi complex did not affect IL-1β release, thus pointing to the involvement of secretory lysosomes. Secretory lysosomes are unusual organelles found principally in hematopoietic cells with a dual-function, degradative and secretory (Blott and Griffiths, 2002). The exocytic process can be triggered by different stimuli among which ATP, possibly via the increase in the intracellular Ca^{2+}

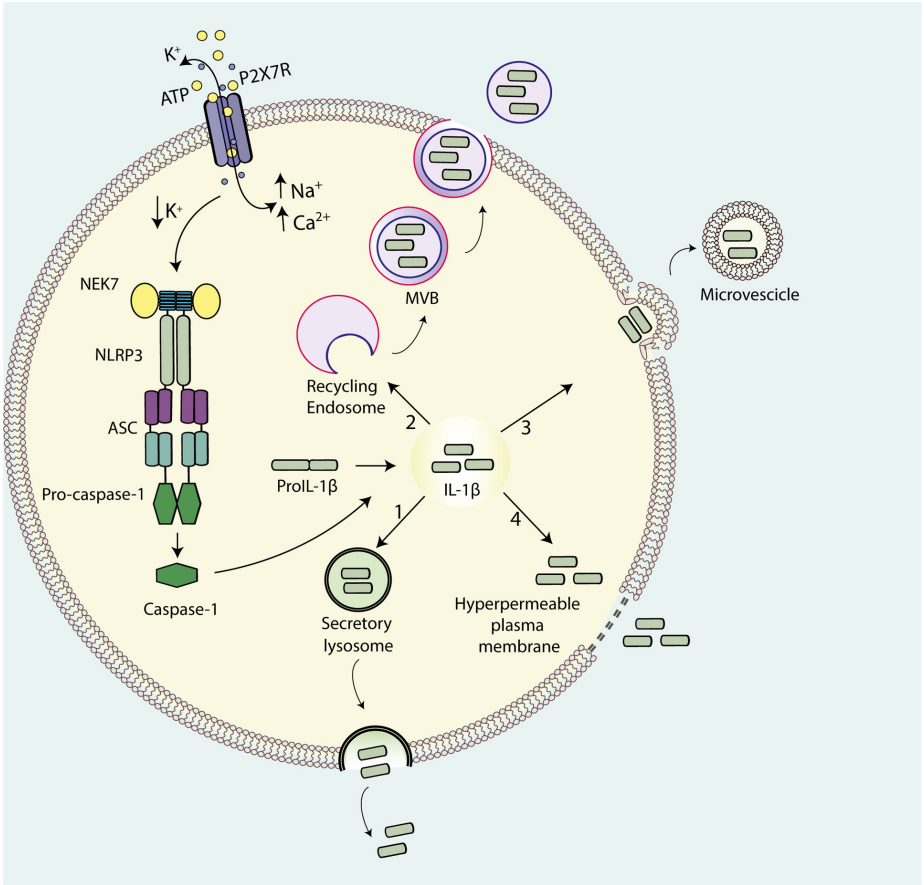

FIGURE 1 | Pathways for IL-1β release from activated immune cells. IL-1β maturation is catalyzed by ATP-mediated stimulation of the P2X7R that drives NLRP3 inflammasome assembly and casp-1 recruitment. Four models have been proposed for IL-1β release: (1) exocytosis of secretory lysosomes; (2) shedding of plasma membrane-derived microvesicles; (3) exocytosis of multivesicular body (MVB)-derived exosomes; (4) passive efflux across hyperpermeable plasma membrane during pyroptotic cell death.

concentration. Migration of exocytic lysosomes to the plasma membrane is a microtubule-dependent process that brings the lysosomes close to the plasma membrane allowing fusion and release of their content into the extracellular space. This model for IL-1β secretion is mainly based on morphological evidence from ATP-stimulated monocytes where IL-1β was found to be trapped within organelles akin to late endosomes and early lysosomes (Andrei et al., 1999). In human monocytes and mouse macrophages, ATP-stimulated, P2X7R-dependent release of mature IL-1β and casp-1 strongly correlated with secretion of the lysosomal markers cathepsin B, cathepsin D and lysosomal-associated membrane protein 1 (LAMP1) (Andrei et al., 1999; Carta et al., 2006). Both IL-1β and casp-1 are found in the extracellular medium 20 min after ATP stimulation, suggesting a similar time course. According to Rubartelli and coworkers a fraction of intracellular pro-IL-1β is co-stored together with pro-casp-1 within the secretory lysosomes, ready to be secreted in response to P2X7R stimulation (Rubartelli et al., 1990). The triggering stimulus is thought to be the P2X7R-induced loss of intracellular K^+, which activates a phosphatidylcholine-specific phospholipase C, which in turn causes an increase in cytosolic Ca^{2+}, Ca^{2+}-dependent phospholipase A_2 activation and finally exocytosis of the IL-1β-containing lysosomes. These events are blocked by inhibitors of phospholipase A_2 or phosphatidylcholine-specific phospholipase C. This model suggests that, whereas the massive K^+ efflux due to P2X7R activation has a key role in the maturation of pro-IL-1β, the intracellular Ca^{2+} increase is more directly responsible for IL-1β secretion.

SHEDDING OF IL-1β-CONTAINING PLASMA MEMBRANE MICROVESICLES

Surprenant and coworkers proposed a different vesicular mechanism for IL-1β release from THP-1 monocytes (MacKenzie et al., 2001). According to this mechanism, P2X7R stimulation induces mature IL-1β accumulation at discrete sub-plasmalemmal sites, where from it is then trapped into small plasma membrane blebs that are finally rapidly shed as microvesicles into the extracellular space (**Figure 2**). Microvesicle shedding is preceded by flip of phosphatidylserine

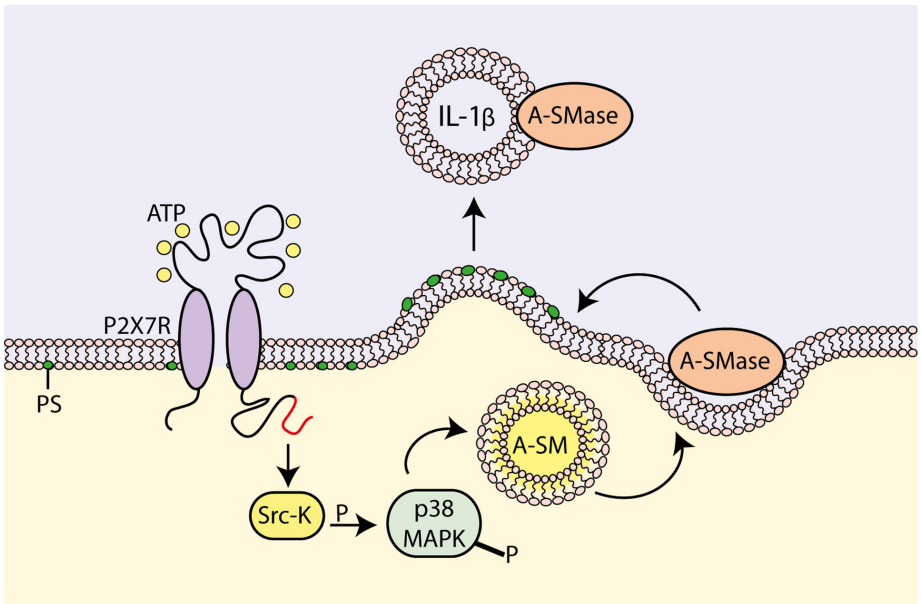

FIGURE 2 | Molecular mechanism for P2X7R-dependent microvesicle shedding. P2X7R activation promotes interaction of the C-terminal domain with a src-protein tyrosine kinase (Srk-K), which in turn phosphorylates P38 MAP kinase (P38 MAPK). P38 MAPK induces flip of acidic sphingomyelinase (A-SMase) from the inner to the outer plasma membrane leaflet. On the outer plasma membrane leaflet, A-SMase hydrolyzes sphingomyelin to generate ceramide that in turn alters membrane fluidity, drives formation of plasma membrane blebs and promotes shedding of IL-1β-containing microvesicles (modified from Bianco et al., 2009).

(PS) to the outer leaflet of the plasma membrane. Microvesicles size ranges from 200 nm to 1 μm, which makes them distinct from the much larger apoptotic bodies derived from apoptotic cells (1–4 μm size), and the smaller exosomes derived from intraluminal vesicles of endosomal multivesicular bodies (MVBs). A similar mechanism for IL-1β release has also been observed in human monocyte-derived dendritic cells (DCs) and mouse microglia (Bianco et al., 2005; Pizzirani et al., 2007). Shed microvesicles contain (a) plasma membrane phospholipids, e.g., PS (MacKenzie et al., 2001), (b) membrane intrinsic proteins, such as P2X7R, CD63, CD39, MHC-II, LAMP1 (Andrei et al., 1999; Pizzirani et al., 2007) and (c) cytoplasmic proteins, such as pro-IL-1β, pro-casp-1, IL-1β, casp-1, casp-3 and cathepsin D (Gudipaty et al., 2003; Andrei et al., 2004; Bianco et al., 2005; Carta et al., 2006; Pizzirani et al., 2007; Qu et al., 2007). It is not clear how and if IL-1β finally effluxes out of the microvesicles, thus fulfilling its role as an extracellular signaling molecule, or alternatively is delivered intracellularly following microvesicle fusion with the plasma membrane of target cells. Verderio and coworkers provided ample evidence showing that microvesicles released from P2X7R-stimulated microglia fuse with the plasma membrane of target cells (e.g., neurons), deliver their content and affect target cell responses (e.g., synaptic activity) (Antonucci et al., 2012; Turola et al., 2012; Verderio et al., 2012). We reported some time ago that microvesicles shed from P2X7R-stimulated DCs express the P2X7R and are lysed by exposure to extracellular ATP, thus releasing their cargo of IL-1β (Pizzirani et al., 2007). This observation led us to propose that IL-1β is released in the vicinity of the target cell plasma membrane by ATP-stimulated and P2X7R-dependent microvesicle rupture (Pizzirani et al., 2007). In fact, it is known that due to continuous ATP release into the extracellular space, cells are surrounded by an "ATP halo" that generates an ATP concentration higher in the vicinity of the plasma membrane than in the bulk solution. Thanks to this ATP gradient, microvesicle journey across the interstitial space should be relatively safe until they reach the target cell surface where they are supposed to find an ATP concentration sufficient to activate the P2X7R and trigger lysis.

EXOCYTOSIS OF IL-1β-CONTAINING EXOSOMES

In mouse bone marrow-derived macrophages (BMDMs) the main mechanism for non-classical IL-1β release has been reported to be neither secretory lysosomes nor microvesicle shedding, but rather P2X7R-stimulated MVBs formation and exosome release (Qu et al., 2007). Exosomes are small vesicles (30–100 nm) released upon fusion of MVBs with the cell plasma membrane. Exosomes originate as intraluminal vesicles during the process of MVBs formation. MVBs or late endosomes are components of the endocytic pathway that range from 250 to 1000 nm in diameter. MVBs can either be degraded or fuse with the plasma membrane, releasing the intraluminal vesicles into the extracellular space. Intraluminal vesicles are then referred to as exosomes following their extracellular release. During the process of formation, transmembrane and peripheral membrane proteins are incorporated into the exosome membrane, while cytosolic components are enclosed within the vesicles. Exosomes released from macrophages, DCs or B-lymphocytes contain

soluble proteins present in the cytosol, such as pro-IL-1β, pro-casp-1 and the respective mature form IL-1β and casp-1, and plasma membrane proteins such as MHCI and MHCII, a feature of exosomes derived from antigen presenting cells. From P2X7R-stimulated BMDMs two distinct types of membrane-bound vesicles are shed: (a) plasma membrane-derived microvesicles carrying P2X7R and LAMP1, and (b) MVB-derived exosomes lacking both P2X7R and LAMP1. However, both types of vesicles are able to present peptide-MHCII complexes to T cells (Ramachandra et al., 2010). Secretion of IL-1β and MHCII are strongly inhibited in mice deleted of ASC and NLRP3, suggesting the possibility that inflammasome complex regulate the formation of MVBs and the accumulation of IL-1β and casp-1, although the mechanism remains unclear (Qu et al., 2009).

IL-1β RELEASE AS A CONSEQUENCE OF PLASMA MEMBRANE DAMAGE AND CELL DEATH

A model for IL-1β release involving plasma membrane damage and cell death (whether by necrosis or apoptosis) has been proposed several years ago (Hauser et al., 1986; Hogquist et al., 1991). A major obstacle for the acceptance of this model is the need for proteolytical activation of pro-IL-1β, which is assumed to occur coordinately with its secretion, and the consistent observation that cytoplasmic mature IL-1β levels are very low (Perregaux et al., 1992). Of course, it is possible that extracellular proteases, e.g., trypsin or cathepsins might do the job, but *in vivo* relevance of extracellular pro-IL-1β maturation is dubious. However, in a recent paper, Pelegrin and co-workers have re-visited the cell permeabilization/cell death model for IL-1β release from BMDMs taking advantage of novel, highly sensitive, fluorescence-based technique to measure IL-1β secretion and of a novel inhibitor, punicalagin (Martin-Sanchez et al., 2016). Rigorous analysis of release of the cytoplasmic marker lactic dehydrogenase and of IL-1β revealed that the kinetics of two processes were closely over-imposed. Furthermore, punicalagin, a polyphenolic compound that efficiently prevents plasma membrane permeabilization in response to a number of membrane-perturbing agents, fully abolished ATP-dependent IL-1β secretion but not its processing, thus showing that pro-IL-1β cleavage and mature IL-1β secretion can be dissociated, and that a "leaky membrane" is needed for IL-1β release. Since casp-1 activation is also a major driver of pyroptotic cell death, Pelegrin and co-workers suggested that in macrophages IL-1β secretion occurs via a non-specific increase in plasma membrane permeability associated to cell death (Martin-Sanchez et al., 2016).

IS THE P2X7R-TARGETING A THERAPEUTICALLY LIVE OPTION?

Several studies show that P2X7R blockade efficiently antagonize IL-1β release in different disease experimental models (Bartlett et al., 2014). However, similar evidence from human studies is lacking. Measurement of serum IL-1 in autoimmune and autoinflammatory diseases is seldom significantly elevated, and is not thought to be a reliable indicator of inflammation (Dinarello, 2005). Therefore, it is not possible to verify in humans whether P2X7R blockade has any effect on IL-1β release. Assessing the *in vivo* effect of P2X7R blockade on IL-1, and in general, all cytokines, release, is made even more complex by the disappointing results of most clinical trials so far carried out (De Marchi et al., 2016; Jacobson and Muller, 2016).

CONCLUSION

Extracellular ATP is now acknowledged to be one of the earliest most ubiquitous DAMPs (Di Virgilio, 2013; Kepp et al., 2014; Hammad and Lambrecht, 2015; Venereau et al., 2015). Its remarkable efficiency and plasticity as an alarm signal strongly depends on the diverse of ATP-selective plasma membrane receptors expressed by immune cells. Very interestingly, even before all ATP receptors (P2 receptors) expressed by immune cells were cloned and fully characterized, it was clear that stimulation with extracellular ATP was able to cause a dramatic acceleration of pro-IL-1β processing and release from monocytes/macrophages, as well as from microglial cells, and this was very likely a receptor-mediated event (Perregaux and Gabel, 1994; Di Virgilio et al., 1996; Ferrari et al., 1996). About at the same time the P2X7R was cloned (Surprenant et al., 1996), and soon after identified as the molecule responsible for ATP-dependent mature IL-1β release (Ferrari et al., 1997). Thus, the association between IL-1β and the P2X7R is rock solid and long standing. However, this has not led to the introduction of any P2X7R-targeted anti-inflammatory therapy, despite large effort by virtually all major Pharma Industries. Are we missing some crucial information of P2X7R and IL-1β biology, or is there a recurrent fault in P2X7R-targeting drug design and development, or both?

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FDV coordinated writing and reviewed the MS. AG wrote sections of the MS. AS wrote sections of the MS. SF wrote sections of the MS.

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frontiers in Pharmacology

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Different Regulation of Interleukin-1 Production and Activity in Monocytes and Macrophages: Innate Memory as an Endogenous Mechanism of IL-1 Inhibition

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Production and activity of interleukin (IL)-1β are kept under strict control in our body, because of its powerful inflammation-promoting capacity. Control of IL-1β production and activity allows IL-1 to exert its defensive activities without causing extensive tissue damage. Monocytes are the major producers of IL-1β during inflammation, but they are also able to produce significant amounts of IL-1 inhibitors such as IL-1Ra and the soluble form of the decoy receptor IL-1R2, in an auto-regulatory feedback loop. Here, we investigated how innate immune memory could modulate production and activity of IL-1β by human primary monocytes and monocyte-derived tissue-like/deactivated macrophages *in vitro*. Cells were exposed to Gram-negative (*Escherichia coli*) and Gram-positive (*Lactobacillus acidophilus*) bacteria for 24 h, then allowed to rest, and then re-challenged with the same stimuli. The presence of biologically active IL-1β in cell supernatants was calculated as the ratio between free IL-1β (i.e., the cytokine that is not bound/inhibited by sIL-1R2) and its receptor antagonist IL-1Ra. As expected, we observed that the responsiveness of tissue-like/deactivated macrophages to bacterial stimuli was lower than that of monocytes. After resting and re-stimulation, a memory effect was evident for the production of inflammatory cytokines, whereas production of alarm signals (chemokines) was minimally affected. We observed a high variability in the innate memory response among individual donors. This is expected since innate memory largely depends on the previous history of exposure or infections, which is different in different subjects. Overall, innate memory appeared to limit the amount of active IL-1β produced by macrophages in response to a bacterial challenge, while enhancing the responsiveness of monocytes. The functional re-programming of mononuclear phagocytes through modulation of innate memory may provide innovative approaches in the management of inflammatory diseases, as well as in the design of new immunization strategies. In this respect, the interindividual variability in innate memory suggests the need of a personalized assessment.

Keywords: cytokines, inflammation, innate memory, interleukin-1, monocytes, macrophages

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INTRODUCTION

During the last several years, our knowledge on the interleukin (IL)-1 family molecules, as central mediators of innate immunity/inflammation and as "guilty" molecules of the development of autoinflammatory, autoimmune, infectious and degenerative diseases, has increased (Sims and Smith, 2010; Dinarello, 2011a,b, 2013; Dinarello and van der Meer, 2013; Garlanda et al., 2013a) The IL-1 family encompasses 11 cytokines/ligands and 10 related receptors (Dinarello et al., 2010; Boraschi and Tagliabue, 2013). Among the IL-1 family ligands, IL-1β is produced by mononuclear phagocytes in response to infectious or other stressful events, and initiates a potent defensive inflammatory response, while the structurally similar IL-1α is released only upon cell death and functions as an alarmin (Dinarello, 2011a; Rider et al., 2013). The IL-1-induced inflammation is regulated by a complex interaction of receptors and soluble inhibitors, whose concerted action determines the timing of activity and its shut-off. Both IL-1α and IL-1β bind to IL-1R1 and form an activating complex with the signaling chain IL-1R3. The receptor antagonist IL-1Ra binds to IL-1R1 receptor with high affinity, thereby competing with IL-1α and IL-1β, and does not recruit IL-1R3 (thus the complex is inactive). The other IL-1-binding receptor, the decoy receptor IL-1R2, can bind IL-1β and less efficiently IL-1α and IL-1Ra, and can recruit IL-1R3. However, the complex is inactive due to the lack of signal-initiating sequences in the intracellular domain of IL-1R2. The soluble forms of these receptors (sIL-1R1, sIL-1R2, sIL-1R3) have an inhibitory function by acting as ligand traps, and ensure a balance between amplification/activation of defensive responses and uncontrolled inflammation (Boraschi and Tagliabue, 2013; Garlanda et al., 2013b).

IL-1β is primarily produced by hematopoietic cells in response to various microbial stimuli, activated complement components, other inflammatory cytokines (e.g., TNF-α) and IL-1 itself. IL-1β is synthesized as a long inactive pro-form, which needs cleavage within the context of the inflammasome for being activated and then secreted (by non-conventional mechanisms) (Burns et al., 2003; Martinon et al., 2009; Monteleone et al., 2015). Activation of the inflammasome and of the IL-1 cleaving enzyme caspase-1 is therefore an additional mechanism controlling IL-1β-induced inflammation.

IL-1β production may vary, in innate immune cells, depending on the activation status of such cells. The concept of innate immune memory, i.e., the variation of innate reactivity in cells previously exposed to various stimuli, is a concept well known in invertebrates and also in vertebrates, which has been recently re-confirmed in higher vertebrates and humans (Kleinnijenhuis et al., 2012; Netea et al., 2016). A re-programming of innate immune cells can lead to decreased (tolerance) or enhanced (training) reactivity against reinfection by the same or different pathogens. Tolerance aims to avoid extensive tissue damage, whereas training aims to improve tissue surveillance, necessary to protect weakened tissues (Ifrim et al., 2014; Töpfer et al., 2015). It has been known for several decades that priming of mononuclear phagocytes with lipopolysaccharide (LPS) inhibits cellular functions in a process called LPS-induced tolerance (Dobrovolskaia and Vogel, 2002; Fan and Cook, 2004), whereas only recently it has been shown that priming with *Candida albicans* or the fungal cell wall component β-glucan can induce enhanced responses (Quintin et al., 2012). The effect of different types of bacteria (Gram-negative *vs*. Gram-positive) on the development of innate immune memory still remains unclear. While Gram-negative bacterial components such as LPS can lead to innate tolerance and a decreased response, exposure to Gram-positive Bacillus Calmette–Guérin leads to trained immunity and a more effective host immune response, accompanied by a reduced mortality to non-related infections (Quintin et al., 2014; Blok et al., 2015).

A series of recent *in vitro* and *in vivo* experiments has shown that pathogen-associated molecular patterns and a number of danger-associated molecular patterns induce innate immune memory (Netea et al., 2011; Crisan et al., 2016). Molecular and cellular mechanisms involved in this phenomenon are still not fully understood. It is believed that the main mechanism underlying enhanced responses involves epigenetic reprogramming, which in turn may entail altered pattern recognition receptors' expression, metabolic reprogramming, or/and altered cytokines release (Saeed et al., 2014; Bekkering et al., 2016b). Long-term epigenetic changes in monocytes and macrophages apparently involve both histone methylation and acetylation, as for instance H3K4 monomethylation and H3K27 acetylation induced by LPS (Ostuni et al., 2013; Saeed et al., 2014), and histone H3K4 trimethylation and H3K27 acetylation caused by β-glucan (Quintin et al., 2012; Saeed et al., 2014). Recently, it has been hypothesized that innate memory could also involve the modulation of expression of "latent and *de novo*" enhancers, microRNAs and/or long non-coding RNAs (Netea et al., 2016).

Unraveling the mechanisms at the basis of innate memory could lead to a better understanding of innate host defense and development of new immunization strategies and immunotherapies (Töpfer et al., 2015).

The aim of the present study was to investigate how innate memory can change the inflammatory reactivity of human monocytes and macrophages. Our study particularly focuses on changes in the levels of active available IL-1β produced by cells exposed and re-stimulated with Gram-positive and Gram-negative bacteria and bacterial components. This could set the stage for understanding how innate memory could be exploited for regulating IL-1 family ligands and receptors for innovative therapies of IL-1-mediated diseases, as well as new immunization strategies.

MATERIALS AND METHODS

Human Monocyte Isolation and Macrophage Differentiation

Human monocytes were isolated from fresh buffy coats of healthy donors recruited at the Blood Transfusion Center of Policlinico Hospital in Napoli. The national legislation does not require informed consent or ethical approval for the use of the anonymous, discarded buffy coats. Peripheral blood mononuclear cells (PBMC) were obtained by density

gradient centrifugation on Ficoll-Hypaque (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). Monocytes were isolated from PBMC by positive selection with human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (>95% pure) were cultured in RPMI-1640 medium (GIBCO, Life Technologies, Paisley, United Kingdom) containing 5% heat-inactivated human AB serum (Lonza, Walkersville, MD, United States) and 50 μg/ml gentamicin (Sigma-Aldrich, Inc., St. Louis, MO, United States) (culture medium). Cells were cultured at a density of 0.75×10^6 cells/ml/well in 12-well culture plates (Costar, Corning Inc., Corning, NY, United States) at 37°C in humid air with 5% CO_2. Monocyte stimulation was performed after overnight resting.

The average percentage of $CD14^{++}CD16^{-}$ (85.4%), $CD14^{++}CD16^{+}$ (2.8%), and $CD14^{dim}CD16^{+}$ (7.7%) monocyte subsets after purification by magnetic sorting fully reflected the percentage of the same subpopulations found in PBMC (78.1, 5.5, and 9.0%, respectively). Thus, the monocyte population used in our experiments was representative of the monocyte heterogeneity as present in the circulation and was similar for all the donors.

Freshly isolated monocytes were differentiated into tissue-like macrophages following a previously published protocol (Mia et al., 2014) with slight modifications. Monocytes were cultured in culture medium containing 50 ng/ml macrophage colony-stimulating factor (M-CSF) for 6 days (with one medium change on the third day). After 6 days, cells were additionally exposed for 24 h to M-CSF (50 ng/ml), IL-10 (20 ng/ml), and TGF-β (10 ng/ml), to generate tissue-like/deactivated macrophages (we will refer to these cells as "macrophages" throughout the text).

We have used IL-10 and TGF-β *in vitro* for reproducing the tissue microenvironment in which gut macrophages develop and reside. Gut resident macrophages contribute to maintaining tissue homeostasis by producing robust amounts of IL-10 (Saraiva and O'Garra, 2010; Maheshwari et al., 2011; Bain and Mowat, 2014; Kamada and Núñez, 2014). IL-10 also promotes the differentiation and maintenance of regulatory T (Treg) cells along with TGF-β produced by the intestinal epithelium upon contact with commensal bacteria. Similar conditions are present in other mucosal districts, and for this reason we generically define these *in vitro* differentiated macrophages as "tissue-like." The concentrations of IL-10 and TGF-β used were selected from dose–response experiments.

All cytokines and factors were obtained from R&D Systems (R&D Systems, Minneapolis, MN, United States). Macrophages were exposed to stimuli immediately after the 7-day differentiation process.

Phenotypical Characterization of Mononuclear Phagocytes

Freshly isolated human monocytes and *in vitro* generated macrophages were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD14, phycoerythrin (PE)-conjugated anti-CD64, anti-CD80, anti-CD206 (BD Biosciences, San Jose, CA, United States) and FITC-conjugated anti-CX3CR1 (BioLegend, San Diego, CA, United States). Monocytes were incubated with antibodies for 30 min at room temperature (RT), then fixed with 4% paraformaldehyde for 15 min at RT, and kept overnight in 4°C prior to analysis. Macrophages were harvested using Macrophage Detachment Solution DXF (PromoCell GmbH, Heidelberg, Germany), stained with antibodies for 30 min at RT and analyzed immediately. Appropriate isotype controls were used as negative controls. Samples were acquired with the BD FACSCanto™ II system (BD Biosciences). Prior to analysis, monocytes and macrophages were gated based on size forward scatter (FSC) and granularity side scatter (SSC) in order to eliminate other cell types, dead cells, or debris. Analysis was performed using the FlowJo v10.0.7 software (Tree Star, Inc., Ashland, OR, United States). Monocytes were $CD14^{+}$ $CD64^{+}$ $CX3CR1^{+}$ $CD206^{-}$ $CD80^{-}$, whereas macrophages were heterogeneous, with about 50% expressing CD14 and CD64, whereas in general they were positive for CX3CR1 and CD206, and negative for CD80.

In Vitro Stimulation of Monocytes and Macrophages

Monocytes and macrophages were exposed to different bacteria at a bacteria:cell ratio of 10:1. The bacteria:cell ratio was chosen from dose–response experiments. Bacteria used were heat-inactivated Gram-negative *Escherichia coli* (strain BL12-pLysE) and commensal Gram-positive *Lactobacillus acidophilus* (strain LPLANE20174). As positive control, LPS (10 ng/ml) from *E. coli* serotype O55:B5 (Sigma-Aldrich, Inc.) was used. Supernatants were collected after 24 h, centrifuged at $500 \times g$ for 5 min and stored at −80°C until analysis.

In Vitro Model of Innate Memory

Monocytes and macrophages were incubated with a low dose of LPS (1 ng/ml) or a low ratio of *E. coli* or *L. acidophilus* to monocytes/macrophages (0.1:1) for 24 h (priming), then supernatants were collected and cells maintained in culture medium for 6 additional days. Medium was changed after 3 days for monocytes and every second day for macrophages. After the resting period, supernatants were collected and cells were challenged with a higher dose of the same stimulus (10 ng/ml LPS, bacteria at a ratio of 10:1) for 24 h. Controls included unprimed cells (exposed only to the challenge) unchallenged cells (exposed only to the priming) and unprimed/unchallenged cells. Supernatants were centrifuged at $500 \times g$ for 5 min and stored at −80°C until analysis.

Cytokine Measurements

Levels of IL-1β, IL-1Ra, TNF-α, IL-8/CXCL8, and MCP-1/CCL2 were measured in monocyte and macrophage supernatants by ELISA (R&D Systems). The IL-1 family cytokines (IL-1α, IL-1β, IL-18, IL-33), and receptors and accessory proteins (sIL-1R1, sIL-1R2, sIL-1R3, sIL-1R4, IL-18BP) were measured using a multiplex assay technology and software custom-developed by Quansys Biosciences, Inc. (Logan, UT, United States). All measurements and analyses were performed according to the manufacturer's instructions. IL-1α, IL-18, IL-33, sIL-1R4, and

IL-18BP were minimally produced (data are not shown in Section "Results").

Assessment of Free and Active IL-1β

The calculation of free IL-1β in cell culture supernatants was done by applying the law of mass action, similarly to the calculation of free IL-18 (Novick et al., 2001; Migliorini et al., 2010). The calculation considers that in culture supernatants the levels of sIL-1R1 and sIL-1R3 are stable (data not shown) and that the concentrations of IL-1α are minimal. In these circumstances, the major soluble ligand of IL-1β is sIL-1R2, a molecule that has good affinity for IL-1β (2.7 nM) but low affinity for IL-1Ra (25 μM).

The law of mass action was therefore rearranged to account for the free ligand concentration ($[L_F]$, see below) according to Clark's theory (i.e., one ligand, one receptor, specific binding).

$$[L_F] = \frac{-[R_T] + [L_T] - K_d + \sqrt{([R_T] - [L_T] + K_d)^2 + 4[L_T] \times K_d}}{2}.$$

Where:

R_T: concentration of sIL-1R2 in pg/ml (MW 47 kDa).

L_T: concentration of IL-1β in pg/ml (MW 17 kDa).

K_d: dissociation constant (2.7 nM; Symons et al., 1995).

Active IL-1β was calculated as a ratio between free IL-1β (calculated as above) and IL-1Ra, multiplied by 1000.

Active IL-1β = (free IL-1β/IL-1Ra) × 1000.

Statistical Analysis

All values were expressed as mean ± SD. Mann–Whitney *U*–test or independent samples *t*-test were employed to compare results from different treatments. Statistics was analyzed using the GraphPad Prism 6 software. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Reactivity of Human Monocytes and Macrophages to Gram-Positive and Gram-Negative Bacteria

The response of human monocytes and macrophages to stimulation with bacteria *in vitro* was measured in terms of production of inflammatory cytokines (IL-1β, TNF-α) and chemokines (CXCL8, CCL2). As shown in **Figure 1**, monocytes produced higher amounts of cytokines compared to macrophages from the same donor after exposure to bacterial LPS, and whole bacteria *E. coli*, and *L. acidophilus*. More specifically, response to LPS was 4.1- and 7.3-fold higher in terms of TNF-α and IL-1β production, respectively, while responses to *E. coli* were 3.5- and 14.1-fold higher, and those to *L. acidophilus* were 21.1- and 86.0-fold higher. Thus, macrophages produce much less IL-1β than monocytes, whereas the difference in terms of TNF-α production is less evident. Even less evident is the difference in chemokine production, with monocytes producing 3.5- and 1.4-fold more CXCL8 and CCL2 in response to LPS, 2.0-fold more in response to *E. coli*, and 2.0- and 3.5-fold more in response to *L. acidophilus*. Thus, while overall the responsiveness of tissue-like/deactivated macrophages to bacterial stimuli is limited, when compared to monocytes, it is obvious that the capacity of producing alarm signals such as chemokines is less affected than the ability to produce inflammatory/destructive factors.

Innate Memory Is Not Stimulus-Specific

An *in vitro* model based on human primary mononuclear phagocytes was set up, in order to study the development of innate memory upon microbial stimulation. Priming was performed by exposing cells to a low concentration of the stimulus that was later used at higher concentrations as challenge (**Figure 2**). To confirm the notion that innate memory is not stimulus-specific (i.e., that the priming stimulus does not need to be the same as the challenge agent) we have performed preliminary experiments by assessing the generation of innate memory *in vitro* upon homologous *vs*. cross-stimulation. In the representative experiment shown in **Figure 3**, the generation of memory was assessed by measuring the production of TNF-α by monocytes primed with either one of two microbial agents (bacterial LPS, yeast Zymosan) in response to challenge with the same or with the other agent. Data in **Figure 3** show that, compared to unprimed monocytes, primed cells respond to challenge with either agent with a decrease of TNF-α production. Such decrease depends on the dose of the priming agent but it is independent of its nature. Thus, priming with LPS induced TNF-α decrease in response not only to the homologous LPS challenge but also to the unrelated challenge with Zymosan, and *vice versa*. Having confirmed the notion of lack of specificity in innate memory, in this study we have performed the memory experiments using the same stimulus for both priming and challenge.

Development of Innate Immune Memory: Re-programming of Inflammatory Reactivity

The generation of memory was assessed by measuring the production of TNF-α and CCL2 by primed cells in comparison to the levels of cytokines produced by unprimed cells using the same stimulus for priming and challenge (i.e., LPS, *E. coli*, and *L. acidophilus*; **Figure 4**).

Primed monocytes secreted less than half the amount of TNF-α compared to unprimed cells, independently of the stimulus (LPS or whole bacteria). In the case of CCL2, the donor-to-donor variation in the monocyte response was evident, with a decrease of production observed in response to LPS (donor 1), and little/no variation (bacteria in donor 1, LPS and *E. coli* in donor 2), or a significant increase (*L. acidophilus* in donor 2) in other cases. The situation is different in macrophages, in which the decrease in cytokine production due to memory is much less evident, and in general clear only in the case of LPS and practically absent with *L. acidophilus*.

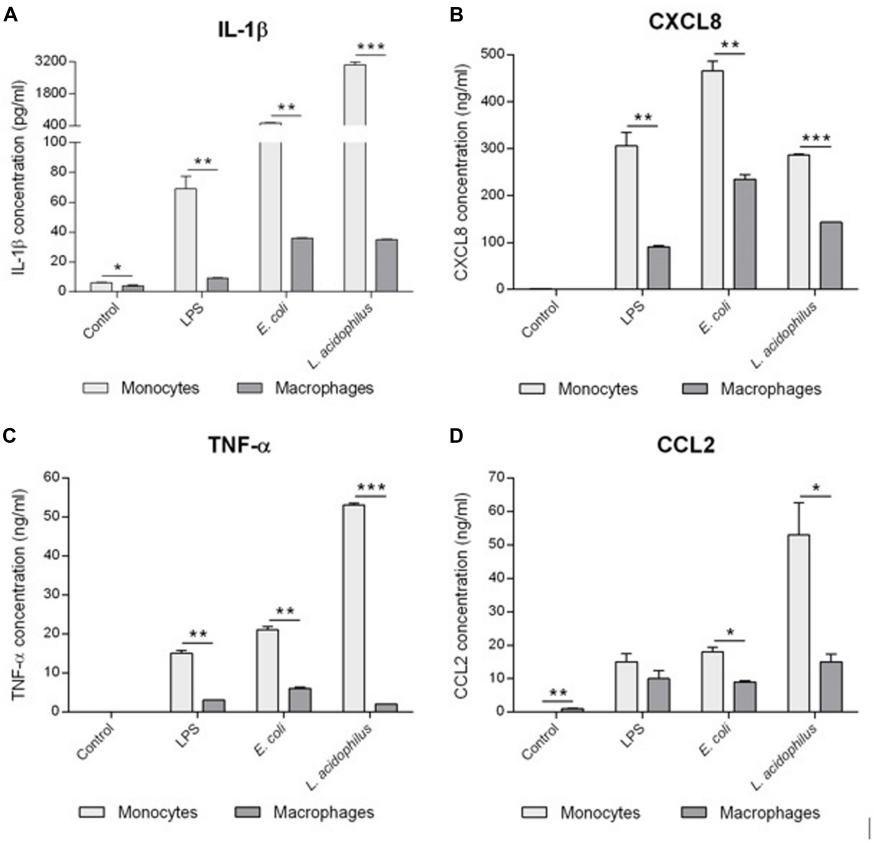

FIGURE 1 | Reactivity of human monocytes and macrophages to bacteria. Human monocytes and monocyte-derived macrophages were cultured in the absence or presence of LPS (10 ng/ml), *E. coli* or *L. acidophilus* (bacteria:cell ratio 10:1) for 24 h. The production of IL-1β **(A)**, CXCL8 **(B)**, TNF-α **(C)**, and CCL2 **(D)** was determined in the supernatant by ELISA. Data from one representative donor. Values shown are means of two independent determinations. The independent samples *t*-test was used to detect significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The difference between controls and treatments are all statistically significant. The *P*-value is not indicated to avoid overwriting the figure.

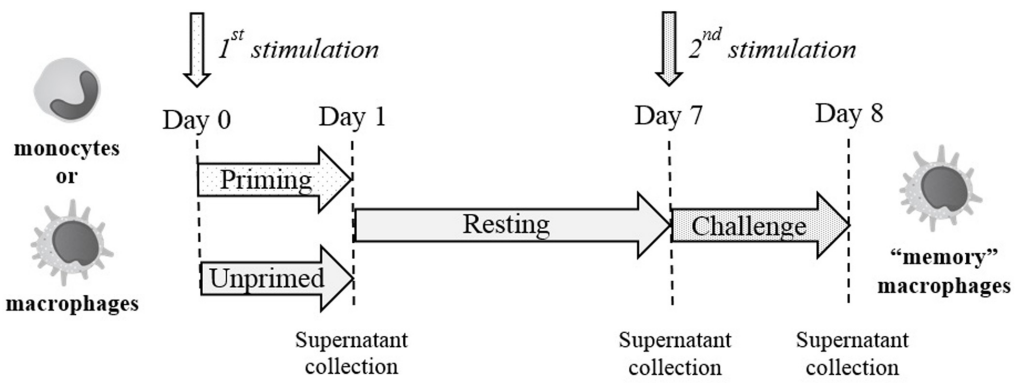

FIGURE 2 | Schematic representation of the *in vitro* model for generating innate memory.

Innate Memory Can Modulate IL-1 Production

We have examined more in detail the effect of innate memory on the production of cytokines of the IL-1 family by monocytes and macrophages. Here we mainly focus on the IL-1 system, i.e., the agonist cytokines IL-1α and IL-1β, their receptor antagonist IL-1Ra, and the soluble receptors sIL-1R1, sIL-1R2, and sIL-1R3. IL-1α was minimally produced in our culture conditions (data not shown). Indeed this cytokine may be detected in cell culture supernatants of stressed cells that undergo necrosis or

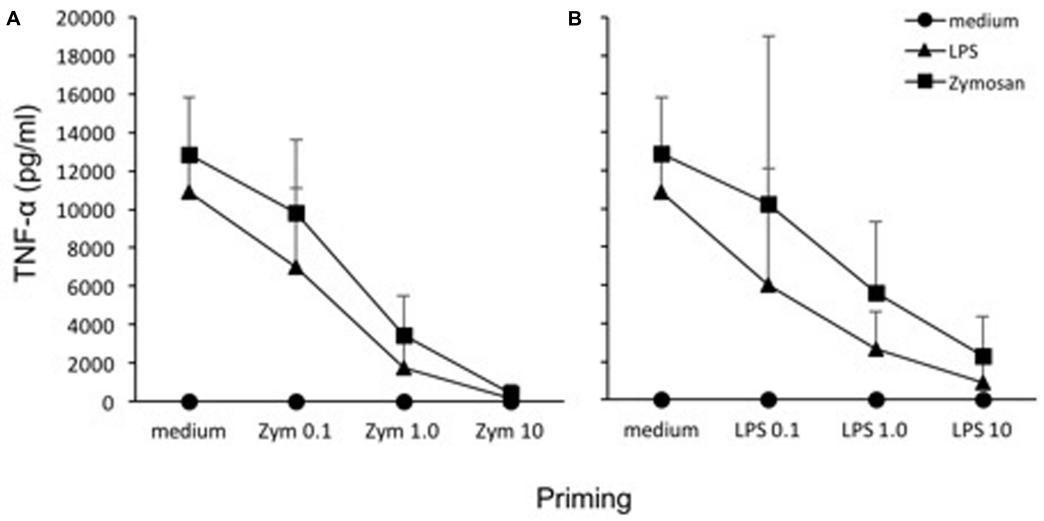

FIGURE 3 | Lack of specificity of innate memory. Monocytes were primed with different doses of Zymosan (0.1, 1, 10 μg/ml) **(A)** or LPS (0.1, 1, 10 ng/ml) **(B)** for 24 h, rested for 6 days, and challenged for 24 h with high doses (10 ng/ml LPS, 10 μg/ml Zymosan) of the same or different stimulus. Production of TNF-α was measured by ELISA. The values were expressed as average ± SD of $n = 3$ donors. The Mann–Whitney *U*-test was used to detect significant difference. The difference between controls and treatments are all statistically significant ($P < 0.001$), except at the highest priming concentration.

pyroptosis (Dinarello, 2013). IL-1β is also minimally produced by unstimulated mononuclear phagocytes (data not shown). On the other hand, macrophages constitutively produced around 35-fold higher amounts of IL-1Ra than monocytes (data not shown). Regarding the soluble receptors, monocytes released amounts of sIL-1R1 and sIL-1R3 comparable to those of macrophages, whereas macrophages released more sIL-1R2 than monocytes (**Table 1** and data not shown). Priming of monocytes or macrophages with low doses of stimuli did not significantly affect the release of soluble IL-1R1 and IL-1R2 after challenge (**Table 1**). A lack of memory was also observed for sIL-1R3, although its levels were significantly variable between donors (data not shown).

Production of IL-1Ra was strongly affected by priming of monocytes with bacterial stimuli (**Figure 5**). In monocytes primed with LPS, a challenge with a higher LPS concentration resulted in 80–90% inhibition of IL-1Ra production as compared to unprimed cells. The "tolerance" was evident also with *E. coli* as stimulus, whereas with *L. acidophilus* there was variability among donors (with 1/3 actually showing increased IL-1Ra production). In contrast, no substantial changes in IL-1Ra production were observed in macrophages, except for pre-treatment with LPS for one donor (**Figure 5**).

To better evaluate the functional significance of modulation of IL-1 family cytokines and receptors consequent to memory generation, we have calculated the actual presence of free biologically active IL-1β. First, we have measured by ELISA the production of immunoreactive IL-1β produced by monocytes and macrophages (**Figures 6A,B**), and in the same samples we have measured the concentration of produced sIL-1R2 (data not shown) and of IL-1Ra (**Figure 5**). Free IL-1β was calculated according to the law of mass action, based on a stoichiometric 1:1 ratio between the cytokine and sIL-1R2 and a binding affinity of 2.7 nM. To have a measure of active IL-1β, i.e., the amount of free IL-1 that is not blocked by IL-1Ra, we have used the ratio between free IL-1β and IL-1Ra (**Figures 6C,D**).

Unprimed monocytes produced low levels of active IL-1β (**Figure 6C**). Upon challenge, active IL-1β did not change significantly in monocytes primed with LPS, whereas priming with *E. coli* induced an increase of active IL-1β in 2/3 donors (no change in donor 3). Eventually, memory induced by *L. acidophilus* resulted in variable donor-dependent effects on active IL-1β levels (either decreased or increased production).

Unprimed macrophages produced in general similar or higher levels of active IL-1β than monocytes in response to the various stimuli (**Figure 6D**). Memory induced by LPS caused a significant decrease of active IL-1β. Priming with *E. coli* significantly decreased the levels of active IL-1β in one donor (the one that showed a high reaction to challenge), whereas macrophages from the other donors had a low reactivity that was not significantly changed by priming. Similar to monocytes, memory induced by *L. acidophilus* in macrophages resulted in variable donor-dependent effects on active IL-1β levels. Thus, LPS-induced memory can decrease active IL-1β in macrophages but not in monocytes, while *E. coli*-induced memory increased active IL-1β in monocytes and could decrease it in macrophages, and *L. acidophilus*-induced memory variably affected the levels of active IL-1β in both cell types.

It is noteworthy that when looking at IL-1β (**Figures 6A,B**), LPS-induced tolerance is evident in both monocytes and macrophages of donors 1 and 2 (values of donor 3 were too low). But when the levels of active IL-1β were calculated, i.e., when considering the concomitant production of inhibitors, this decrease is not evident any longer except in one case (macrophages of donor 2). Thus, the control of IL-1β activity is more complex than simple decrease of cytokine production

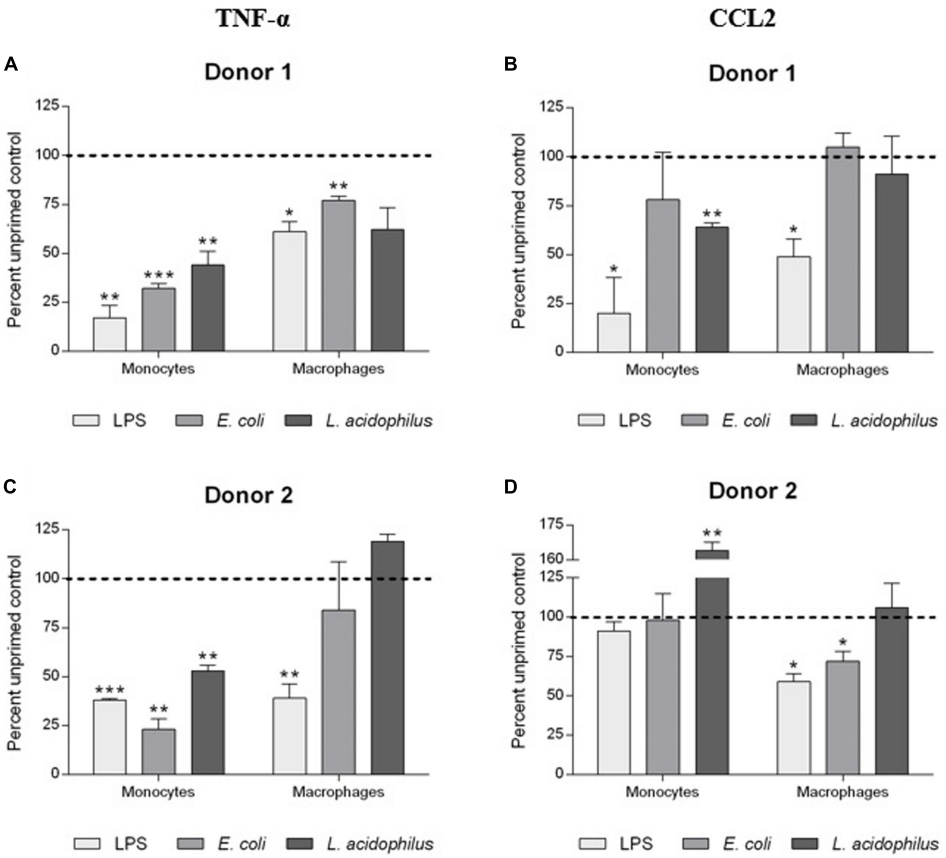

FIGURE 4 | Development of innate memory in monocytes and macrophages. Monocytes and monocyte-derived macrophages were primed with low doses of LPS (1 ng/ml), *E. coli* (0.1:1), or *L. acidophilus* (0.1:1) for 24 h, rested for 6 days, and challenged for 24 h with high doses of the same stimuli (10 ng/ml LPS, *E. coli* and *L. acidophilus* at 10:1). Production of TNF-α **(A,C)**, and CCL2 **(B,D)** was measured by ELISA. Data are presented as percentage of the cytokine amount produced by unprimed cells (dotted line). Representative data from two different donors are shown. Values shown are means of two independent determinations for each donor. Absolute mean TNF-α values upon stimulation with LPS were 1.56 and 2.56 ng/ml for monocytes of donors 1 and 2, respectively; and 1.15 and 20.92 ng/ml for macrophages of the two donors. Absolute mean CCL2 values were 14.91 and 11.17 ng/ml for monocytes of donors 1 and 2, while for macrophages values were 14.95 and 7.91 ng/ml. The independent samples *t*-test was used to analyze statistically significant differences. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and depends on the concomitant modulation of other regulatory factors.

DISCUSSION

Regulation of the activity of IL-1β is of paramount importance for obtaining optimal defensive reactions without causing excessive tissue damage. Indeed, the mechanisms of IL-1 regulation are multiple and at all levels, from regulation of its maturation/secretion to regulation of its activity both at the receptor binding level and at the level of post-receptor signaling. This kind of regulation reflects the powerful biological activity of IL-1β that, if not properly controlled, can be at the basis of numerous inflammatory and degenerative diseases (Dinarello, 2011a,b).

In this study, we have investigated a novel mechanism of IL-1β regulation, through the generation of innate memory.

Several studies have recently revived the old concept of innate immune memory, i.e., the ability of innate immune cells to "remember" previous encounters with microorganisms or foreign agents by changing their reactivity to a subsequent challenge with the same or with a different agent (Netea et al., 2011; Saeed et al., 2014; Gardiner and Mills, 2016). The concept, well known in invertebrates and also in mice, applies also to human innate immune cells *in vivo* and *in vitro* (Quintin et al., 2012; Arts et al., 2015; Bekkering et al., 2016a; van der Valk et al., 2016). In our study, we have used an *in vitro* system, based on human primary monocytes and tissue-like/deactivated monocyte-derived macrophages from the same donors, to examine the role of innate memory in determining the production of active IL-1β. We have used as stimuli two bacteria, the Gram-negative *E. coli* and the Gram-positive *L. acidophilus*, and a molecule derived from *E. coli*, i.e., its cell wall LPS. Data presented here were obtained by using the same stimulus for both priming and challenge of cells in culture. However, preliminary data of cross-stimulation confirm the notion that innate memory is not stimulus-specific (**Figure 3**).

TABLE 1 | Production of sIL-1R1 and sIL-1R2 by monocytes and macrophages upon challenge with bacterial stimuli.

Stimulus	sIL-1R1 (pg/ml)		sIL-1R2 (pg/ml)	
	Unprimed	Primed	Unprimed	Primed
Monocytes				
medium	63.1 ± 2.6		156.1 ± 39.0	
LPS	53.9 ± 16.5	47.9 ± 8.3	155.7 ± 77.4	130.8 ± 83.2
E. coli	56.1 ± 23.5	48.1 ± 19.9	144.7 ± 51.9	174.3 ± 56.9
L. acidophilus	51.6 ± 13.8	53.5 ± 19.7	136.3 ± 49.6	196.3 ± 33.5
Macrophages				
medium	44.9 ± 14.6		1409.9 ± 952.3	
LPS	61.9 ± 9.1	54.7 ± 11.4	1101.6 ± 482.5	596.9 ± 192.6
E. coli	58.5 ± 14.9	57.1 ± 18.1	998.7 ± 191.9	1041.4 ± 131.9
L. acidophilus	53.4 ± 12.1	58.7 ± 9.1	1092.9 ± 544.9	1126.9 ± 630.4

The values were expressed as average ± SD of n = 3 donors. Two independent determinations for each donor were performed. No statistically significant differences were detected with Mann–Whitney U-test.

The first observation we made is that monocytes are more reactive than macrophages to microbial stimuli in terms of production of inflammatory cytokines and chemokines (**Figure 1**). Although this was expected, it is noteworthy that while the difference in the production of inflammatory cytokines (IL-1β, TNF-α) is significant (up to almost 100×), there is much less difference in the production of chemokines (CXCL8, CCL2). Weaker response of macrophages in terms of inflammatory effector cytokines but almost normal response in terms of alarm signals (such as chemokines) is in line with the sentinel role of tissue-resident macrophages, which should be active in producing chemokines such as CXCL8 and CCL2, aiming at recruiting effector cells (e.g., neutrophils and blood circulating monocytes), but not directly involved in the very early effector phase of inflammation, in order to avoid uncontrolled tissue damage (Davies et al., 2013).

The innate immune memory appears as a decreased (tolerance) or increased response (trained innate immunity) to a second immune challenge. Both tolerance and trained innate immunity seem to be the result of long-term epigenetic changes in monocytes and macrophages, and the ability of these cells to "remember" is actually dependent on epigenetic changes. All the epigenetic changes and molecular mechanisms at the basis of memory eventually result in lower or higher transcription levels in several genes, such as pathogen recognition receptors, signaling molecules, and cytokines, in a short window of time. For example, priming of human monocytes with IFN-γ or IL-10, or the interaction with microbial components, alters the receptor repertoire expressed by monocytes/macrophages, e.g., changes in TLR4 and MD2, and in Dectin-1 and MARCO in macrophages (Bosisio et al., 2002; Willment et al., 2003), or in signaling molecules such as MyD88 (Bosisio et al., 2002). These changes in the number of receptors and the regulation of signaling drive a different production of effector molecules, such as inflammatory and anti-inflammatory cytokines. Although in this study we did not investigate the molecular mechanisms underlying the difference of responsiveness between monocytes and macrophages in terms of cytokine production, it is possible to speculate that the two cell types may undergo different epigenetic changes. In fact, although macrophages in this study are derived from monocytes, the *in vitro* differentiation protocol with deactivating cytokines and growth factors yields a cell population that differs from monocytes also in terms of the level of innate receptors (Ifrim et al., 2014). It is plausible

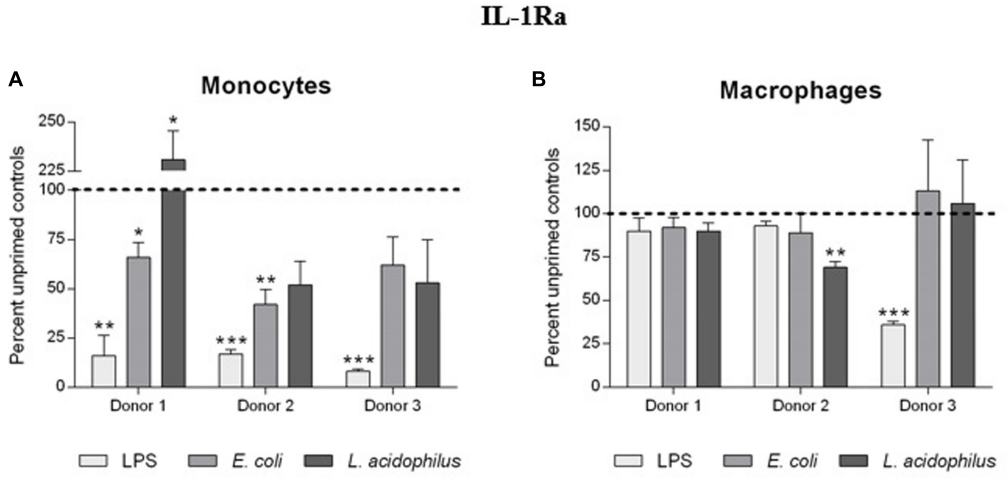

FIGURE 5 | Influence of innate memory on the production of IL-1Ra by monocytes and macrophages. Monocytes and monocyte-derived macrophages were primed with low doses of LPS (1 ng/ml), *E. coli* (0.1:1) or *L. acidophilus* (0.1:1) for 24 h, rested for 6 days, and challenged for 24 h with high doses of the same stimuli (10 ng/ml LPS, *E. coli* and *L. acidophilus* 10:1). Production of IL-1Ra by monocytes **(A)** and macrophages **(B)** was measured by ELISA. Data are presented as percentage of the cytokine amount produced by unprimed cells (dotted line). Representative data from three different donors are shown. Values shown are means of two independent determinations for each donor. Absolute mean values of IL-1Ra production upon LPS stimulation were 43.73, 36.78, and 14.21 ng/ml for monocytes of donors 1, 2, and 3, respectively; IL-1Ra produced by macrophages of the same donors in response to LPS was 66.62, 146.67, and 43.99 ng/ml. The independent samples *t*-test was used to analyze statistically significant differences. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

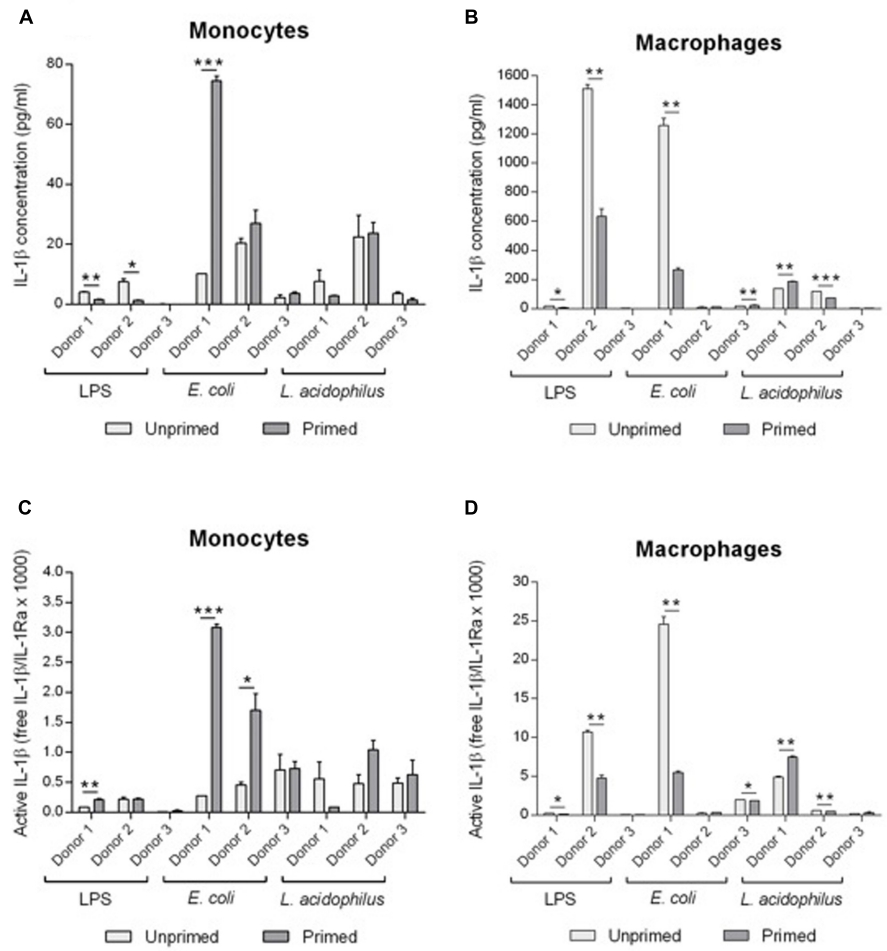

FIGURE 6 | Modulation of active IL-1β levels by innate memory. Monocytes and monocyte-derived macrophages were primed with low doses of LPS (1 ng/ml), *E. coli* (0.1:1) or *L. acidophilus* (0.1:1) for 24 h, then rested for 6 days, and challenged for 24 h with high doses of the same stimuli (10 ng/ml LPS, *E. coli* and *L. acidophilus* 10:1). The concentrations of IL-1β **(A,B)** were: for donor 1, unprimed monocytes have a value of about 0.28 pg/ml and macrophages 24.59 pg/ml; for donor 2 monocytes have 0.46 pg/ml and macrophages 0.23 pg/ml; for donor 3 monocytes have 0.71 pg/ml and macrophages 2.39 pg/ml. Free IL-1β was calculated by applying the law of mass action, and active IL-1β **(C,D)** was determined as a ratio between free IL-1β and its antagonist IL-1Ra. Representative data from three different donors are shown. Values shown are means of two independent determinations for each donor. The independent samples *t*-test was used to detect significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

that chromatin modifications occur during the differentiation process, and, when macrophages are primed and challenged, this is translated in a transcription programs different from that of monocytes.

In our *in vitro* model of innate memory, we observed two important facts. First, that also in the case of memory there is little effect on chemokine production, suggesting that the ability of cells to send alarm signals should not be hampered. A second observation is that memory is not induction of tolerance or induction of enhanced responses. In fact, the same stimulus can induce decreased or increased responses in primed cells depending on the endpoint measured (production of one or another of the various inflammation-related factors) and on the cell type examined (monocytes *vs.* macrophages). Thus, innate memory is a complete re-programming of the reactivity of cells rather than a decreased or enhanced responsiveness.

We focused on how innate memory could affect the production of active IL-1β. For doing this we have separately measured the production of IL-1β and of a number of soluble factors, concomitantly produced by monocytes and macrophages, that could affect its biological activity. The inflammatory activity of IL-1β is similar to that of IL-1α, which, however, is a cytokine mostly active intracellularly or as a cell-associated factor in the cell–cell communication (Kim et al., 2013; Di Paolo and Shayakhmetov, 2016; Bertheloot and Latz, 2017). Indeed, we found that IL-1α was practically absent in the supernatants of monocyte and macrophage cultures stimulated with the various stimuli (data not shown). Thus, the IL-1-like activity in these supernatants can be exclusively attributed to IL-1β. It is known that the soluble form of IL-1R2 is a major inhibitor of IL-1β, as it can capture it in solution with high affinity (2.7 nM), while unable to bind with sufficient affinity

either IL-1α (1.6 μM) or IL-1Ra (25 μM) (Symons et al., 1995). Thus, sIL-1R2 can be considered as a specific inhibitor of IL-1β. Monocytes and macrophages also produce significant levels of the soluble forms of the other two IL-1 receptor chains, i.e., sIL-1R1 and sIL-1R3. The IL-1-binding sIL-1R1 possibly binds IL-1Ra with higher affinity than IL-1α and IL-1β (Arend et al., 1994), while sIL-1R3 might form complexes with sIL-1R2 of higher affinity for IL-1α and IL-1β but not IL-1Ra (Smith et al., 2003). In these circumstances, since the levels of both sIL-1R1 and sIL-1R3 were constant in monocyte and macrophage supernatants and did not depend on stimulation or memory (**Table 1** and data not shown), we decided to exclude them from the calculation of IL-1β activity. Thus, we have limited the calculation to two elements, IL-1β and sIL-1R2, binding to each other at a 1:1 stoichiometric ratio and with a known affinity (2.7 nM). It was therefore possible to apply the law of mass action to such interaction and to calculate the amount of free IL-1β, i.e., the amount of cytokine not blocked by sIL-1R2 and therefore available for activating target cells by binding to membrane receptors. However, since the presence of IL-1Ra is expected to compete with free IL-1β for receptor binding, we have also taken into account its presence, in order to have a more thorough evaluation of biologically active IL-1β. IL-1Ra is abundantly produced by monocytes (0.9–3.0 ng/ml) and more abundantly by macrophages (9–150 ng/ml), is upregulated upon stimulation (2–7×; data not shown) and it varies depending on memory (**Figure 4**). Thus, its presence is relevant for determining the final capacity of IL-1β to exert its inflammatory effects. In the absence of reliable data for accurately calculating possible competition between the two ligands in a complex system such as the membrane of a responding cells (in which activation is regulated not only by the quantitative presence of the receptor chains but also by the changes in affinity due to the presence of accessory chains and inhibitory receptors), we have indicatively expressed the levels of active IL-1β as the ratio between free IL-1β and IL-1Ra. We have observed several very interesting phenomena. The first observation is, as expected, that each individual subject responds differently, both in quantitative terms (the amount of cytokine produced) and also in terms of type of response (enhanced reaction *vs*. decreased response). This behavior most likely depends on the past history of exposure of the donor, i.e., age, vaccinations, diseases, etc. Another important observation is the different behavior of macrophages (the tissue-resident mononuclear phagocytes) as opposed to monocytes (the blood-borne inflammatory cells). As an example, monocytes of donor 1 reacted to challenge with *E. coli* with a sharp increase in active IL-1β, whereas macrophages of the same donor significantly down-regulated active IL-1β production upon challenge with *E. coli*. This underlines the different role of the two cell types in inflammatory reactions, with monocytes being the inflammatory effector cells that must be ready and reactive, whereas tissue macrophages must avoid excessive reaction that would cause unwanted tissue damage. Another observation that warrants attention is the different response raised by whole *E. coli* bacteria as opposed to the bioactive *E. coli* LPS molecule. For instance, in the case of donor 2, active IL-1β produced by unprimed monocytes in response to LPS is lower than that induced by *E. coli* and is not changed by priming, whereas response to *E. coli* is significantly reduced by priming. Macrophages of the same donor are responsive to LPS but practically unresponsive to *E. coli*, and their response is reduced by priming with LPS but not by priming with *E. coli*. Also, it is notable that macrophages seem to respond either to whole *E. coli* or to LPS, but not to both (see donors 1 and 3: no response to LPS *vs*. significant response to *E. coli*; and donor 2: good response to LPS and undetectable response to *E. coli*).

Our conclusions are based on a preliminary assessment on three donors, and the very high inter-individual variability of the findings may lead to over-interpretation of the data. On the other hand, since the reactivity of the immune system is tailored on previous experiences, it is in a way expected that each subject could respond differently, based on his/her individual immunological history. Thus, increasing the number of subjects may not overcome the issue of inter-individual variability. What we would like to underline with these data is the importance of an individual evaluation of the innate memory status, in view of future approaches of precision medicine that could help us improving/optimizing immunotherapeutic strategies. In cases as that of innate memory, it seems clear that no general conclusions can be drawn, and in these circumstances a case-by-case assessment becomes crucial.

Overall, it could be concluded that innate memory limits the amount of active IL-1β produced by tissue macrophages in response to a challenge, in line with the hypothesis that these cells are sentinels and not effector cells, and should avoid damaging the tissue by initiating a potentially destructive inflammatory response. On the other hand, memory tends to enhance the reaction of monocytes, which are the effector cells in inflammatory reactions, thereby making them more efficacious in combating the potential danger. Thus, the induction of innate memory could help increasing the host resistance to infections without causing excessive local tissue damage. This could be among the reasons for the efficacy of adjuvants in enhancing protective immunity, in addition to facilitating the establishment of adaptive memory. It is exciting the hypothesis that manipulation of innate memory may also become an important therapeutic strategy in chronic inflammatory and degenerative diseases.

AUTHOR CONTRIBUTIONS

MM performed the experiments and wrote the paper; ET performed cross-stimulation experiments; PI and DB designed the study and wrote the manuscript.

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Revisiting the Role of Interleukin-1 Pathway in Osteoarthritis: Interleukin-1α and -1β, and NLRP3 Inflammasome Are Not Involved in the Pathological Features of the Murine Menisectomy Model of Osteoarthritis

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Background: Innate immune response components such as toll-like receptors (TLRs) and NLRP3-inflammasome act in concert to increase IL-1α/β secretion by synovial macrophages. Previous results suggest that IL-1α/β could be an important mediator involved in the pathogenesis of osteoarthritis (OA).

Objectives: The aim of our study was to evaluate the role of NLRP3, IL-1β, and IL-1α in the menisectomy (MNX) model of murine OA.

Methods: Murine chondrocytes (CHs) and bone marrow-derived machrophages (BMDM) were stimulated with hydroxyapatite (HA) crystals, a form of calcium-containing crystal found in human OA, and IL-1β and IL-6 secretion assayed by ELISA.Conversely, the ability of IL-1β and IL-6 to induce CHs calcification was assessed *in vitro* by Alizarin red staining. Knees from 8 to 10 weeks old C57Bl/6J wild-type (WT) ($n = 7$), NLRP3$^{-/-}$ ($n = 9$), IL-1α$^{-/-}$ ($n = 5$), and IL-1β$^{-/-}$ ($n = 5$) mice were menisectomized, using the sham-operated contralateral knee as control. 8 weeks later, knee cartilage degradation and synovial inflammation were evaluated by histology. In addition, apoptotic chondrocytes, metalloproteases activity, and collagen-type 2 expression were evaluated in all mice. Joint calcification and subchondral bone parameters were quantified by CT-scan in WT and IL-1β$^{-/-}$ menisectomized knees.

Results: *In vitro*, HA crystals induced significant increased IL-6 secretion by CHs, while IL-1β remained undetectable.Conversely, both IL-6 and IL-1β were able to increase chondrocytes mineralization. *In vivo*, operated knees exhibited OA features compared to sham-operated knees as evidenced by increased cartilage degradation and synovial inflammation. In menisectomized KO mice, severity and extent of cartilage lesions were similar (IL-1α$^{-/-}$ mice) or exacerbated (IL-1β$^{-/-}$ and NLRP3$^{-/-}$ mice) compared to that of menisectomized WT mice. Metalloproteases activity, collagen-type 2 expression,

chondrocytes apoptosis, and synovial inflammation were similar between KO and WT mice menisectomized knees. Moreover, the extent of joint calcification in osteoarthritic knees was comparable between IL-1β$^{-/-}$ and WT mice.

Conclusions: MNX knees recapitulated features of OA, i.e, cartilage destruction, synovial inflammation, cell death, and joint calcification. Deficiency of IL-1α did not impact on the severity of these features, whereas deficiency of IL-1β or of NLRP3 led to increased cartilage erosion. Our results suggest that IL-1α and IL-1β are not key mediators in this murine OA model and may explain the inefficiency of IL-1 targeted therapies in OA.

Keywords: NLRP3 inflammasome, interleukin-1β, cartilage, knock-out mice, animal model of OA

INTRODUCTION

Osteoarthritis (OA) is a progressive disease of the joint tissues, characterized by cartilage degradation (Goldring and Goldring, 2007; Loeser et al., 2012), mild synovial inflammation (Scanzello and Goldring, 2012), subchondral bone sclerosis, osteophyte formation, and calcium crystal deposition (calcification) on cartilage (McCarthy and Cheung, 2009). Several OA risk factors have been identified including joint trauma, aging, sex, genetics, obesity (Abramson and Attur, 2009), and basic calcium phosphate (BCP) crystals in joints (McCarthy and Cheung, 2009). BCP crystals include octacalcium phosphate (OCP), hydroxyapatite (HA), and carbonated-apatite (CA) crystals (Ea and Liote, 2009), the latter being the most abundant (Gibilisco et al., 1985). Although OA is the most common form of joint disease and a leading cause of disability in the elderly (Goldring, 2006), no drug exists to slow the progression, or reverse the OA disease process (Iqbal and Fleischmann, 2007).

There is evidence that articular tissues in OA produce proinflammatory cytokines such as IL-1 and IL-6. IL-6 is synthesized and secreted in an active form, which binds first to its receptor (IL-6R) and then to the signaling gp130 molecule triggering STAT and ERK pathways. In contrast, both IL-1α and IL-1β exist as an intracellular proform of about 31 kDa, which can be cleaved to a mature form of 17 kDa. In particular, a first signal (such as TLR1/2 agonist PAM3Cys or TLR4 agonist LPS) is needed to trigger an NF-kB–dependent production of pro–IL-1β. A second signal (ATP, BCP crystals, and others) then leads to the assembly and oligomerization of the NLRP3 inflammasome composed of the NLRP3 sensor, the adaptor protein ASC (Apoptosis-associated speck-like protein containing a CARD) and procaspase-1 which is activated in caspase-1 (Broz and Dixit, 2016). Caspase-1 and NLRP3 inflammasome facilitate or are needed for proIL-1β proteolytic processing and release (Gross et al., 2012), but pro-IL-1α is mainly processed by calpain and other proteases (Di Paolo and Shayakhmetov, 2016). Cellular activation due to signaling through the IL1-R1 occurs when either IL-1α and/or IL-1β (which are equally potent cytokines, collectively known as IL-1) bind to the widely expressed IL-1R type 1 (IL-1R1). Binding induces the formation of a high-affinity complex with the IL-1R accessory protein (IL-1RAcP) and the recruitment of the intracellular adaptor protein myeloid differentiation factor 88 (MyD88) and of the IL-1R-associated kinase 1 (IRAK), which are the proximal mediators of IL-1 signaling. Uncontrolled activation of IL-1R1 is prevented by two distinct mechanisms. One is mediated by IL-1Ra that competes with IL-1 for binding to IL-1R1, and blocks intracellular signaling and cell activation. The other is via the type 2 IL-1 decoy receptor (IL-1R2) that acts as a trap for IL-1 but, in contrast to IL-1R1, lacks a cytoplasmic domain and is unable to induce signaling (Re et al., 1996).

In vitro, in joints cells (such as fibroblasts, macrophages, chondrocytes, osteoblasts), IL-6 and IL-1 are responsible for the loss of cell metabolic homeostasis by 1-promoting autocrine induction of cytokines or production of other inflammatory compounds or chemokines, 2-inducing matrix-degrading enzymes such as MMP-1,-3,-9,-13, and -14 and ADAMTS4,5, and 9 (Murphy and Nagase, 2008; Hashizume and Mihara, 2010; Wojdasiewicz et al., 2014) and, 3-inhibiting the expression of a number of genes, such as collagen type 2 gene (Col2a1) (Goldring et al., 1988; Poree et al., 2008), and proteoglycan (van Beuningen et al., 1991; Sui et al., 2009), normally associated to healthy chondrocytes. Additionally, both IL-1 and IL-6 have pro-mineralizing activity in chondrocytes (Johnson et al., 2001; Nasi et al., 2016a). Finally IL-1β and IL-6 exert their catabolic effect also in bone, by inducing differentiation of mononuclear precursors in osteoclasts (Jandinski, 1988; Nakamura and Jimi, 2006; Kim et al., 2009) and by stimulating bone resorption activity by osteoclasts via the receptor-activator of NF-kB ligand (RANKL) (Jules et al., 2012). Altogether, these *in vitro* effects of IL-1 and IL-6 strongly suggest that these cytokines should have a deleterious role in OA progression *in vivo*, inducing synovitis, favoring cartilage degradation by both catabolic and anabolic effects, and promoting cartilage calcification.

In synovial fluids of either human or experimental models of OA, both cytokines were found to be increased and correlated with radiographic knee OA (Livshits et al., 2009; McNulty et al., 2013). However, IL-1 was not significantly overexpressed in moderate OA compared to mild OA (McNulty et al., 2013). In experimental murine OA, IL-6 neutralization, its genetic deficiency or inhibition of its signaling molecule Stat3 with a non-peptidic small molecule prevented cartilage damage (Ryu et al., 2011; Latourte et al., 2016). In addition, in menisectomized

Abbreviations: MyD88, Myeloid differentiation primary response gene 88; IL-1α, Interleukin-1α; IL-1β, Interleukin-1β; NLRP3, NOD-like receptor protein-3; OA, Osteoarthritis; MNX, Medial partial menisectomy.

mice, increasing deposits of BCP-crystals were observed around the joint and correlated with cartilage degradation and IL-6 expression (Nasi et al., 2016a). While most of the studies clearly report a deleterious role of IL-1 in *in vitro* models of OA, many discrepancies exist about IL-1 effects and IL-1 blockade in experimental OA (see summary in **Table 2**). In particular, using genetically deficient mice, Glasson et al. reported that IL1β$^{-/-}$ mice were protected in a surgical induced instability model of OA (Glasson, 2007), whereas Clements found in a similar model that cartilage damage was exacerbated in Caspase1$^{-/-}$ and IL1β$^{-/-}$ mice (Clements et al., 2003). More recently, in the collagenase-induced model of OA (CiOA), IL-1αβ$^{-/-}$ mice were not protected against synovial inflammation and cartilage destruction if compared to WT mice. Moreover, intra-peritoneal injection of IL-1Ra in WT osteoarthritic mice did not ameliorate OA features (van Dalen et al., 2016).

Due to the confusing published results concerning the role of IL-1 in experimental models of OA, we have further reexplored its role and the role of NLRP3 inflammasome in IL-1 activation in the context of surgically induced murine OA.

METHODS

Mice and Induction of Experimental Osteoarthritis

IL-1α$^{-/-}$, IL-1β$^{-/-}$, and NLRP3$^{-/-}$ female mice, all in the C57Bl/6J background (obtained by Prof Fabio Martinon, Epalinges, Switzerland), were compared with WT littermates. Body weight, fertility and viability were similar among different genotypes. Mice between 8 and 10 weeks were anesthetized and knee joint instability was induced surgically by medial partial meniscectomy of the right knee, as previously described (Nasi et al., 2014). The contralateral knee joint was sham-operated and used as internal control. The animals were allowed unrestricted activity, food and water *ad libitum* in a pathogen-free housing facility. This study was carried out in accordance with the guidelines set by the "Service de la consommation et des affaires vètèrinaires du Canton de Vaud." The protocol was approved by the Federal Veterinary Office and the work complied with the Directive 2010/63/EU.

Histology of Total Knee Joints

Total knee joint of mice were fixed, decalcified and embedded in paraffin, and sagittal sections were cut from the whole medial compartment of the joint (three sections/mouse) as previously described (Nasi et al., 2014). Sections were then stained with Safranin-O-fast green to examine the OA-like cartilage and bone changes according to the scoring method recommended by OARSI (Glasson et al., 2010). Finally, synovial inflammation was scored using the following scale: 0=no inflammation; 1=mild inflammation; 2=moderate inflammation; 3=major inflammation. Synovial histological changes included synovial hypertrophy and hyperplasia and an increased number of lining cells, accompanied sometimes by infiltration of the sublining tissue. Histological scorings were assessed by two observers who were blinded with regard to the mice genotypes.

MicroCT-Scan

MicroCT-scans analysis were performed using a SkyScan 1076® X-ray μCT scanning system (SkyScan, Belgium) and the following parameters: 18 μm resolution, 60 kV, 167 μA, 0.4° rotation step over 360°, 0.5 mm Aluminum filter, 1180 ms exposure time. *Ex vivo* samples acquisition was made using formol fixed knees. Images were reconstructed using NRecon Version 1.6.6.0 (Skyscan, Belgium) considering the following parameters: gray-values = 0.0000–0.105867, Ring Artifact Reduction = 3, Beam Hardening Correction = 40%. In the menisectomized knees, quantitative analyses of crystal content (μg), and quantitative analysis of tibial subchondral bone parameters (bone mineral density (BMD g/cm^3), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp) were performed using CTAnalyzer Version 1.10 (SkyScan, Belgium) for different Volumes Of Interest (VOIs).

Immunohistochemical Detection of VDIPEN, Type II Collagen and Apoptosis

MMP-induced neoepitope VDIPEN staining was performed with affinity-purified anti-VDIPEN IgG and type II collagen synthesis was evaluated using an anti-collagen type II, biotinylated monoclonal antibody (MD Bioproduct, 1041007B) (Nasi et al., 2014). Apoptotic chondrocytes were detected in paraffin sections using the Apoptag kit (ApopTag plus Peroxidase *In situ*, Millipore) as previously described (Nasi et al., 2014).

Calcium Phosphate Crystals

Hydroxyapatite (HA) crystals were synthesized as previously published (Prudhommeaux et al., 1996). HA crystals were sterilized by gamma-radiation and assessed as pyrogen-free. Prior to experimentation, crystals were resuspended in sterile PBS and sonicated for 5 min.

Bone Marrow Derived Macrophage (BMDM) Preparation

Bone marrow cells were isolated from the tibia and femur of C57BL/6 mice. For their differentiation into BMDM, the extracted cells were incubated for 7 days in Petri dishes with 30% L929 conditioned media (source of M-CSF) and 20% FBS in Dulbecco's Modified Eagle Media (DMEM). The resulting BMDM were detached using cold PBS, plated in complete DMEM medium (Gibco), [10% FBS and 1% Penicillin Streptomycin (Sigma)] or incomplete DMEM (1% Penicillin Streptomycin only) and primed or not with 100 ng/ml PAM3Cys overnight. The following day, crystal stimulation was performed in incomplete DMEM.

Joint Chondrocyte (CHs) Preparation

Chondrocytes were isolated from new-born C57Bl/6J mice as described previously, with slight modifications (Gosset et al., 2008). Briefly, the joint cartilage (articular and epiphyseal) was harvested from the knee and hip joints of mice aged between 4 and 6 days. The cartilage was degraded by a three step digestion process by using decreasing concentrations of Liberase (Roche). The day after, the digested tissue was passed through a 70 μm filter (BD biosciences) to obtain immature chondrocytes.

The cells were plated into a culture plate at high density (3.5×10^4 cells/cm^2) and amplified for 7 days in complete DMEM (10% FBS, 1% Penicillin Streptomycin). Prior to crystal stimulation experiments, cells were detached using Trypsin-EDTA (Amimed). The resulting chondrocytes were plated in complete DMEM medium (Gibco), [10% FBS and 1% Penicillin Streptomycin (Sigma)] or incomplete DMEM (1% Penicillin Streptomycin only) and primed or not with 100 ng/ml PAM3Cys overnight. The following day, crystal stimulation was performed in incomplete DMEM. For chondrocyte mineralization analysis, cells were cultured for 7 days in complete BJGb medium (Gibco) (10% FBS, 50 μg/ml ascorbic acid, 20 mM β-glycerol phosphate), stimulated or not with 10 ng/ml of IL-6 (Gibco PMC0064) or with 1 ng/ml IL-1β (Gibco PMC0814). Medium was changed for the last 4 days.

Calcium Phosphate Crystal Stimulation

Cells were primed overnight with100 ng/ml Pam3Cys, where indicated, and stimulated with 500 μg/ml HA crystals. Supernatants were collected for cytokine ELISAs, and cells placed in TRIZOL for RT-PCR analysis.

Crystal Detection from Chondrocyte Cultures

Articular chondrocytes cultured for 7 days were washed in PBS and crystal deposition analyzed as previously described (Gregory et al., 2004).

PCR Analysis

RNA was extracted and PCR or qRT-PCR with gene specific primers (**Table 1**) was performed as previously described (Ea et al., 2013).

Cytokine Quantification

Supernatants were assayed using murine IL-1β and IL-6 ELISA kit (eBioscience) following the manufacturer's protocol. Results were read at 450 nm using the Spectrax M5e (Molecular devices).

Statistical Analysis

In vitro experiments were performed using pools of primary cells from at least 3 different mice (either chondrocytes or bone marrow derived macrophages). Moreover, all experiments were performed with triplicates and reproduced independently at least two times. Statistical analysis was performed using the Student's *t*-test or one- or two-way ANOVA test corrected with *post-hoc* tests for multiple comparisons, where appropriate. Data was analyzed with GraphPad Prism software (GraphPad, San Diego).

TABLE 1 | Gene specific primers for PCR and qRT-PCR analysis.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Asc	CCA GTG TCC CTG CTC AGA GT	TCA TCT TGT CTT GGC TGG TG
Casp1	CCG TGG AGA GAA ACA AGG AG	ATG AAA AGT GAG CCC CTG AC
Gapdh	CTC ATG ACC ACA GTC CAT GC	CAC ATT GGG GGT AGG AAC AC
Il-1b	CCA CCA ACA AGT GAT ATT CTC CAT G	GTG CGG TCT TTC ATT ACA CAG
Nlrp3	TGC TCT TCA CTG CTA TCA AGC CCT	ACA AGC CTT TGC TCC AGA CCC TAT
Tbp	CTT GAA ATC ATC CCT GCG AG	CGC TTT CAT TAA ATT CTT GAT GGT C

RESULTS

IL-1α$^{-/-}$, IL-1β$^{-/-}$, and NLRP3$^{-/-}$ Mice Are Not Protected against Cartilage Damage and Synovial Inflammation Induced by Menisectomy

At 8 weeks after surgery, sham-operated knee joints showed intact cartilage with smooth cartilage surfaces and conserved proteoglycan (PGs) staining in both tibia and femur (**Figure 1A**). Chondrocytes organization was typical of that of healthy cartilage, with one or two layers of flat cells in the superficial zone and columns of round cells in the middle and deep zones. By contrast, operated knees (MNX) from WT mice exhibited cartilage damage, PGs loss and disorganized chondrocytes arrangement. In IL-1α$^{-/-}$, IL-1β$^{-/-}$, and NLRP3$^{-/-}$ menisectomized mice, cartilage degradation was evidenced and similar (for IL-1α$^{-/-}$) or more pronounced (for IL-1β$^{-/-}$ and NLRP3$^{-/-}$) to that of WT mice (**Figure 1A**). We also observed in all mice genotypes, chondrocyte morphology changes, chondrocytes loss in the superficial and intermediate cartilage layers, and chondrocytes hypertrophy in the deep zone.

We then scored the histological sections and found that the severity (grade) and the extent (stage) of cartilage degradation were similar between WT and IL-1α$^{-/-}$, but significantly increased in IL-1β$^{-/-}$ knees, both for the tibia and the femur cartilage (**Figure 1B**). Femur cartilage in NLRP3$^{-/-}$ mice was similarly damaged but tibial cartilage was significantly increased if compared to that of WT mice (**Figure 1B**). Finally, levels of Safranin-O loss were similar amongst the genotypes, indicating similar levels of PG loss in cartilage of these mice. Altogether these results demonstrate that a single deficiency of IL-1α, IL-1β, or of NLRP3 does not prevent cartilage damage.

We next examined synovial inflammation in sham operated and MNX mice. We found mild synovial inflammation in all WT menisectomized mice compared to sham-operated. We also found similar synovial inflammation in IL-1α$^{-/-}$, IL-1β$^{-/-}$, and NLRP3$^{-/-}$ MNX mice (**Figure 1C**). Synovial histological features induced by OA development included synovial hypertrophy and hyperplasia. **Figure 1D** shows the synovial inflammation score, which confirmed no significant differences between genotypes.

NLRP3$^{-/-}$, IL-1α$^{-/-}$, and IL-1β$^{-/-}$ Mice Are Not Protected against Catabolic Changes and Chondrocyte Apoptosis Induced by Menisectomy

Signs of cartilage catabolism, in particular of MMP-mediated aggrecan degradation, were evidences by VDIPEN staining.

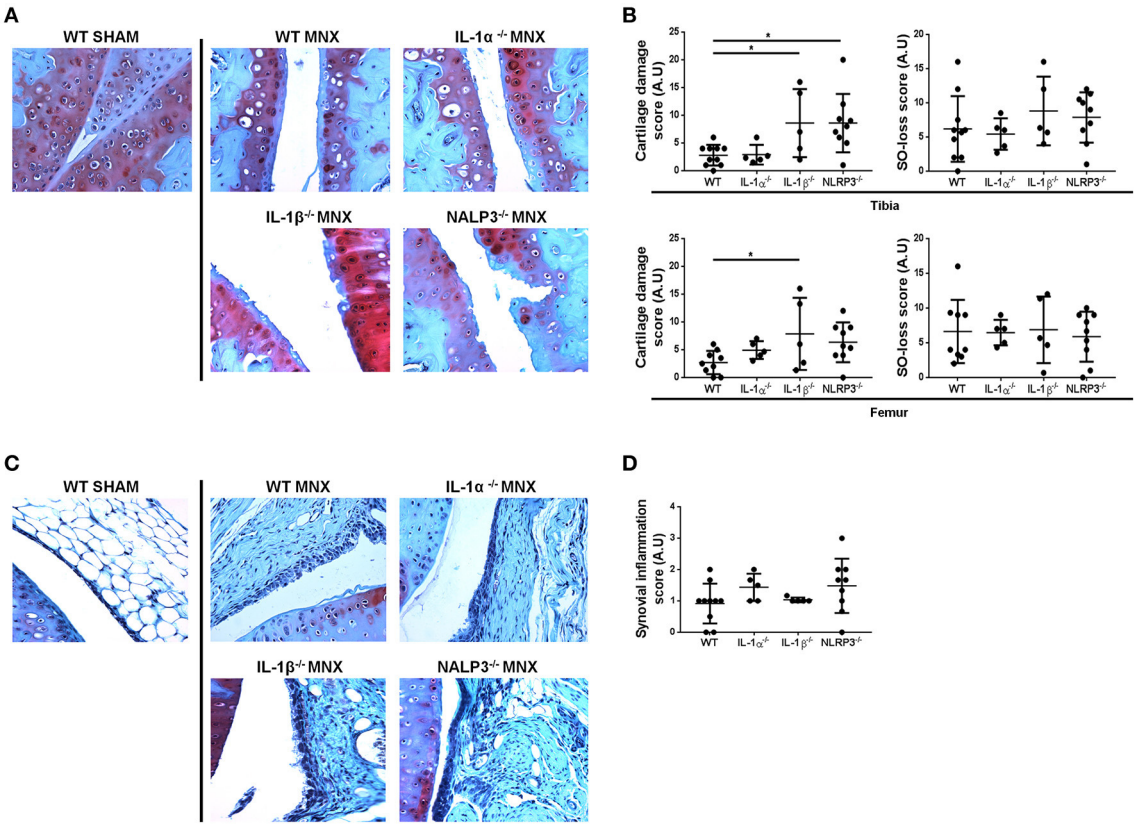

FIGURE 1 | IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, and NLRP3$^{-/-}$ mice develop OA features similar to WT mice after menisectomy. **(A)** Representative histologies, stained with Safranin-O, and **(B)** respective histological scoring of sham operated (WT SHAM) and menisectomized (WT MNX, IL-1$\alpha^{-/-}$ MNX, IL-1$\beta^{-/-}$ MNX and NLRP3$^{-/-}$ MNX) knee joint sections, at 8 weeks after surgery. Note the similar level of cartilage destruction and loss of proteoglycans in WT and IL-1$\alpha^{-/-}$ menisectomized knees, and exacerbated cartilage damage in IL-1$\beta^{-/-}$ and NLRP3$^{-/-}$ MNX mice. **(C)** Representative histologies, stained with Safranin-O, and **(D)** respective synovial inflammation scoring of sham operated and menisectomized knee joint sections. Note the similar level of synovial inflammation in mice of different genotypes. Mice number: WT MNX $n = 10$, IL-1$\alpha^{-/-}$ MNX $n = 5$, IL-1$\beta^{-/-}$ MNX $n = 5$, and NLRP3$^{-/-}$ MNX $n = 9$. *$p < 0.05$.

MMP-generated neoepitopes were markedly increased in WT MNX knees if compared to that of sham-operated mice, especially in the middle and deep zones of cartilage (**Figure 2A**). Moreover, both chondrocytes and pericellular matrix were associated with marked VDIPEN neoepitope immunostaining in a comparable way in WT, and NLRP3$^{-/-}$, IL-1$\alpha^{-/-}$, and IL-1$\beta^{-/-}$ operated mice (**Figure 2A**).

In addition to catabolic signs, also anabolism and in particular collagen type 2 immunostaining has been investigated. Our observations identified weak but homogeneous extracellular distribution of collagen type 2 in sham-operated mice (**Figure 2B**). On the contrary, osteoarthritic joints showed sites of activated collagen type 2 synthesis, in a similar way in WT and KO mice. Even if subtle differences were seen in collagen type 2 expression between the different KO mice, positivity was overall detected at a similar level and especially in the superficial damaged region as well as in the deeper layer close to the tidemark with the bone. Another prominent feature of OA is increased chondrocyte apoptosis, that we tested by TUNEL staining (**Figure 2C**). Only few apoptotic chondrocytes were detected in sham-operated mice whereas an increased number of randomly distributed apoptotic chondrocytes was noticeable in menisectomized mice. Chondrocyte aptosis was similar between WT and KO mice (**Figure 2C**).

MNX IL-1$\beta^{-/-}$ Mice Have Similar Knee Joint Calcification but Increased Subchondral Bone Osteoporosis Compared with WT Mice

We previously demonstrated that, 2 months after menisectomy, mice exhibited new calcific formation at the place of the removed meniscus (Nasi et al., 2016a). Calcification of the joint structures is a typical OA feature, also found in human OA, and IL-1 can be a trigger of joint calcification as discussed above. We therefore examined if menisectomy-induced calcific deposits were different between WT and IL-1$\beta^{-/-}$ mice. MicroCT-scan examination conducted 8 weeks after surgery revealed similar joint calcification (**Figure 3A,** white circles) and crystal content (**Figure 3B**) between the two mice genotypes, suggesting that the lack of IL-1β did not protect against ectopic mineralization induced by joint instability. Moreover, analysis

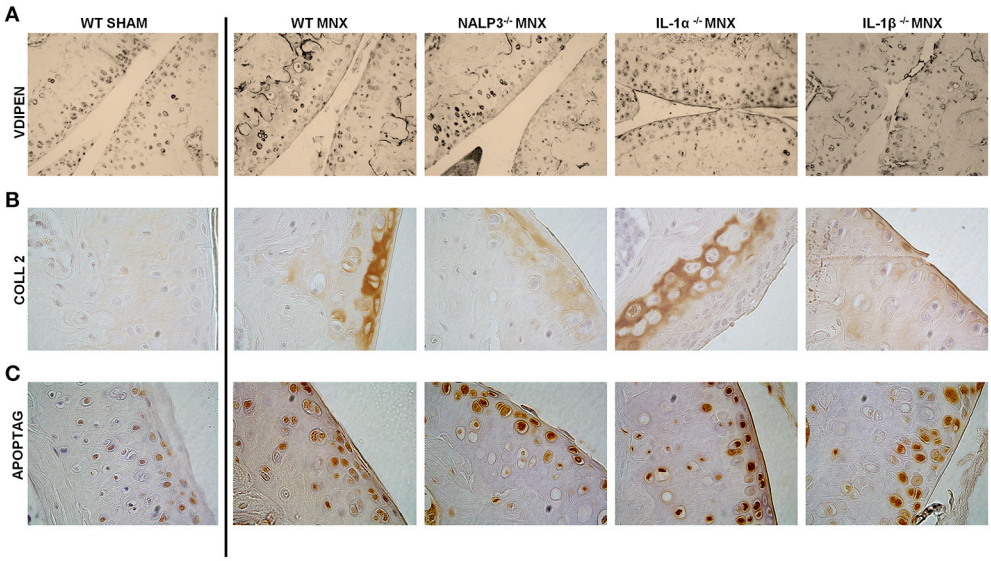

FIGURE 2 | Knock-out mice are not protected against catabolic changes and chondroptosis caused by OA induction. **(A)** Representative immunohistochemical sections of knee joints, stained for MMP-mediated proteoglycan degradation (VDIPEN staining). Note markedly increased VDIPEN in MNX knees if compared to sham-operated mice and no protection in knock-out mice. **(B)** Representative immunohistochemical sections of knee joints, stained with anti collagen type 2 antibody. Note increased collagen type 2 synthesis in the superficial and deep cartilage layers of MNX mice compared to the sham operated mice and the similar expression of collagen type 2 in WT and KO menisectomized knees. **(C)** Representative immunohistochemical sections of knee joints, stained with ApopTag. Note increased apoptosis in MNX mice compared to sham operated mice and the similar level of chondrocyte aptosis in WT and KO menisectomized knees. Mice number: WT MNX $n = 4$, IL-1$\alpha^{-/-}$ MNX $n = 4$, IL-1$\beta^{-/-}$ MNX $n = 4$, and NLRP3$^{-/-}$ MNX $n = 4$.

of subchondral trabecular bone parameters revealed that IL-1$\beta^{-/-}$ mice have similar tibial bone mineral density (BMD) and trabecular thickness (Tb.Th) to WT mice. However, they showed significantly decreased trabecular number (Tb.N) and increased trabecular separation (Tb.Sp), suggesting a high subchondral bone remodeling with increased bone resorption (Chiba et al., 2012) (**Figure 3C**).

IL-1β Induces Chondrocyte Mineralization, but Mineralization Does Not Induce IL-1β Secretion

We previously demonstrated that BCP crystals stimulated IL-6 secretion by murine chondrocytes. Conversely, exogenous IL-6 promoted chondrocyte mineralization, thus building an amplification loop leading to OA (Nasi et al., 2016a). We hypothesized that the absence of IL-1 effect in the MNX model could be due to the absence of this amplification loop. To test this hypothesis, we stimulated primary murine chondrocytes (CHs) with exogenous IL-1β. After 7 days of culture, calcium containing crystals, detected by Alizarin red staining were significantly increased compared to unstimulated cells (**Figure 4A**). As a positive control, we used IL-6 incubated for 7 days, which showed an even stronger promineralizing activity (**Figure 4A**). We next stimulated primed CHs and BMDM with HA crystals. In these conditions, chondrocytes did not secrete mature IL-1β, but an abundant secretion of IL-6 could be detected. By contrast, increased levels of IL-1β and IL-6 were measurable in HA-stimulated BMDM (**Figure 4B**). To explain the ELISA results, we analyzed the effect of the PAM3Cys priming on IL-1β and IL-6 genes. qRT-PCR analysis revealed that PAM3Cys strongly induced *IL-1*β gene expression in BMDM, but only marginally in CHs, whereas priming had an opposite effect on *IL-6* gene expression, being strongly up-modulated in CHs, but almost not in BMDM (**Figure 4C**). Therefore, the lack of IL-1β detection in primed CHs, under basal and HA-stimulated conditions, could be due to lower *IL-1*β gene expression in CHs ($35<Ct<40$) compared to BMDM ($28<Ct<30$). Additionally, the absence of IL-1β detection could also be accounted for by lower expression of NLRP3 inflammasome components in CHs compared to BMDM (**Figure 4D**). Our results are in agreement with already reported lower NLRP3 inflammasome expression in human OA chondrocytes and synoviocytes compared to the monocytic THP1 (Jin et al., 2011).

DISCUSSION

As *in vitro* IL-1 stimulation of joint cells led to inflammation, catabolism and oxidative stress, and IL-1 blockade by IL-1Ra or IL-1R2 reverted these IL-1-induced deleterious effects (Roessler et al., 1995; Attur et al., 2000, 2002; Palmer et al., 2002, 2004), it has been suggested for more than two decades that *in vivo* IL-1 could be of paramount significance in OA (Fernandes et al., 2002). In the present study, we demonstrated that IL-1$\beta^{-/-}$ and NLRP3$^{-/-}$ mice were not protected against cartilage damage and synovial inflammation induced by menisectomy. Rather, cartilage damage was exacerbated by these deficiencies, thereby

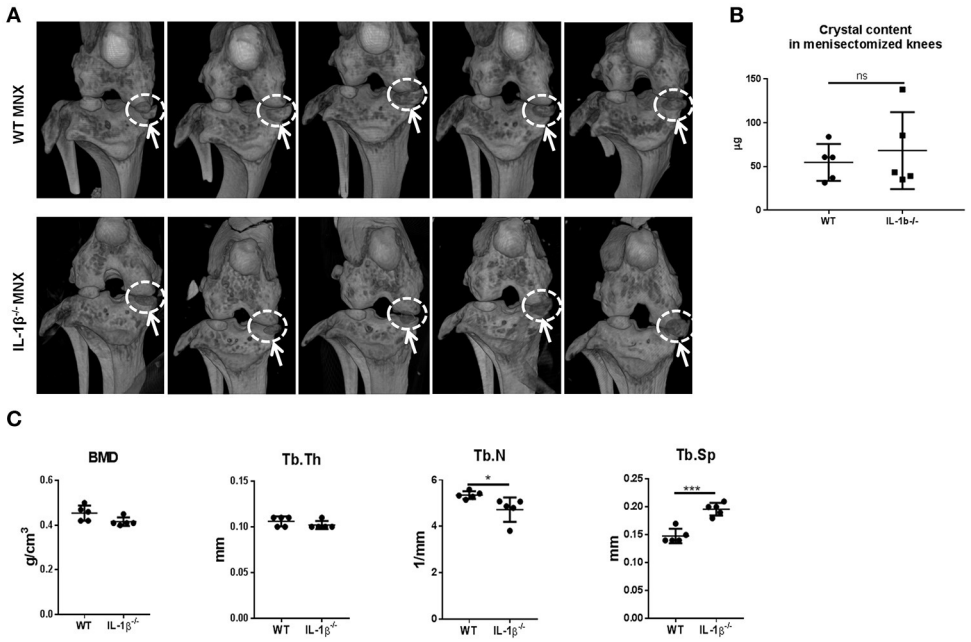

FIGURE 3 | Calcific deposits and tibial subchondral trabecular bone parameters in osteoarthritic knee joints of WT and IL-1β$^{-/-}$ mice. **(A)** Micro-CT scan images of menisectomized murine knee joints 2 months after surgery. White circles show new periarticular crystal deposits in menisectomized knees. **(B)** Crystal content in the menisectomized knees and **(C)** tibial subchondral trabecular bone parameters were measured in the same animals using CTAnalyzer. Mice number: WT $n = 5$, IL-1β$^{-/-}$ $n = 5$. $^{*}p < 0.05$; $^{***}p < 0.001$.

confirming previous results obtained in a similar MNX model, in which cartilage was more damaged in IL-1β$^{-/-}$ and caspase-1$^{-/-}$ mice compared to WT mice (Clements et al., 2003). The mechanisms explaining the protective role of IL-1β and NLRP3 inflammasome on cartilage in the surgically induced model of OA remain to be explored. We also reported here that IL-1α is not involved in cartilage damage and synovial inflammation as, in the MNX model, the phenotype of IL-1α$^{-/-}$ was similar to that of WT mice. In agreement with the lack of a protective role of IL-1 deficiency in experimental OA, in another model of experimental OA, the collagenase-induced model of OA, mice deficient for both IL-1α and IL-1β (IL-1αβ$^{-/-}$) developed cartilage destruction and synovial inflammation similar to WT mice (van Dalen et al., 2016). Interestingly, histological scoring of the cartilage lesion showed a trend toward increased damage in IL-1 deficient mice, although this increase did not reach significancy. The lack of a pathogenic role of IL-1 has been further confirmed in the collagenase-induced model by the lack of effect of IL-1Ra treatment in WT osteoarthritic mice (van Dalen et al., 2016). We previously reported that intra-articular BCP crystals can elicit synovial inflammation and cartilage degradation suggesting that BCP crystals have a direct pathogenic role in OA. We also found that these effects are independent of IL-1 and NLRP3 inflammasome, as knee joint inflammation and damage was similar in crystal-injected IL-1α$^{-/-}$, IL-1β$^{-/-}$, ASC$^{-/-}$, or NLRP3$^{-/-}$ mice and as IL-1Ra treatment did not prevent OA features of WT mice (Ea et al., 2013). Finally, the fact that deficiency of MyD88, the adaptor molecule for IL-1R1, did not impact on the severity of experimental OA strongly suggests that IL-1 is not a key mediator in the development of OA (Nasi et al., 2014). Altogether our results strongly suggest that IL-1 is not involved in cartilage degeneration in murine models of OA. By contrast, in a spontaneous model of OA characterized by joint calcification (Ank$^{-/-}$ model), NLRP3 inflammasome deficiency partially (∼30%) protected against joint pathology (Jin et al., 2011). However, no proof of IL-1 involvement was provided in this study. Of note, one single report claimed that IL-1β$^{-/-}$ mice had reduced cartilage erosion, but the number of mice and detailed procedures used to reach this conclusion were not mentioned and therefore caution should be taken when quoting this work (Glasson, 2007).

In non-murine experimental models of OA the role of IL1-mediated pathway seemed to be deleterious (see **Table 2** for a summary of the works published). In the transection of the anterior cruciate ligament (ACL) dog model of OA, recombinant human interleukin-1-receptor antagonist (rHuIL-1Ra) either injected intra-articularly or locally expressed by synovial cells transduced with HuIL-1Ra gene protected against OA lesions, partially via a reduction of collagenase-1 expression (Caron et al., 1996; Pelletier et al., 1997). Similarly, in rabbit and equine surgically-induced models of OA, intra-articular overexpression of IL-1Ra resulted in a significant improvement in disease activity, cartilage degradation and synovitis (Fernandes et al., 1999; Frisbie et al., 2002). In a spontaneous model of OA (aged Hartley guinea pigs), diminished IL-1 signaling by RNA interference-based reduction (siIL-1β), or by mILRa led to

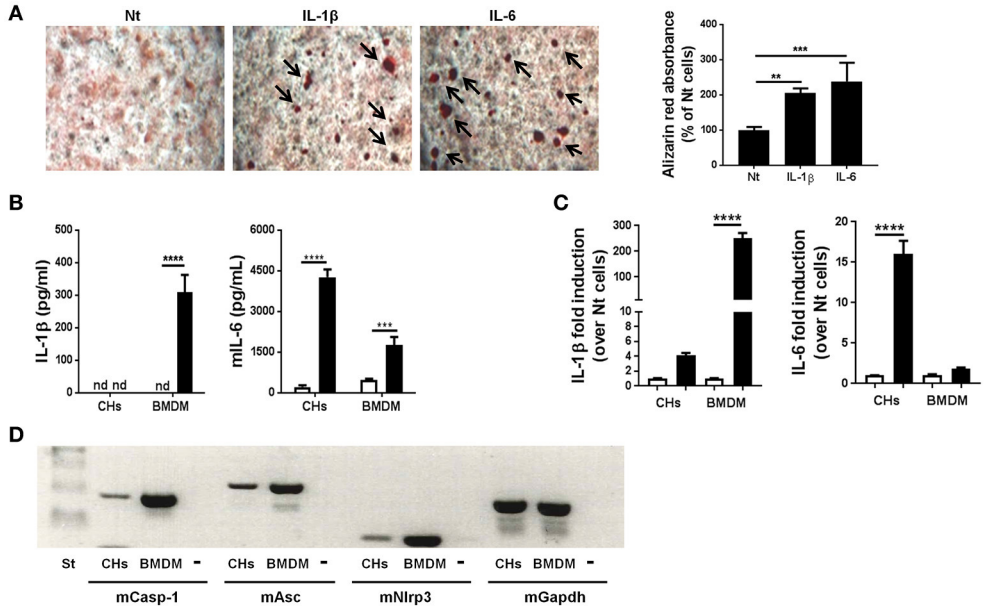

FIGURE 4 | Murine primary chondrocytes express the components of the NLRP3 inflammasome but do not produce IL-1β upon HA crystal stimulation. **(A)** Alizarin red staining and quantification in murine chondrocytes culture, stimulated or not with 5 ng/ml IL-1β or with 10 ng/ml IL-6 for 7 days in BGJb medium. Values represent means ± *SD* of triplicates from one representative experiment out of three. **(B)** IL-1β and IL-6 secretion by primed CHs or by BMDM, stimulated (black bars) or not (white bars) with HA crystals for 6 h. Values represent means ± *SD* of triplicates from one representative experiment out of three. **(C)** qRT-PCR analysis of the indicated genes in CHs and BMDM stimulated (black bars) or not (white bars) with 100 μg/ml PAM3Cys for 2 h. Results are expressed as the fold increase of gene expression in PAM3Cys treated over not treated (Nt) cells, using the mean ± *SD* of triplicate independent RNA samples. **(D)** Gel electrophoresis for the analysis of Caspase-1, Asc and Nlrp3 expression in primary murine chondrocytes (CHs) and primary bone marrow derived macrophages (BMDM). Minus (-) is the negative control (water). All experiments were performed using pools of primary cells from at least 3 different mice. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

decreases expression of mediators implicated in OA pathogenesis such as IL-1β, TNF-α, IL-8, INF-γ, MMP-13, and increased TGF-β1 (Santangelo et al., 2012). Finally, in a murine model of post-traumatic arthritis induced by articular fracture, intra-articular inhibition of IL-1 by IL-1Ra exerted protective effects in terms of cartilage degradation and synovial inflammation (Furman et al., 2014).

The conflicting results about the role of IL-1, but also other cytokines such as IL-6 (de Hooge et al., 2005; Ryu et al., 2011; Latourte et al., 2016; Nasi et al., 2016a) in experimental models of OA, could reflect species difference, differences in age and sex of the animals used, variability in the method of OA induction (different progression of joint degeneration, degrees of inflammation, degrees of unpaired loading for each experimental OA model)."

It is important to keep in mind that in the context of inflammatory arthritis, such as collagen-induced arthritis (Joosten et al., 1996) and antigen-induced arthritis (Kolly et al., 2009), IL-1 deficiency or IL-1 neutralization leads to cartilage protection. This suggests that the mechanisms involved in cartilage degradation in an inflammatory context may be dependent on IL-1 whereas in a less inflammatory setting (such as in OA) this could not be any more true.

Discordant results about the role of IL-1 in OA have been reported not only in animal studies but also in human studies. It has been reported that patients subjected to ACL transection have increased IL-1β and IL-1Ra in synovial fluid, if compared to healthy individuals, while IL-1α remained undetectable (Marks and Donaldson, 2005). In accordance, the expression of IL-1β in synovial membrane positively correlated with OA grade (Smith et al., 1997) and with joint space width (Ning et al., 2011), and negatively correlated with joint alignment and physical disability (Ning et al., 2011). Moreover, IL-1α and IL-1β expression was detected by immunohistochemical analysis in human OA cartilage, especially in early stage OA (Towle et al., 1997). In a study by Denoble and co-workers, IL-1β level in synovial fluid of OA patients was increased and correlated with synovial fluid uric acid. The author concluded that uric acid could be a danger signal that contributes to increasing risk for OA through inflammasome activation and subsequent IL-1β production (Denoble et al., 2011). On the contrary, in a study conducted at different time-points after ACL injury, synovial fluid level of IL-1β was not increased and IL-1Ra was decreased compared to healthy controls (Bigoni et al., 2013). In a study in symptomatic knee OA patients, plasma levels of IL-1Ra were modestly associated with the severity and progression of the disease independent of other risk factors (Attur et al., 2015). High innate *ex vivo* production of IL-1β and IL-1Ra by whole blood samples from OA patients was associated with an increased risk of familial OA at multiple sites (Riyazi et al., 2005). However, in a separate study, *ex vivo* production of IL-1β and IL-1Ra by whole blood samples were not significantly associated with

TABLE 2 | Summary of studies in animal models of OA where IL-1 role has been tested.

Species	Model	Conditions	Duration of experiment	Conclusion about IL-1 role in OA	Citation
Dog	ACL transection	I.a injection of 4 mg rHuIL-1Ra, at the moment of surgery	4 weeks	Deleterious	Caron et al., 1996
Dog	ACL transection	I.a injection of autologous synoviocytes transduced with HuIL-1Ra, 2d post-surgery	4 weeks	Deleterious	Pelletier et al., 1997
Rabbit	Partial medial menisectomy	Three i.a injection at 24 h intervals of 1,000 μg DogIL-Ra plasmid, 4w post-surgery	8 weeks	Deleterious	Fernandes et al., 1999
Horse	Osteochondral fragment	I.a injection of 20 × 10^{10} VP AdEqIL-1Ra, 2w post-surgery	10 weeks	Deleterious	Frisbie et al., 2002
Guinea pig	Spontaneous	I.a injection of 10^{12} DRPs siIL-1β, at 8w of age	24 weeks	Deleterious	Santangelo et al., 2012
		I.a injection of 2x10^{11} IFUs mAd-IL-1Ra, at 8w of age	24 weeks	Deleterious	
Mouse	Destabilization medial meniscus	IL-1β$^{-/-}$ mice	8 weeks	Deleterious	Glasson, 2007
Mouse	Ank$^{-/-}$	Caspase-1$^{-/-}$ mice	12 weeks	Deleterious	Jin et al., 2011
		NLRP3$^{-/-}$ mice			
Mouse	Articular fracture of the knee	IL-1Ra administration intra-articularly or sistemically	4 weeks	Deleterious	Furman et al., 2014
Mouse	I.a injection of BCP crystals	IL-1α$^{-/-}$, IL-1β$^{-/-}$, ASC$^{-/-}$, NLRP3$^{-/-}$ mice	4, 17, 30 days	No role No role	Ea et al., 2013
		IL-1Ra	4 days		
Mouse	Partial medial menisectomy	IL-1β$^{-/-}$ mice	4 weeks	Protective	Clements et al., 2003
		Caspase-1$^{-/-}$ mice	4 weeks	Protective	
Mouse	Partial medial menisectomy	MyD88$^{-/-}$ mice	8 weeks	No role	Nasi et al., 2014
Mouse	Partial medial menisectomy	IL-1α$^{-/-}$ mice	8 weeks	No role	This study
		IL-1β$^{-/-}$ mice	8 weeks	Protective	
		NLRP3$^{-/-}$ mice	8 weeks	Protective	
Mouse	Collagenase-induced	IL-1αβ$^{-/-}$ mice	4 weeks	No role	van Dalen et al., 2016
		rIL-1Ra administration at the moment of the surgery and for 2w	2 weeks		

progression of knee OA over a 2-year period (Botha-Scheepers et al., 2008).

In addition to the contrasting results about the role of IL-1 in OA obtained in a number of experimental studies *in vitro*, *in vivo*, and *ex vivo*, the anti-IL-1 approach in patients has not yet proven significative improvement in OA symptoms and as a disease-modifying therapy. Various treatment strategies have been tested in human such as administration of monoclonal antibodies against IL-1 or IL-1R1 to block IL-1 signaling, administration of IL-1Ra to antagonize IL-1 action, and blockade of the formation of active IL-1β. In a first double-blind, placebo controlled, multiple-dose study by Cohen and colleagues (Cohen et al., 2011), a monoclonal antibody (AMG108) directed against IL-1R1, therefore inhibiting both IL-1α and IL-1β activity, was administered systemically (IV or SC) to knee OA patients (KOA). AMG108-treated group did not show statistically significant improvements in pain compared with the placebo group, as shown by pain scores (Cohen et al., 2011). Recombinant human IL-1 receptor antagonist proteins (IRAPs) are competitive antagonists of IL-1. In placebo-controlled clinical trials conducted in KOA patients, intra-articular injection of either Anakinra or Orthokine, two available IRAPs, didn't lead to significantly different pain score compared to placebo-treated patients (Chevalier et al., 2005, 2009; Auw Yang et al., 2008). In another study in KOA patients, the clinical effects of intra-articular injection of Orthokine was compared with those of hyaluronan (HA) or placedo injection (Baltzer et al., 2009). Preliminary results showed that the effects of Orthokine were higher than those of HA or saline in terms of pain, stiffness and joint function (about 30% improvement) (Baltzer et al., 2009). Further confirmation and additional studies on the mechanism of action of Orthokine (i.e., DMOAD, chondroprotective, others) are required.

From the above mentioned data obtained both in experimental and clinical OA, we can conclude that IL-1

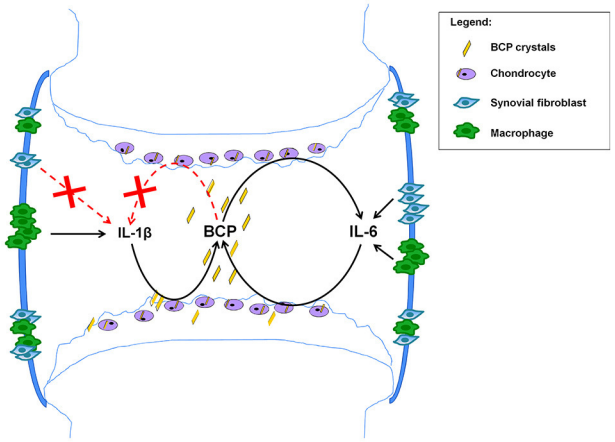

FIGURE 5 | Proposed mechanism based on the obtained results. BCP crystals found in 100% of OA patients at the moment of joint replacement (Ea et al., 2011), can activate NLRP3 inflammasome in macrophages leading to IL-1β secretion (Pazar et al., 2011), but this process does not occur in chondrocytes and synovial fibroblasts (Kolly et al., 2010; Jin et al., 2011). In addition BCP crystals induce IL-6 in macrophages, fibroblasts and chondrocytes. In these latter cells, BCP crystals induce IL-6, which in turn induce mineralization, thus creating a vicious circle and a chronification of the disease. Strategies interrupting this vicious circle could ameliorate this degenerative disease.

has not yet proven to be a good target for OA. Based on previous published papers by us (Nasi et al., 2016a,b) and by others (Ryu et al., 2011; Latourte et al., 2016), we suggest that IL-6-targeted strategies could lead to new therapeutic options for OA, by interrupting the vicious circle between BCP crystal formation and IL-6 production by chondrocytes (see **Figure 5**).

This proposed mechanism, built on chondrocytes grown in monolayers and *in vivo* observations in the menisectomy model after 2 months, needs further validations using additional *in vitro* models, such 3D-models of chondrocyte cultures, and additional *in vivo* OA models, considering different experimental points and looking to different read-outs.

ETHICS STATEMENT

The experiments complied with the Guidelines for Animal Experimentation issued by the local Ethics Committee on Animal Care and Experimentation.

AUTHOR CONTRIBUTIONS

SN, HE, and NB designed, performed, and evaluated all experiments. The entire work was supervised by NB. The figures were prepared and the manuscript was written by SN, HE, AS, and NB. All authors discussed and commented on the manuscript.

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Rescued Chondrogenesis of Mesenchymal Stem Cells under Interleukin 1 Challenge by Foamyviral Interleukin 1 Receptor Antagonist Gene Transfer

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Background: Mesenchymal stem cells (MSCs) and their chondrogenic differentiation have been extensively investigated *in vitro* as MSCs provide an attractive source besides chondrocytes for cartilage repair therapies. Here we established prototype foamyviral vectors (FVV) that are derived from apathogenic parent viruses and are characterized by a broad host range and a favorable integration pattern into the cellular genome. As the inflammatory cytokine interleukin 1 beta (IL1β) is frequently present in diseased joints, the protective effects of FVV expressing the human interleukin 1 receptor antagonist protein (IL1RA) were studied in an established *in vitro* model (aggregate culture system) of chondrogenesis in the presence of IL1β.

Materials and Methods: We generated different recombinant FVVs encoding enhanced green fluorescent protein (EGFP) or IL1RA and examined their transduction efficiencies and transgene expression profiles using different cell lines and human primary MSCs derived from bone marrow-aspirates. Transgene expression was evaluated by fluorescence microscopy (EGFP), flow cytometry (EGFP), and ELISA (IL1RA). For evaluation of the functionality of the IL1RA transgene to block the inhibitory effects of IL1β on chondrogenesis of primary MSCs and an immortalized MSC cell line (TERT4 cells), the cells were maintained following transduction as aggregate cultures in standard chondrogenic media in the presence or absence of IL1β. After 3 weeks of culture, pellets were harvested and analyzed by histology and immunohistochemistry for chondrogenic phenotypes.

Results: The different FVV efficiently transduced cell lines as well as primary MSCs, thereby reaching high transgene expression levels in 6-well plates with levels of around 100 ng/ml IL1RA. MSC aggregate cultures which were maintained in chondrogenic media without IL1β supplementation revealed a chondrogenic phenotype by means of strong positive staining for collagen type II and matrix proteoglycan (Alcian blue). Addition of IL1β was inhibitory to chondrogenesis in untreated control pellets. In contrast, foamyviral mediated IL1RA expression rescued the chondrogenesis in pellets

cultured in the presence of IL1β. Transduced MSC pellets reached thereby very high IL1RA transgene expression levels with a peak of 1087 ng/ml after day 7, followed by a decrease to 194 ng/ml after day 21, while IL1RA concentrations of controls were permanently below 200 pg/ml.

Conclusion: Our results indicate that FVV are capable of efficient gene transfer to MSCs, while reaching IL1RA transgene expression levels, that were able to efficiently block the impacts of IL1β *in vitro*. FVV merit further investigation as a means to study the potential as a gene transfer tool for MSC based therapies for cartilage repair.

Keywords: mesenchymal stem cell, chondrogenesis, pellet culture, foamy virus, virus vectors, IL1RA, interleukin 1 receptor antagonist, arthritis

INTRODUCTION

The repair capacity of articular cartilage is very limited, among others due to the lack of vascularization that could provide progenitor cells to the injured tissue (Caplan et al., 1997; Patra and Sandell, 2012; Orth et al., 2014). Therapies so far are based on the implantation of autologous chondrocytes at the site of the lesion, or marrow-stimulating approaches for the recruitment of bone-marrow derived mesenchymal stem cells (MSCs) (Brittberg et al., 1994; Steinert et al., 2007). MSCs can be isolated readily from several sources, like for instance bone marrow, blood, and mesenchymal tissues (Nöth et al., 2010). Moreover, they are able to self-renew and differentiate into multiple tissues, which makes *ex vivo* expanded MSCs an attractive alternative cell source to chondrocytes (Pittenger et al., 1999). MSCs are already intensively investigated and applied in clinical trials for regenerative therapies in the musculoskeletal system (Steinert et al., 2012). However, such demands failed so far and did not result in the desired sustained regeneration of hyaline cartilage *in vivo*, as the newly formed tissue resulted widely in fibrocartilage like structures (Steinert et al., 2007; Orth et al., 2014). A major problem seems thereby the insufficient delivery of soluble factors for driving the chondrogenic differentiation of the transplanted cells *in vivo* (Steinert et al., 2007; Madry et al., 2011). To overcome this problem, gene transfer technologies have been intensely used to study diverse candidate genes such as bone morphogenic proteins, Indian hedgehog (Ihh) and the SOX (SRY [sexdetermining region Y]-related HMG [high-mobility group] box) family of transcription factors for modulation of the chondrogenic differentiation *in vitro* (Ikeda et al., 2004; Steinert et al., 2009; Haddad-Weber et al., 2010). Among the currently used viral vector systems, are human immunodeficiency virus (HIV)-based orthoretroviral-, Moloney leukaemia virus (MLV)-, adenoviral-, and recombinant adenoassociated virus (rAAV) vectors (Gouze et al., 2002; Palmer et al., 2003; Pagnotto et al., 2007; Steinert et al., 2008; Frisch et al., 2015). Single stranded and self-complementary rAAV vectors are among the most promising vectors for gene therapy so far (Kay et al., 2009; Watson et al., 2013; Cucchiarini and Madry, 2014; Rey-Rico et al., 2015).

Here we studied the use of foamyviral vectors (FVV) for gene delivery to human MSCs. FVV are derived from apathogenic parental viruses and might be a safe and efficient alternative for stable gene transfer (Rethwilm, 2007; Armbruster et al., 2014). They are naturally self-inactivating and have a big packaging capacity due to their large (∼13 kb) proviral genome (Lindemann and Rethwilm, 2011). As therapeutic target in this setup we choose delivery of the interleukin 1 receptor antagonist protein (IL1RA), as the inflammatory cytokin interleukin 1β (IL1β) is highly expressed in diseased and injured joints and a known mediator of cartilage breakdown, synovial inflammation, as well as a known inhibitor of chondrogenesis (Wehling et al., 2009; Kraus et al., 2012; Martínez de Albornoz Torrente and Forriol, 2012; Schett et al., 2016).

MATERIALS AND METHODS

Recombinant DNA

All used foamy vector (FV) plasmids were derived from the plasmid MD9 (Heinkelein et al., 2002; Peters et al., 2005; Wiktorowicz et al., 2009) that expresses the enhanced green fluorescent protein (EGFP) markergene driven by the spleen focus forming virus (SFFV)-U3 promoter (**Figure 1A**). The FV plasmids NA1 and KG84 (**Figures 1A**, **2A**) were already described earlier (Gartner et al., 2009; Armbruster et al., 2014). For cloning of the FV vector plasmid construct NA4 that expresses EGFP via an internal ribosomal entry site (IRES) of the encephalomyocarditis virus driven by the human elongation factor 1α (EF1α) promoter (Uetsuki et al., 1989), EF1α was PCR amplified with specific primers using the pEF-GW-51-lacZ plasmid as PCR template (Gateway Vektor System, Invitrogen) and inserted into the pBF014 plasmid via the AfeI and AscI restriction site (Armbruster et al., 2014) (**Figure 1A**). The plasmid JK1 was derived from a pTW01 based FV plasmid, namely pTW22, coding for mCherry with a fused 2A cleavage site and EGFP (Ryan et al., 1991; Wiktorowicz et al., 2009). The EGFP was replaced with the PCR amplified human IL1RA cDNA, which was inserted via RsrII and SalI (**Figure 2A**). The structure of each plasmid was verified by restriction mapping and sequencing prior to use. FV vectors were abbreviated with the respective promoter and insert (followed by the name of the corresponding vector plasmid), namely FV.CMV-EGFP

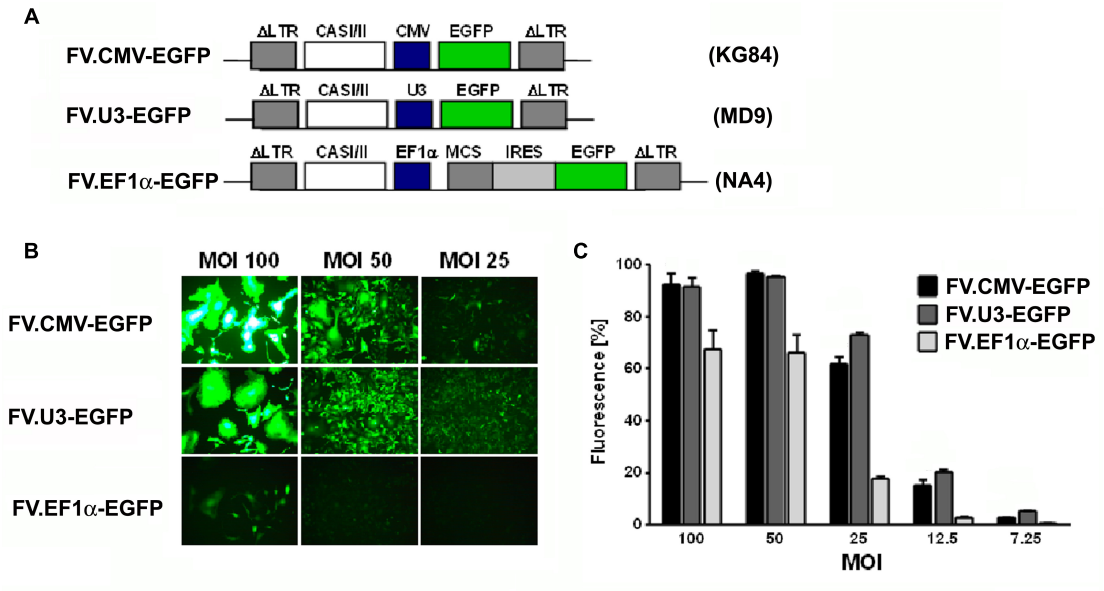

FIGURE 1 | Vector optimization. Part 1: Promoter study. **(A)** Schematic illustration and designation of foamyviral vectors (FVV) expressing enhanced green fluorescent protein (EGFP) with different promoters. The expression of the EGFP genes were under the control of the constitutively active cytomegalovirus immediate early promoter, the SFFV U3 promoter, or the human elongation factor (EF) 1α promoter, and the corresponding foamyvirus vectors (FVV) (and vector plasmids) were disignated FV.CMV-EGFP (KG84), FV.U3-EGFP (MD09), and FV.EF1α-EGFP (NA4). IRES (internal ribosomal entry site), CASI/II (cis-acting sequences; required for vector transfer), MCS (multiple cloning site), LTR (long terminal repeat). **(B)** Fluorescence microscopy of HT1080 transduced fibroblasts 3 days post-transduction with the different FVV at different multiplicities of infection (MOI). 100× magnification. **(C)** Quantification of transduction rates analyzed by EGFP expression in FACS analyses 3 days post-transduction at different vector dilutions (MOI). MOCK controls revealed no EGFP positive cells (data not shown). Data are shown as mean + SD with $n = 2$ experiments and three replicates per condition.

(KG48), FV.U3-EGFP (MD9), FV.EF1α-EGFP (NA4), FV.U3-IL1RA-EGFP (NA1), FV.U3-mcherry-IL1RA (JK1) (illustrated in **Figures 1A**, **2A**).

Foamyviral Vector Production

Viral vector stocks were generated by transient transfection of HEK 293T cells as descriebd earlier (Stange et al., 2005; Wiktorowicz et al., 2009; Armbruster et al., 2014). Briefly, 8×10^6 cells were seeded on 10 cm-dishes and were transfected the next day using 20 μg of total plasmid DNA with the following ratio 10:5:1:1 of vector plasmid and packaging plasmids (pCZIgag2, pCZIpol, and pCZ-HFVenvEM002) using polyethylenimine (Polysciences) (Stange et al., 2005). MOCK controls were also performed, using a transfection mix lacking the env plasmid and empty pcDNA to adjust the DNA amount. One day after transfection cellular transcription was induced by addition of 10 mM Na-butyrate for 8 h. After 2 days the supernatants were harvested, passed through a 0.45-μm filter (Millipore) and stored in aliquots at −80°C. Vectors were optionally concentrated to higher titers by centrifugation (polyallomer tubes, 12000 g, 4°C, 2 h). The infectious titer of the FVV preparations were determined using vector dilutions and an immunofluorescence assay as previously described (Peters et al., 2008). Vector preparations were adapted to 3.5×10^7 infectious particles/ml and stored at −80°C. (Peters et al., 2008). FVV and transduced cells were handled in the laboratories of the Department of Virology, University of Wuerzburg under appropriate biosafety level 2 conditions according to German law (Gentechniksicherheitsverordnung-GenTSV).

Cell Culture, Vector Transfer, Transgene Expression

The vector-containing supernatant was assayed functionally by transfer to 1×10^4 cells by using different doses of infectious virus, as given per multiplicities of infection (MOI) in the respective experiments. For characterization studies the expression of EGFP was monitored by fluorescence-activated cell sorting (FACS) using a FACSCalibur flow cytometer (Beckton Dickinson) if not otherwise mentioned 72 h after transduction. Levels of cell culture infectious dose 50 ($CCID_{50}$) were determined using the GraphPad prism 4.0 software (GraphPad Software). The vector transfer assays were done at least three times with different plasmid preparations. As recipient cells the human fibroblastic cell line HT1080, the human TERT4 MSC line and primary human MSCs were used (Abdallah et al., 2005). Primary MSCs were obtained from bone marrow of several human donors undergoing total hip replacement surgery after informed consent and as approved by the by institutional review board of Wuerzburg University. MSCs were isolated by adherence of cells harvested from the patient's spongiosa to plastic and maintained as described previously (Nöth et al., 2002).

For evaluation of IL1RA transgene expressions conditioned media from cell cultures were collected over a 24-h period

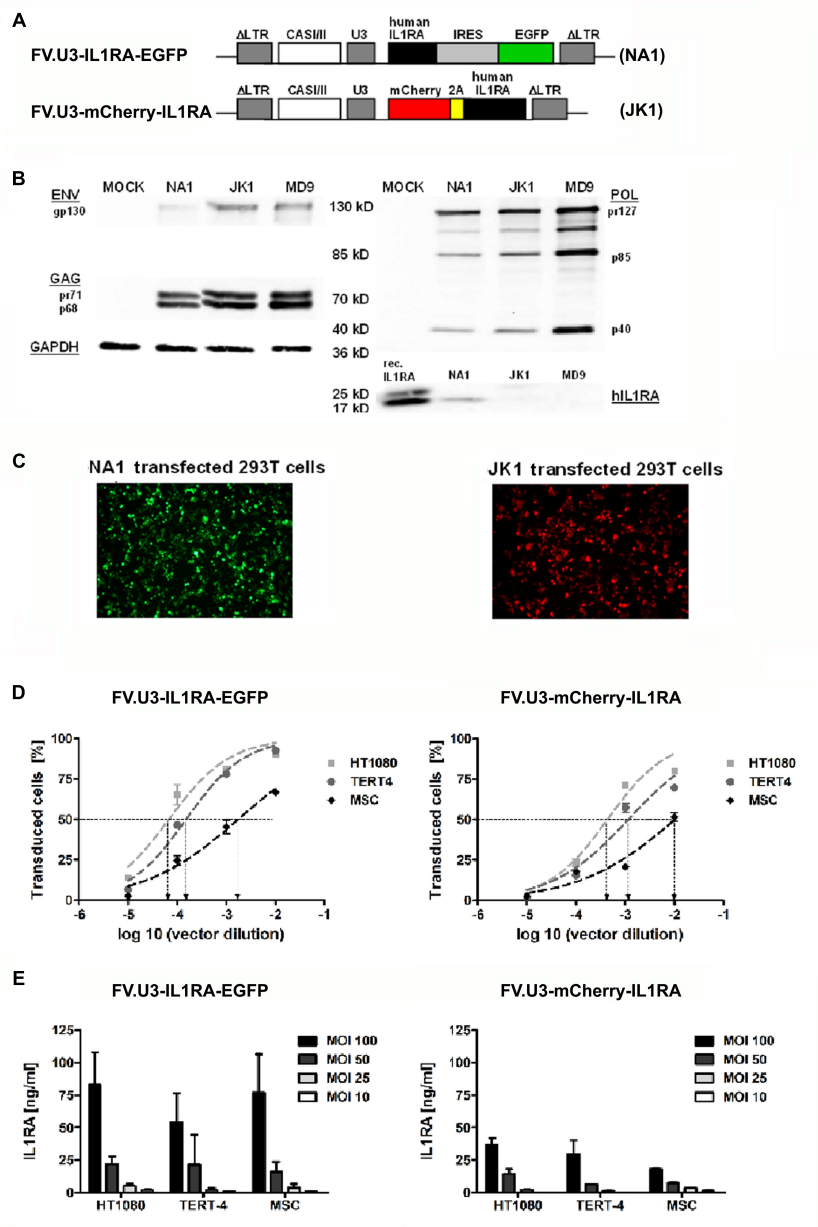

FIGURE 2 | Vector optimization. Part 2: Vector verification study. **(A)** Schematic illustration of the prototype foamy virus vectors under control of the SFFV U3 (SFFV-U3) promoter expressing the therapeutic gene interleukin 1 receptor antagonist (IL1RA) along with a marker gene via two different modes. The FVV were designated (with corresponding name of the vector plasmid) FV.U3-IL1RA-EGFP (NA1) and FV.U3-mCherry-IL1RA (JK1). In the bicistronic FV.U3-IL1RA-EGFP vector, the internal ribosomal entry site (IRES) of the encephalomyocarditis virus allows the co-expression of IL1RA and EGFP from a single promoter and two open reading frames (ORF). In the FV.U3-mCherry-IL1RA vector, the linking of mCherry and IL1RA with the self-cleaving small foot-and-mouth disease virus (FMDV) 2A peptide results in expression of the two proteins derived from a single ORF and also a single promoter. **(B)** Exemplary western blot analysis. Detection of prototype foamy virus (PFV) GAG, POL, and ENV proteins as well as IL1RA in cells cotransfected with the respective FVV plasmid and GAG-, POL-, and ENV-encoding constructs. Glyceraldehyde-3-dehydrogenase (GAPDH) served as loading control. Recombinant human hIL1RA was used as positive control for the IL1RA blot. **(C)** Fluorescence microscopy of 293T cells 24 h after transfection with the respective vector plasmids NA1 (left; green fluorescence) and JK1 (right; red fluorescence). 100× magnification. **(D)** Transduction rates following infection of different mesenchymal cell types with FV.U3-IL1RA-EGFP (left) and FV.U3-mCherry-IL1RA (right). HT1080 fibroblast cell line, TERT4 mesenchymal stem cell (MSC) line, and primary MSCs were incubated with dilution series of vector preparations for dosing and calibration of vector solutions. EGFP+ cells were determined after 48 h by flow cytometry. Using a sigmoidal curve function, CCID50 (cell culture infectious dose 50 – the vector dilution at which the half-maximal transduction rate was visible) concentration values were determined by nonlinear regression. **(E)** Levels of secreted human IL1RA measured by a specific ELISA in cell culture supernatants from transduced cells with different doses of FV.U3-IL1RA-EGFP (left) and FV.U3-mCherry-IL1RA (right). MOCK controls (data not shown) were below the detection limit. Data are shown as means + or ± SD with $n = 3$ experiments and triplicate measurements per condition. MOI = multiplicity of infection.

and stored at –20°C. The human IL1RA concentrations were measured by ELISA (DuoSet Elisa Development Quantikine kit, R&D Systems). The minimum detectable dose of the human IL1RA ELISA is 6.26 pg/ml according to the manufacturer. All measurements were performed in triplicates.

Aggregate Culture

Primary MSCs and the mesenchymal TERT4 cells were transduced with FVVs at 1000 MOI in T-125 flasks at ∼40% confluency to obtain transduction efficiencies of around 50%, which were confirmed by detection of green fluorescence. Three days after transduction EGFP+ cells were sorted using a FACSDiVa (Beckton Dickinson). Selection efficiency was determined to be approximately 99 %. After one week, expansion sorted primary MSCs and mesenchymal TERT4 cells were trypsinized and placed in aggregate cultures as described earlier (Johnstone et al., 1998; Penick et al., 2005). Briefly, cells were distributed in 15 ml polypropylene tubes (Falcon) at a concentration of 3×10^5 cells/pellet in 500 µl medium [serum-free DMEM, 37.5 mg/ml ascorbate, 1 mM pyruvate, 10^{-7} M dexamethasone, 1% ITS (insulin, transferrin, and selenous acid containing culture supplement), all Sigma] to promote aggregate formation. To induce chondrogenesis 10 ng/ml recombinant TGFβ1 protein (R&D Systems) was added. Further, aggregates were additionally supplemented with 5 ng/ml IL1β (R&D Sytems) to inhibit chondrogenesis. The aggregates were cultured at 37°C, 5% CO2 and formed spherical pellets within 24 h. Changes of media were performed every 2 to 3 days. The aggregates were harvested after 21 days for further analysis. At least three different aggregates per group and bone marrow preparations from three different preparations were analyzed if not otherwise mentioned. MSC passages for aggregate cultures ranged from passage 4-6.

Cell Proliferation and Glycosaminoglycan Assay

Cell proliferation in aggregates was assessed by quantitative detection of adenosine 5′-triphosphate (ATP), which correlates with the number of viable cells present, using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, pellets were homogenized mechanically using a pellet pestle and mixed with 100 µl of CellTiter-Glo® reagent (CellTiter-Glo® substrate + CellTiter-Glo® buffer). After incubation for 10 minutes at room temperature luminescence was measured using a plate-reading luminometer.

For analysis of glycosaminoglycan (GAG) content, aggregates were washed with phosphate buffered saline (PBS), digested with 200 µl of papain digest solution (1 µg/ml, Sigma), and incubated for 16 hours at 65°C. Samples were stored at –20°C. Total GAG content was measured by reaction with 1,9-dimethylmethylene blue using the Blyscan™ Sulfated Glycosaminoglycan Assay (Biocolor Ltd) as directed by the supplier. For normalization, DNA content of aggregates was also determined fluorometrically using the Quant-iT™ PicoGreen® kit as directed by the supplier (Invitrogen).

Histological and Immunohistochemical Analyses

For histological analyses, aggregates were fixed in 4% paraformaldehyde for one hour afterward dehydrated in graded alcohols, embedded in paraffin and sectioned to 5 µm. Representative sections were stained using haematoxylin and eosin (H&E) for evaluation of cellularity and alcian blue (Sigma) for the detection of matrix proteoglycan.

For immunohistochemical analyses sections were washed for 20 min in Tris-buffered saline (TBS) and incubated in 5% bovine serum albumin (BSA) (Sigma). Following washing in TBS, sections were pre-digested with pepsin at 1 mg/ml in Tris–HCl (pH 2.0) for 15 min at room temperature. Sections were then incubated overnight at 4°C with a monoclonal anti-COL II primary antibody (diluted in 0.5% BSA, Acris Antibodies GmbH) for detection of collagen type II (Collagen II). Immunostaining was visualized by treatment with peroxidase-conjugated antibodies (Dako) followed by diaminobenzidine staining (DAB kit; Sigma). The slides were finally counterstained with hemalaun (Merck).

Immunoblotting

Analysis of viral protein expression was done essentially as described previously (Peters et al., 2005). In brief, cell lysates were prepared using lysis buffer (RIPA plus protease inhibitors). Viral proteins were probed with anti-Gag (Heinkelein et al., 2002), anti-Pol and anti-Env (Imrich et al., 2000) mouse monoclonal antibodies after separation in sodium dodecyl sulfate-containing 8% polycrylamide gels and semi-dry blotting onto Hybond ECL membranes (Pharmacia). Protein bands were detected using horseradish-coupled secondary antibodies (Dako) and employing the ECL detection system (Pharmacia). For detection of human ILRA protein, the antibody SC-25444 from Santa Cruz was used (1/200 dilution). As loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used (0.5 µg/ml, IMG-5143A, Imgenex).

Total RNA Extraction, Semi-quantitative, and Real-Time RT-PCR

Total RNA from cultured cells was isolated using Trizol (Invitrogen) and reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad). For quatitative polymerase chain reaction (qPCR) analyses, the iCycler iQ system (BioRad) with QuantiFast SYBR Green PCR Master Mix was used. PCR was conducted in tripicates for each sample. Isoform-specific primers were used for the expression of human IL1RA (Sigma–Aldrich) and βActin (QuantiTect Primer Assay, Qiagen) was used for normalization. The primer sequences were as follows: human IL1RA forward 5′-ggcctccgcagtcacctaatcactct-3′, reverse 5′-ttgacacaggacaggcacat-3′. The amplified transcripts were quantified using the comparative ΔΔCT method (Pfaffl et al., 2002).

Statistical Analyses

The numerical data were expressed as mean values plus standard deviation (SD). All experiments were performed in triplicates on $n = 2$–8 different samples as indicated in the respective

experiments. Where indicated, numerical data were subjected to variance analysis (one or two factor ANOVA) and statistical significance was determined by student's *t*-test with $p < 0.05$ considered statistically significant, $p < 0.01$ considered very significant and $p < 0.001$ considered extremely significant. All measurements were done with a minimum of three technical replicates if not otherwise mentioned.

RESULTS

Foamyviral Vector Optimization

We first aimed to compare three different promoters in their ability to drive the EGFP expression in FVV constructs by transducing the human fibrosarcoma cell line HT1080 (**Figure 1**). For this, the EGFP transgene in the FVV was under the control of either the constitutively active SFFV-U3 promoter, the cytomegalovirus immediate-early promoter (CMV) or the human EF1α promoter via an IRES site (**Figure 1A**). Accordingly, the cells were cultured and transduced with different vector doses. Three days thereafter fluorescence microscopy and FACS analysis revealed a dose-dependent effect of EGFP expression by all FVV types at different dilutions (**Figure 1C**). The two viral promoters were thereby similarly strong, whereas the EF1α promoter/IRES construct showed less EGFP+ cells, and fluorescence also appeared weaker under the fluorescence microscope (**Figures 1B,C**). To obtain a more detailed and representative comparison, the majority of the transduced cells should contain at best one viral integration per cell. At the low FV concentrations used, namely MOI 7.25, 12.5, and 25 in our experiments, FV fabricated from the SFFV-U3 construct were stronger than the CMV and EF1α promoter construct vectors (**Figure 1C**). We further verified a centrifugation protocol which we used for the concentration of our vector preparations. Fluorescence microscopy and CCID50 determination of the standard control vector 3 days after the respective transduction, revealed a 26-fold concentration factor compared to the uncentrifuged FVV preparation (data not shown).

As recently published, we exploited already FVV carrying the SFFV-U3 promoter for driving the transgene expression in rat knee joints (Armbruster et al., 2014). We therefore were interested in comparing our FV.U3-IL1RA-EGFP (NA1) in which an IRES site allows the co-expression of IL1RA and EGFP from the SFFV-U3 promoter, with a new FVV called FV.U3-mCherry-IL1RA (JK1) (**Figure 2A**). The FV.U3-mCherry-IL1RA vector is based on a FVV with a 850 bp shorter *cis*-acting sequence (CAS) that was previously characterized and is sought to improve the safety and packaging capacitiy of available FVV (Wiktorowicz et al., 2009). Further the linkage of mCherry and IL1RA with the self-cleaving small foot-and-mouth disease virus (FMDV) 2A peptide in the FV.U3-mCherry-IL1RA construct results in expression of the two proteins derived from a single open reading frame (ORF) (**Figure 2A**). Western blot analysis of 293T whole cell lysates transfected with the respective FVV plasmid and gag-, pol-, and env-encoding constructs showed the presence of the FV precursor and processed proteins (**Figure 2B**). The IL1RA levels from the FVV (NA1 and JK1) transfected cells were unexpectedly low compared to the loaded recombinant hIL1RA control. Two faint bands, corresponding to a 25 kD glycosylated and a 17 kD unglycosylated IL1RA form were visible within the JK1 transfected cells, whereas one 17 kD IL1RA band was detectable with the NA1 transfected cell lysate. EGFP and mCherry expression of the two constructs were further verified by fluorescence microscopy 24 h after transfection (**Figure 2C**).

We next determined CCID50 values (the vector dilution at which the half-maximal transduction rate was visible) of the FV.U3-IL1RA-EGFP (NA1) and FV.U3-mCherry-IL1RA (JK1) vector (**Figure 2D**). In order to compare the two different vectors, HT1080 fibroblasts, mesenchymal TERT4 cells and primary MSCs were incubated with a dilution series of the same vector preparation. As depicted in **Figure 2D**, primary MSCs were less susceptible (CCID50: JK1 = 0.01; NA1 = 0.003) to FVV transductions than TERT4 (CCID50: JK1 = 0.001; NA1 = 0.0002) and HT1080 cells, that were more susceptible (CCID50: JK1 = 0.0004; NA1 = 0.00007). This corresponded for instance to a 24- and 38-fold-lower susceptibility for primary MSCs compared to HT1080 cells for the FV.U3-IL1RA-EGFP and FV.U3-mCherry-IL1RA vector, respectively. Overall the the FV.U3-IL1RA-EGFP CCID50 values were about one log ratio smaller than the FV.U3-mCherry-IL1RA values, implying a better transduction efficiency of the FV.U3-IL1RA-EGFP vector. In parallel we analyzed the secreted IL1RA levels in the cell culture supernatants from the transduced cells with a specific ELISA (**Figure 2E**). Here we obtained corresponding results to the FACS analysis with a higher IL1RA secretion from the FV.U3-IL1RA-EGFP than from the FV.U3-mCherry-IL1RA transduced cells. Interestingly we also found that although we obtained lower transduction rates with MSCs compared to HT1080 and TERT4 cells, the secreted IL1RA levels from MSCs were comparable to the two cell lines for both FVV vectors. For instance the FV.U3-IL1RA-EGFP transductions with an MOI of 100 resulted in mean IL1RA amounts of 83, 54, and 77 ng/ml for HT1080, TERT4 and MSCs, respectively (**Figure 2E**).

FV.U3-IL1RA-EGFP Vector Verification

We further verified the FV.U3-IL1RA-EGFP (NA1) vector in more detail using different cell lines and primary cells (**Figures 3**, **4**). Fluorescence microscopy 3 days after the respective transductions displayed as already shown, that HT1080, TERT4, and MSCs are highly susceptive to gene delivery with FV.U3-IL1RA-EGFP (NA1) and FV.U3-EGFP (MD9) vectors (**Figure 3A**) at 1000 MOI. To avoid possible variability of expression due to the choice of promoter at low FVV doses, a maximum dose of 1000 MOI was chosen for this experiment. Due to the IRES a weaker EGFP expression from the FV.U3-IL1RA-EGFP vector compared to the FV.U3-EGFP construct was observed. This was not surprising as differences in expression levels due to the use of an IRES from the up- or downstream gene were already reported (Houdebine and Attal, 1999; Mizuguchi et al., 2000).

The numbers of EGFP+ cells in the respective cultures transduced with different dilutions of the FV.U3-IL1RA-EGFP vector were quantified using FACS analyses 3 days after vector exposure, and revealed a dose-dependent effect of EGFP

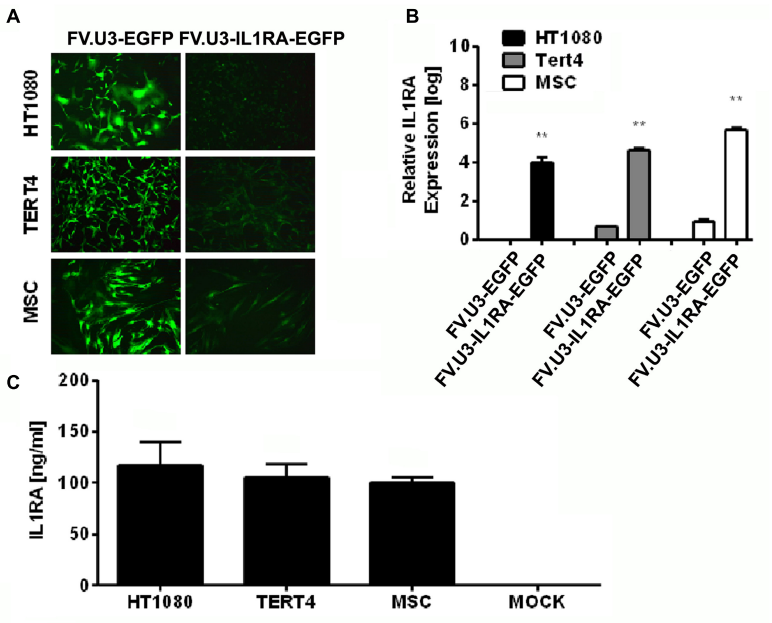

FIGURE 3 | Foamy vector (FV)-mediated gene delivery to human mesenchymal cell lines and primary MSCs. (A) Fluorescence microscopy of fibroblastic HT1080 cell line, MSC cell line TERT4 and primary MSCs (MSC) transduced with a high dose of 1000 MOI of FV.U3-EGFP (left) or FV.U3-IL1RA-EGFP (right) vectors at 3 days post-transduction. 100× magnification. **(B)** Real-time PCR analysis of human IL1RA mRNA expression after transduction with FV.U3-EGFP and FV.U3-IL1RA-EGFP vectors. The values were calculated using the ΔΔCT method as described earlier, with FV.U3-EGFP or FV.U3-IL1RA being the treatment groups, MOCK the control group, IL1RA the target gene and β-Actin the reference gene. Values represent means + SD of three independent experiments ($n = 3$) per group at day 3 three of culture. Levels of significance compared to the controls (Student's *t*-test) are indicated by asterisks ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 001$). **(C)** Levels of secreted human IL1RA in cell culture supernatants were detected by a specific ELISA (MOI 1000). Data are shown as mean + SD with $n = 2$ experiments and three technical replicates per condition.

expression at different dilutions (not shown). FVV-mediated expressions of the human IL1RA transgene in the different cells were analyzed by real-time RT-PCR on RNA (**Figure 3B**), and by ELISA at the protein level (**Figure 3C**). Production of IL1RA mRNA in the respective cultures 3 days after transduction with FV.U3-IL1RA-EGFP or FV.U3-EGFP vectors relative to MOCK controls revealed that only the FV.U3-IL1RA-EGFP cultures expressed IL1RA mRNAs at high levels (**Figure 3B**). Levels of secreted IL1RA protein in cell culture supernatants conditioned by the different cell types transduced with the same MOI (1000) of FV.U3-IL1RA-EGFP were measured by ELISA and displayed significantly elevated levels of IL1RA expression, with mean values of 105, 117, and 100 ng/ml, respectively (HT1080, TERT4, and MSCs) (**Figure 3C**).

Mesenchymal progenitor cells present an attractive source as an alternative to chondrocytes in cell-based approaches for cartilage repair. After verifying the ability of the FV.U3-IL1RA-EGFP to transduce primary MSCs we analyzed the long-term mediated transgene expression of this vector in monolayer cultures. MSCs were transduced in monolayer, FACS sorted for EGFP+ cells and placed in 6-well plates with medium exchange every 3–4 days. Fluorescence microscopy at several time points over 137 days in culture exposed a sustained EGFP expression over time (**Figure 4A**). Also, a final FACS analysis at day 137 showed that 89% of cells were viable and 68 % of the living cells were EGFP+ (**Figure 4B**). Most strikingly an ELISA analysis over time confirmed IL1RA mean protein levels between 46 and 149 ng/ml over the 137 days in culture, which represents accumulated IL1RA protein concentration in the supernatant over a 24 h period at the respective timepoints. The sustained FVV mediated transgene expression of an anti-inflammatory transgene seems interesting, as MSC *in vitro* cultures are known to adopt a state of permanent cell-cycle arrest and are thought to undergo cellular senescence almost from the moment of *in vitro* culturing (Bonab et al., 2006; Steinert et al., 2007, 2012).

Aggregate Cultures of FVV Transduced Mesenchymal TERT4 Cells and Primary MSCs

We next aimed to evaluate the functionality of the IL1RA transgene with studying its ability to block the inhibitory effects of IL1β on chondrogenesis of primary MSCs and the TERT4 MSC line in 3D pellet cultures. Therefore, primary MSCs and the TERT4 MSC cell line were stimulated along the chondrogenic pathway with a standard dose of 10 ng/ml TGFβ1 (Johnstone et al., 1998; Steinert et al., 2009). The cells were maintained following their transduction with FV.U3-IL1RA-EGFP at 1000 MOI in monolayer as pellet cultures in standard chondrogenic media in the presence or absence of 5 ng/ml IL1β (**Figure 5A**). The schematic of the experimental set-up using pellet cultures is depicted in **Figure 5A**, and a macroscopic image of a TERT4

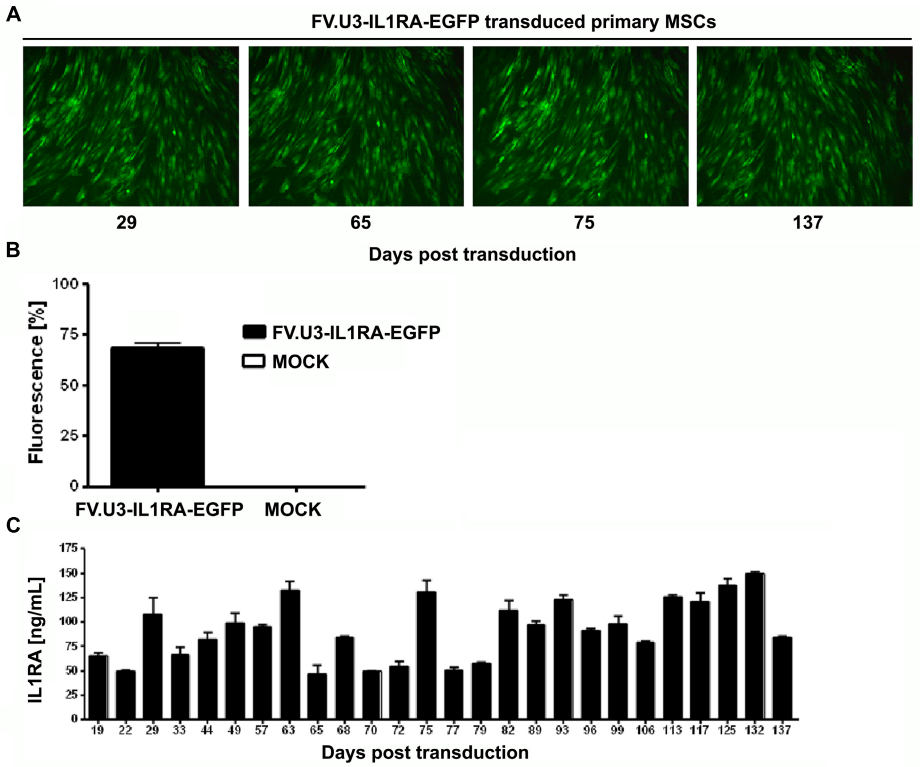

FIGURE 4 | Long-term FV-mediated transgene expression in primary MSCs. (A) Fluorescence microscopy of human marrow-derived primary MSCs transduced with FV.U3-IL1RA-EGFP at 1000 MOI. Images taken over time are shown at 100× magnification. **(B)** Final FACS analysis of green fluorescence at 137 days post-transdcution of the FV.U3.IL1RA-EGFP cultures compared to MOCK controls. Values are given as mean + SD of $n = 3$ measurements. **(C)** IL1RA levels in the supernatant were measured by ELISA over time, and 24-hour-accumulations were analyzed. The experiment shown was done with MSCs from one donor and values represent means + SD from triplicate measurements per timepoint.

control pellet in a 15-ml-conical-tube after three days is shown on the left, and representative images upon fluorescence microscopy at 100× of MOCK control and FV.U3-IL1RA-EGFP aggregates are shown at the bottom (**Figure 5A**).

After three weeks of TERT4-aggregate culture, the pellets were harvested and analyzed for chondrogenic phenotypes (**Figures 5B,C**). MOCK control TERT4 aggregates supplemented with TGFβ1 showed a significant chondrogenic response shown by a strong metachromic staining for matrix proteoglycans with alcian blue (**Figure 5C**). On the other hand, aggregates treated with IL1β, showed an inhibited chondrogenesis with negative alcian blue staining and smaller appearing pellets (**Figures 5A,B**; left images). Moreover, the transduction with the FV.U3-IL1RA-EGFP and respective IL1RA expression was able to rescue the inhibiting effect of IL1β on the chondrogenic differentiation, shown by restored positive alcian blue stainings (**Figures 5A,B**; right images).

In addition, for a quantitative comparison of the extracellular matrix synthesis between the different treatment groups over time, GAG levels in the TERT4 pellets were determined (**Figure 5D**). Pellets treated with IL1β showed significantly decreased GAG contents compared to the other treatment groups. Besides, already at day 3 significantly elevated GAG synthesis levels in the FV.U3-IL1RA-EGFP and FV.U3-IL1RA-EGFP + IL1β treated pellet groups became apparent and lasted over the 3 weeks of culture (**Figure 5D**). At distinct time points we also quantified the cell proliferation using an ATP cell proliferation assay. The IL1β treated pellets showed the highest proliferation levels among the treatment groups at day 3, but then stayed approximately equal over time. Within all the other treatment groups the cell proliferation levels increased gradually over time (**Figure 5E**).

In another experiment a similar experimental set-up (see **Figure 5A**) has been applied to aggregate cultures of primary MSCs that were genetically modified with FV.U3-IL1RA-EGFP (NA1) at 100 MOI or not (MOCK controls) (**Figure 6**). First, we analyzed the EGFP expression levels of primary MSCs after transduction with FV.U3-IL1RA-EGFP and EGFP-FACS-sorting in monolayer and aggregate culture compared to MOCK controls and representative images upon fluorescence microscopy are presented in **Figure 6A**.

Then we compared the IL1RA expression levels of primary MSC aggregates that were transduced with FV.U3-IL1RA-EGFP in monolayer and either FACS sorted or not (**Figure 6B**). After 1 week expansion both experimental groups were placed in aggregate cultures and supernatants were harvested at distinct

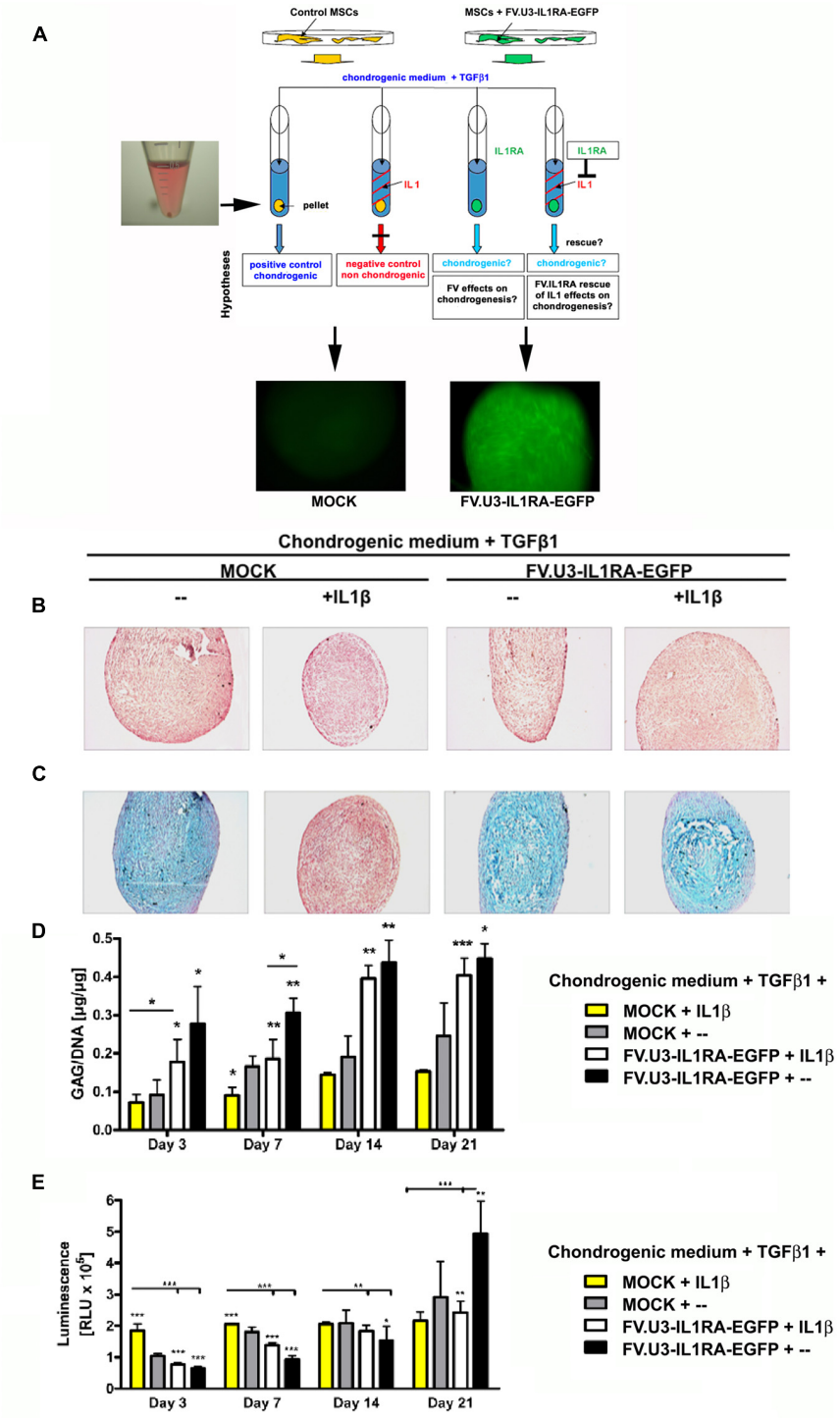

FIGURE 5 | Chondrogenesis assay using the MSC cell line TERT4. (A) Schematic overview of the different treatment groups. Monolayer cultures were MOCK treated or FV.U3-IL1RA-EGFP transduced at 1000 MOI, FACS sorted for EGFP+ cells, and placed into aggreagte cultures with 3×10^5 EGFP+ cells/pellet. Aggregates were treated with TGF1β (10 ng/ml) for chondrogenic induction. For chondrogenesis inhibition media were supplemented with recombinant human IL1β (5 ng/ml). **(B)** Representative paraffin sections with H&E staining for evaluation of cellularity and cell morphology after 21 days of pellet culture. **(C)** Alcian blue staining staining for detection of matrix proteoglycan after 21 days of pellet culture. **(D)** Time-course analysis of glycosaminoglycan (GAG) content normalized to DNA. **(E)** At distinct time points cell proliferation was quantified using the ATP cell proliferation assay. ATP = adenosine 5 triphosphate. The data represent mean values + SD from two experiments and three aggregates per condition and time point. Statistical significance was given by asterisks relative to MOCK controls without IL1β supplementation (MOCK + –) and by bars and asterisks upon multiple comparisons between groups (*$p < 0.05$, **$p < 0.01$, ***$p < 001$).

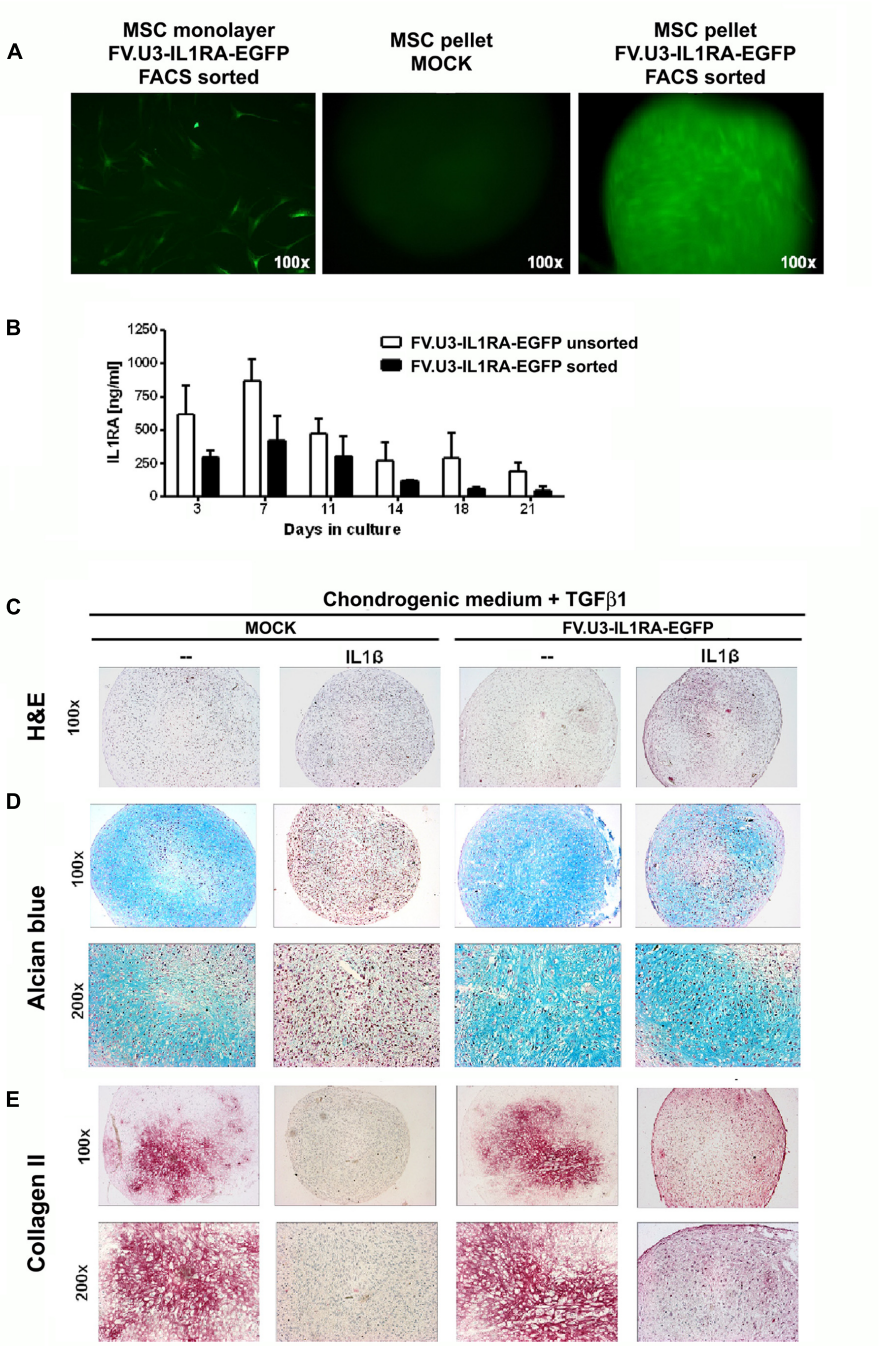

FIGURE 6 | Chondrogenesis assay using primary MSCs. (A) Fluorescence microscopy of FV.U3-IL1RA-EGFP transduced monolayer MSCs 3 days post-transduction and aggregates of untreated control (MOCK) and FV.U3-IL1RA-EGFP transduced aggregates. **(B)** Effect of cell sorting on the transgene expression. MSCs were transduced in monolayer with FV.U3-IL1RA-EGFP and half of the cells were FACS sorted (EGFP) 2 days later. After 1 week expansion, both experimental groups were placed in aggregate cultures (3×10^5 cells/pellet) and supernatants were harvested at distinct time points. Levels of secreted human IL1RA were measured by a specific ELISA. Data are represented as mean + SD. MSCs from two donors with three aggregates per experimental group and time point were analyzed. C-E) MOCK controls and FV.U3-IL1RA-EGFP transduced EGFP+ FACS sorted MSCs were placed in aggregate cultures and treated with TGFβ1 (10 ng/ml) for chondrogenic induction. For chondrogenesis inhibition media were supplemented with recombinant IL1β (5 ng/ml). **(C)** Representative paraffin sections with H&E staining for evaluation of cellularity and cell morphology after 21 days of pellet culture. **(D)** Alcian blue staining for detection of matrix proteoglycan. **(E)** Immunohistochemical analyses for detection of the cartilage matrix protein collagen type II (Collagen II) after 3 weeks. Collagen II staining with positive staining regions that appear red. 100× and 200× magnifications were used.

time points. Within both groups, the transgene expression levels peaked at day 7 and decreased thereafter over time (**Figure 6B**). Possibly due the FACS sorting procedure, the sorted pellets showed thereby at each time point approximately markedly lower IL1RA amounts compared to the levels of the unsorted pellets. Transduced unsorted MSC pellets reached very high IL1RA levels with a peak of 1087 ng/ml after day 7, followed by a decrease to 194 ng/ml after day 21 (**Figure 6B**), while IL1RA concentrations of controls were permanently below 200 pg/ml (not shown).

After 21 days of primary MSC-aggregate culture, the pellets were harvested and analyzed for chondrogenic phenotypes (**Figures 6C–E**). MOCK control pellets supplemented with TGFβ1 showed a significant chondrogenic response shown by increased aggregate size and a strong positive proteoglycan staining with alcian blue, while aggregates treated with IL1β, negative alcian blue staining and smaller appearing pellets (**Figures 6C,D**; left panels). Notably, the transduction with FV.U3-IL1RA-EGFP was able to rescue the inhibiting effect of IL1β on the chondrogenic differentiation, revealed by restored positive alcian blue stainings (**Figures 6C,D**; right panels).

We further conducted the immunohistochemical analysis of collagen type II (Collagen II) in primary MSC aggregates which is the predominant collagen type in hyaline cartilage (**Figure 6E**). MSC aggregates that were stimulated along the chondrogenic pathway with TGF-β1 revealed a strong Collagen II staining that was abrogated with IL1β supplementation (**Figure 6E**; left images). On the other hand, transduction with the FV.U3-IL1RA-EGFP was able to rescue the inhibiting effect of IL1β on the chondrogenic differentiation, shown by a restored Collagen II staining (**Figure 6E**; right images).

DISCUSSION

Foamyvirus vectors are derived from foamy viruses, also known as spumaretroviruses, that constitute a subfamily of retroviruses (Rethwilm, 2007). In our recent work, we evaluated prototype FVV for an *ex vivo* gene delivery approach in rat knee joints (Armbruster et al., 2014). Here we explored their patterns of transgene expression in different mesenchymal cell lines, especially we were interested in studying their ability to also transduce primary human MSCs. We assessed and optimized FVV transduction efficiencies using different vector modifications and examined the functionality of the expressed IL1RA transgene in an *in vitro* model of chondrogenesis.

In a first attempt we compared three FVV constructs with different ubiquitous promoters. We found that the two viral promoters, CMV and SFFV-U3, were significantly stronger in HT1080 cells compared to the cellular EF1α promoter (**Figure 1**). We acknowledge that this was not a fair comparison, as the construct containing the EF1α promoter also had an IRES site as opposed to the other vector constructs included in this study, which is often responsible for attenuated expression of genes downstream of the IRES. As it is known that promoters can vary considerably in their strength between different cell types, and as the CMV promoter was reported recently as very weak in rat MSCs (Qin et al., 2010; McGinley et al., 2011), we further focused on the already *in vivo* verified SFFV-U3 promoter constructs. Interestingly the EF1α promoter has been reported to be superior compared to the other promoters tested (CMV and PGK (phosphoglycerate kinase)) within lentiviral constructs in experiments using rat MSCs (Xia et al., 2007; McGinley et al., 2011). For clinical applications, the use of endogenous promoters seems favorable as viral promoters are often susceptible to transcriptional silencing in particular cell types (Bestor, 2000). The FVV constructs using the human EF1α promoter could therefore be interesting for future studies on human primary MSCs. Furthermore, FVV could be effectively concentrated around 25-fold by ultracentrifugation in polyallomer tubes (data not shown) confirming utility of this vector system similar to previous results (Vassilopoulos et al., 2001; Vassilopoulos and Rethwilm, 2008; Leurs et al., 2003).

Further, we compared for the co-expression of the IL1RA and EGFP or mCherry transgene, by using an IRES or a self-cleaving 2A peptide FVV construct for dual transgene expression (Donnelly et al., 2001; Kim et al., 2011). The comparison between the two FVVs revealed higher IL1RA expression levels and transduction efficiencies with the IRES FVV construct FV.U3-IL1RA-EGFP (NA1) compared to the 2A peptide FVV construct FV.U3-mCherry-IL1RA (JK1) (**Figure 2**). Although the IRES construct seemed superior, the vector with the shorter CAS possesses a higher safety profile and might be more suitable for future clinical applications (Wiktorowicz et al., 2009). Despite the advantage of a shorter viral sequence in the FV.U3-mCherry-IL1RA (JK1) vector, also the replacement of the IRES with the self-cleaving 2A peptide might provide further benefits, hence the IRES can be unreliable and doesn't provide equal levels of expression of the separated genes, with the downstream sequence being usually translated at much lower levels than the upstream sequence (Trichas et al., 2008). Our western blot analysis of lysates from 293T cells transfected with FVV plasmids showed overall unexpectedly faint IL1RA signals, that unfortunately did not allow us to study uncleaved bands of IL1RA-mCherry fused protein amounts (**Figure 2B**). Here, cell lysates and supernatants from FV-transduced cells could provide a more detailed western blot pattern in the future. However, despite the interesting technical specifics detailed above in co-expressing two transgenes, it remains to be seen whether such dual transgene expression approaches via FVV will be of any clinical relevance at all in the future.

Although FVV cannot be pseudotyped (Wu and Mergia, 1999), a very broad tropism makes them a promising tool for gene delivery to various cells (Plochmann et al., 2012). As already mentioned, because of the capability of MSCs to differentiate into several lineages, including chondrocytes, adipocytes and osteoblasts, as well as their role in tissue repair and their ability to home to the site of injury after systemic administration, several clinical trials for a wide range of diseases have been reported and are ongoing (Mahmood et al., 2003; Barry and Murphy, 2004; Steinert et al., 2012; Madrigal et al., 2014; Kim and Cho, 2015; Seebach et al., 2015). We extensively studied the FVV construct FV.U3-IL1RA-EGFP (NA1) compared to a FV.U3-EGFP (MD09) control vector in their ability to transduce mesenchymal cell lines and primary MSCs (**Figure 3**) and provide

sustained long-term transgene expression in primary MSCs over a time period of 137 days which is noticeable (**Figure 4**). The cells were transduced in monolayer (passage 2), FACS sorted for EGFP and kept in 6-well plates without passaging over the time (**Figure 4**). In their state of replicative senescence, the cells maintained the transgene expression, without showing peculiar morphological changes over time (no enlarged cells), as reported by microscopy and FACS analysis. Remarkably, the IL1RA transgene expression persisted at levels around 50 ng/ml until the end of the monolayer culture experiment of more than 4 months (**Figure 4C**). The expression of the anti-inflammatory IL1RA might have had beneficial effects for inhibiting the senescent phenotype of MSCs under such culture conditions (**Figure 4C**).

Further it has been interestingly shown that transductions at late passages result in lower transgene expression levels compared to early passage transductions (McGinley et al., 2011). In our experiments, we were able to detect very high IL1RA amounts from aggregates with later passage cells (passage 7–9) that were transduced, FACS sorted and expanded (**Figure 6B**). The effect of the sorting procedure to the primary MSCs on the other hand resulted in lower IL1RA amount as compared to unsorted MSC pellets (**Figure 6B**), indicating that our sorting procedure might have stressed the cells.

The high levels of transgene expression in the pellet cultures following transduction with FV.U3-IL1RA-EGFP (**Figure 6**) compared to monolayer cultures (**Figures 2E**, **3B**, **4C**) might be attributed to the fact, that only 0.5 ml of media was used for each pellet, which allows strong accumulation of the secreted transgene product. Secondly, the high-density cell culture conditions might have triggered transgene expression, a phenomenon which was also seen in previous studies using adenovirus vectors in similar culture conditions (Steinert et al., 2009).

Because of their low frequency in bone-marrow, the *in vitro* expansion of MSCs prior to clinical use is necessary. Besides, as already mentioned, MSCs become senescent over time in culture, they are not further able to proliferate, show impaired functions and shortened telomers (Baxter et al., 2004; Bork et al., 2010; Duscher et al., 2014). The MSC donor age also significantly affects the rate of *in vitro* senescence in MSC, which also might have an effect on the transgene expression level and duration (Stenderup et al., 2003; Wagner et al., 2009). More detailed experiments to further study the long-term expression of (aged) MSCs as well as molecular senescence markers was beyond the scope of this study, but should be addressed in the future.

TGFβ1 supplementation in a defined serum-free medium in 3D aggregate cultures is routinely used to study the chondrogenic differentiation of MSCs *in vitro*, which has been characterized for cells from different species and also cultures with different biomaterials like alginate, agarose and fibrin alginate hydrogels have extensively been studied (Johnstone et al., 1998). Here we have chosen a biomaterial-free system, the 3D pellet culture system, to further verify IL1RA transgene expression in the MSC cell line TERT4 and primary MSCs (**Figures 5**, **6**).

The telomerase immortalized human MSC cell line TERT4 has been shown to bypass senescence, which has already been verified in several studies (Simonsen et al., 2002; Li et al., 2007; Bocker et al., 2008). Telomerase immortalization of TERT4 cells might also be responsible for the increasing levels of cell proliferation in pellet culture (**Figure 5E**), which is usually not seen in pellet cultures using primary MSCs (Johnstone et al., 1998; Steinert et al., 2009). Interestingly all groups, with exception of the IL1β group, showed a significantly increased cell proliferation especially in the last week of differentiation in the FV.U3-IL1RA-EGFP treatment group compared to the other groups (**Figure 5E**). However, the significance of this finding remains unclear to date and may arise upon further investigation.

Notably, we also detected higher GAG levels over the 21 days of differentiation in the TERT4 pellet groups, that expressed FV.IL1RA, compared to the untransduced chondrogenic positive control (gray bars, **Figure 5D**). This effect might be due to the IL1RA expression or FVV mediated, which also has to be further addressed in future experiments.

Although the IL1RA expression levels of EGFP+ FACS sorted MSCs were lower compared to the levels from unsorted transduced pellets, we performed our aggregate experiments with sorted cells (**Figures 5**, **6**). More experiments for an explicit picture of possible detrimental effects of the sorting procedure on the integrity of the cells should therefore be addressed in the future. We recently showed the feasibility of transduction and FACS sorting of primary rat synovial cells, that succesfully expressed the IL1RA transgene after reimplantation *in vivo* (Armbruster et al., 2014). Moreover, it has to be verified if DNA integration and the resultant transgene expression with high MOIs of FVVs, does adversely affect MSC plasticity, which has already been shown to be absent for lentiviral vectors (Van Damme et al., 2006; McGinley et al., 2011). Though, as we didn't see chondrogenesis to be affected after FVV transduction within our experiments, other differentiation pathways might possibly be similarly unaffected by FVV transdcution itself.

After 21 days of chondrogenic differentiation we analyzed the pellets and found that the IL1β inhibited chondrogenesis could be equally rescued with FV.U3-IL1RA-EGFP transduced TERT4 cell line and primary MSC pellets regarding restored positive cartilage proteoglycan stainings (Alcian blue, **Figures 5C,D**). Correspondingly, the immunohistochemical analyses of FV.U3-IL1RA-EGFP transduced MSC pellets treated with IL1β showed a rescued Collagen II staining (**Figure 6E**). Although the staining was slightly weaker compared to the TGFβ positive controls, it appeared very homogenous with intense stainings being restricted to potentially hypertrophic areas (**Figure 6E**). Noteworthy we also found more homogenous Collagen II stainings in the positive control groups among our different MSC donors (data not shown), which might have been due to differences in the differentiation potential among the donors (Steinert et al., 2007; Mueller and Tuan, 2008; Yu and Kang, 2013).

The evaluation of chondrogensis in this work is limited to only one concentration of the chondrogenic inducer TGFβ1 (10 ng/ml), one concentration of the chondrogenic inhibitor IL1β (5 ng/ml), and only one dose of FV.U3-IL1RA-EGFP vector (100 MOI) which were used in the respective experiments using the TERT4 MSC cell line (**Figure 5**) and the primary MSCs (**Figure 6**), and only one type of 3D culture system

(pellet culture). The inhibitory dose of IL1β of 5 ng/ml was used as this dose reflects a very high dose of this cytokine which is usually not seen as intraarticular concentration, even in severe arthritic or traumatic conditions (Martínez de Albornoz Torrente and Forriol, 2012; Schett et al., 2016). Furthermore, Wehling et al. (2009) studied the effects of IL1β in inhibiting chondrogenesis of primary MSCs in a dose-dependent manner on a molecular level, with the concentrations 1 and 10 ng/ml being most effective in the presence of 10 ng/ml TGFβ1, which argues for the choice of 10 ng/ml TGFβ1 as chondrogenic inducer and 5 ng/ml IL1β as chondrogenic inhibitor in our experimental set-up. Despite FVV dose-response experiments were perfomed using different types of FVV (**Figures 1–3**) for vector optimization, the analysis of different doses of FVV in blocking the inhibitory effects of 5 ng/ml IL1β on chondrogenesis would have been desirable. As our data on chondrogenesis is limited to protein level (**Figures 5**, **6**), future work on RNA level is mandatory, that comprises various aspects of chondrogenesis and inflammation including hypertrophy, osteogenic induction, and MMP regulation, among others.

CONCLUSION

We constructed and verified several prototype foamyviral derived vector constructs and tested them on different mesenchymal cell types. The FVVs efficiently transduced primary human MSCs and a MSC cell line with high and sustainable transgene levels. In a 3D pellet culture model of chondrogenic differentiation the FVV mediated IL1RA expression was able to inhibit the effects of recombinant IL1β and to restore the chondrogenic phenotype of the aggregates. FVV might therefore be suitable to study and improve the outcome of MSC-based approaches for cartilage repair and merits further investigation to experimentally optimize such approaches in the future.

AUTHOR CONTRIBUTIONS

All authors have read and approved the manuscript and contributed to the study design, data analysis, interpretation of data and drafting, and revision of the manuscript. All data have been generated by NA, JK, CS, AS, and a data review committee (NA, JK, MW, CS, AS) analyzed and interpreted the data.

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MINI REVIEW
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How Factors Involved in the Resolution of Crystal-Induced Inflammation Target IL-1β

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One of the main clinical features characterizing crystal-induced inflammation is its spontaneous resolution. The aim of this review is to outline the various factors involved in the self-limiting course of crystal-induced inflammation focusing on their effect on IL-1β production. Endogenous molecules that are induced or locally recruited by the process itself, inhibitory proteins naturally present in the joint and exogenous dietary factors are discussed. Aside from the classical well-known molecules involved in the resolution of crystal-induced acute attack such as TGFβ, IL-10, IL-1Ra, and lipoproteins, particular attention is paid to recently uncovered mechanisms such as the aggregation of neutrophil extracellular traps, the release of ectosomes from neutrophil surface, and alpha-1-anti-trypsin-mediated IL-1 inhibition.

Keywords: crystal-induced inflammation, interleukin-1, transforming growth factor, lipoproteins, neutrophil extracellular traps, dietary factors

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INTRODUCTION

Crystal-induced inflammation is caused by the presence of monosodium urate (MSU) or calcium pyrophosphate (CPP) crystals in articular or periarticular tissues. Although MSU and CPP crystals form in different ways, their effects are very similar and associated with an acute, intense inflammatory reaction characterized by massive leukocyte recruitment and the local release of cytokines, chemokines, reactive oxygen species and proteolytic enzymes (Liu-Bryan and Lioté, 2005).

Since the time cytoplasmic NACHT-LRRPYD-containing protein-3 (NLRP) 3 inflammasome was first identified and its activation by MSU and CPP crystals was demonstrated (Martinon et al., 2006), interleukin (IL)-1β has been considered the most important inflammatory mediator in crystal-induced inflammation, and it represents one of the main targets for new drugs that have been or are being developed to treat gout and calcium crystal-induced arthritis (Dinarello, 2014).

Although the molecular mechanisms leading to the activation of NLRP3 by pathogenic crystals have not been fully elucidated, the two-step process linked to the production of IL-1β has been clearly demonstrated. The first signal is triggered by pattern-recognition receptors (e.g., TLRs) which initiate the transcription of IL-1β; the second signal triggers inflammasome activation, which in turn activates caspase-1 and leads to the cleavage of the IL-1β precursors into the active cytokine (Joosten et al., 2010; Netea et al., 2015; Scanu et al., 2016). Once released, the cytokine promotes the induction of different pro-inflammatory genes amplifying the inflammatory process and leading to long term articular damage. Similarly to IL-1β, IL-18 is produced by caspase-1 activation but its role in crystal-induced inflammation has not been clearly defined.

One of the main clinical features characterizing crystal-induced inflammation is its spontaneous resolution. It is well-known that patients experiencing an acute attack improve within a few days time and that the disease progresses to a chronic state only if untreated (Punzi et al., 2012). The various factors involved in the self-limiting course of crystal-induced inflammation are outlined in this review. Most of these are endogenous molecules that are induced or locally recruited by the process itself or are inhibitory proteins naturally present in the joint. Other factors are exogenous dietary substances that can modulate the resolution of the acute attack (**Figure 1**).

An in-depth analysis of these mechanisms reveals that the final outcome is a direct or indirect action on IL-1β production. In particular, a negative regulation of inflammasome activation and pro-IL-1β expression have been described.

Transforming Growth Factor

Transforming growth factor (TGF) β1 is one of the main molecules involved in the resolution of crystal-induced inflammation. The effect of TGFβ1 was initially described in a subcutaneous air pouch animal model in which it inhibited MSU crystal-induced leukocyte chemotaxis (Lioté et al., 1996).

Some investigators hypothesized that the shift from a pro-inflammatory state to an active production of anti-inflammatory molecules is the mechanism leading to the resolution of an acute attack (Chen et al., 2011). It has been shown, in fact, that the differentiation of monocytes into macrophages and the uptake of crystals by the latter induces TGFβ1 secretion (Chen et al., 2011). The presence of high levels of TGFβ1, IL-1 receptor antagonist (Ra), IL-10 and soluble receptors of tumor necrosis factor (TNF) has, in fact, been demonstrated in the synovial fluid (SF) of patients with gout and has been associated with the upregulation of intracellular negative cytokine regulators such as the suppressors of cytokine signalling (SOCS)3 and the cytokine inducible SH2-containing protein (CIS). While enhancing TGFβ1 expression, these proteins downregulate IL-1β and TNF secretion (Chen et al., 2011).

In addition, the inverse relationship between TGFβ1 and IL-1β was clearly demonstrated by a study examining different inflammatory mediators in the SF collected during different stages of acute gout attack (Scanu et al., 2012).

A tight regulation of IL-1β production by TGFβ1 has also been demonstrated with regard to neutrophils. Activated neutrophils have, in fact, been identified as an additional potential source of local TGFβ1 production. An increased TGFβ1 expression following neutrophil phagocytosis of apoptotic cells, a process which in turn regulates IL-1β production by the neutrophils themselves, has been described (Steiger and Harper, 2013).

The negative regulation of IL-1β production by the crystal-induced TGFβ1 signaling pathway has been confirmed by other investigators. In particular, a higher transglutaminase (TG) 2 expression in crystal-exposed macrophages has been linked to TGFβ production (Yen et al., 2015) which, in turn, downregulates IL-1β release through the inhibition of the Janus kinase (JAK) 2 signaling. TG is indeed highly expressed in gout and has been shown to enhance phagocytosis of apoptotic leukocytes by macrophages limiting neutrophil accumulation and promoting resolution (Rose et al., 2006).

Importantly, TGFβ1 has been shown to inhibit cell surface IL-1 receptor expression (Dubois et al., 1990) supporting an additional role in limiting crystal-induced inflammatory response.

Lipoproteins

Several studies have demonstrated the importance of lipoproteins as modulators of crystal-induced inflammation. It has been hypothesized that changes in SF lipoprotein concentration and in the proteins that coat crystals play an integral role in the self-limiting nature of an acute attack. In this context, lipoproteins containing apolipoproteins (apo) B and E, have demonstrated potential regulatory effects suppressing MSU crystal phagocytosis and alpha-mannosidase release by neutrophils (Terkeltaub et al., 1986, 1991). This is probably due to the ability of these proteins to displace complement-activating IgG that initially cover the crystals (Ortiz-Bravo et al., 1993). It has also been hypothesized that the binding of low density (LDL) and high density lipoproteins (HDL) to the crystal surface reduces MSU- and CPP-induced lactate dehydrogenase release from neutrophils, a marker for cytolysis (Burt et al., 1989). It has been shown that the levels of LDL in the rat air-pouch model surge and remain elevated 24 h after the injection of CPP crystals, while the number of white blood cells and the concentration of β-Glucoronidase and PGE2 significantly fell (Kumagai et al., 2001). In addition, it has been reported that HDL inhibit MSU crystal-induced CCL2 production and expression in human synoviocytes and reduce monocyte/macrophage recruitment (Scanu et al., 2010).

Although several studies have demonstrated that lipoproteins may contribute to limiting crystal induced inflammation, their effects on the production of IL-1β have been evaluated only for HDL. Air pouch experiments have shown that HDL display anti-inflammatory activity reducing the recruitment of leukocytes and affecting pro- and anti-inflammatory cytokine balance after MSU crystal injection. It has been reported, in particular, that HDL reduce the release of IL-1β in pouch exudates and the mRNA levels in membranes. Interestingly, HDL do not affect the production of the crystal-induced anti-inflammatory factor IL-1Ra. Unlike other lipoproteins, HDL may act not only by adhering to the surface of the crystals, but also through a direct interaction with the inflammatory cells (Scanu et al., 2015).

IL-1Ra

Although IL-1Ra is the natural IL-1 inhibitor as it functions as an IL-1 receptor competitor (Dayer, 2002) and its effectiveness in the treatment of gout has been established, few studies have been conducted to assess its role in the spontaneous resolution of crystal-induced inflammation. Anti-inflammatory cytokine assessment in SF demonstrated that IL-1Ra levels are higher in gouty patients than in osteoarthritis patients (Chen et al., 2011). The injection of MSU crystals into a murine air pouch causes IL-1Ra production after 3 h, although the levels detected are not sufficient to inhibit the inflammatory effect of IL-1β at that same time point (Scanu et al., 2015). *In vitro* stimulation of monocytes/macrophages by synthetic MSU, CPP and basic

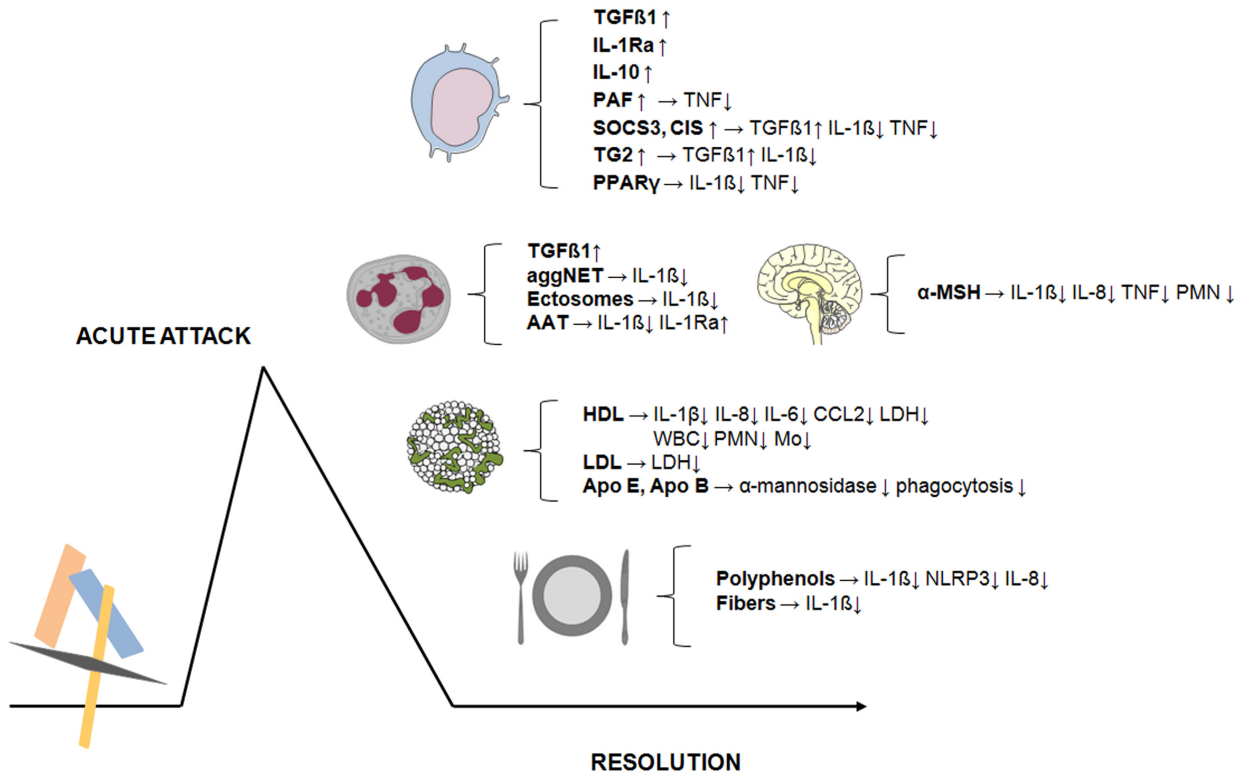

FIGURE 1 | Endogenous and exogenous factors involved in the resolution of crystal-induced inflammation. The acute attack triggered in the joints by MSU or CPP crystals resolves in a few days. Various factors are involved in this self-limiting process. Most of these are endogenous molecules (TGFβ1, IL-1Ra, IL-10, PAF, PPARγ) or intracellular negative cytokine regulators (SOCS3, CIS, TG2) induced in monocytes/macrophages following crystal exposure. Others are hormones (α-MSH) or proteins produced by neutrophils (TGFβ1, AAT) or molecules naturally present in the joints (lipoproteins and apoproteins). Dynamic processes involving neutrophils such as aggNET and ectosomes release are also involved in the resolution of crystal-induced inflammation. Finally, exogenous dietary components (phenolic compounds and fibers) play an additional role in modulating the inflammatory process induced by pathogenic crystals. → Lead to the production of; ↑ increase; ↓ decrease.

calcium phosphate (BCP) crystals induces a rapid increase in pro-inflammatory cytokines such as IL-1β, IL-8 and IL-6, whereas longer periods are required to release high levels of IL-1Ra (a personal observation).

Neutrophil Extracellular Traps and Microvesicles

The aggregation of neutrophil extracellular traps (NETs) induced by pathogenic crystals has been recently associated with the resolution of neutrophilic inflammation that characterizes the acute crystal-induced inflammatory process.

NET formation (NETosis) is accompanied by the release of a variety of pro-inflammatory mediators that orchestrate the local innate immune response. When neutrophils are under conditions of high cell density, crystal-induced NETs form dense aggregates that sequester and degrade neutrophil inflammatory mediators, in particular IL-1β. It has been demonstrated that this process is mediated by ROS which, in this particular case, downregulates inflammation (Schauer et al., 2014).

Another phenomenon that is associated with the resolution of crystal-induced arthritis is the release of phosphatidylserine positive ectosomes from the surface of neutrophils during the inflammatory process. It has been demonstrated that these ectosomes suppress inflammasome and consequently inhibit IL-1β release in C5a primed macrophages (Cumpelik et al., 2016). Although ectosomes trigger the release of TGF by monocyte and macrophages, it was not found that TGF was necessary to suppress inflammasome activation.

Other Endogenous Factors

A variety of other regulatory factors involved in the spontaneous resolution of an acute attack of crystal-induced arthritis have been identified. Among them, peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor, has been shown to be expressed in human monocytes after MSU crystal stimulation. PPARγ ligands reduce the crystal-induced production of IL-1β and TNF *in vitro* (Akahoshi et al., 2003).

Similarly, melacortin receptor (MC-R) agonists can also influence the resolution of an acute gout attack. Selective ligands for MC-R3, such as α-melacortin-stimulating hormone (α-MSH), lower the levels of IL-1β and the chemokine (C-X-C motif) ligand 1 (CXCL1) and the polymorphonuclear cell (PMN) migration in a murine model of MSU crystal-induced peritonitis (Getting et al., 2001, 2006). In addition, it was found that α-MSH inhibits,

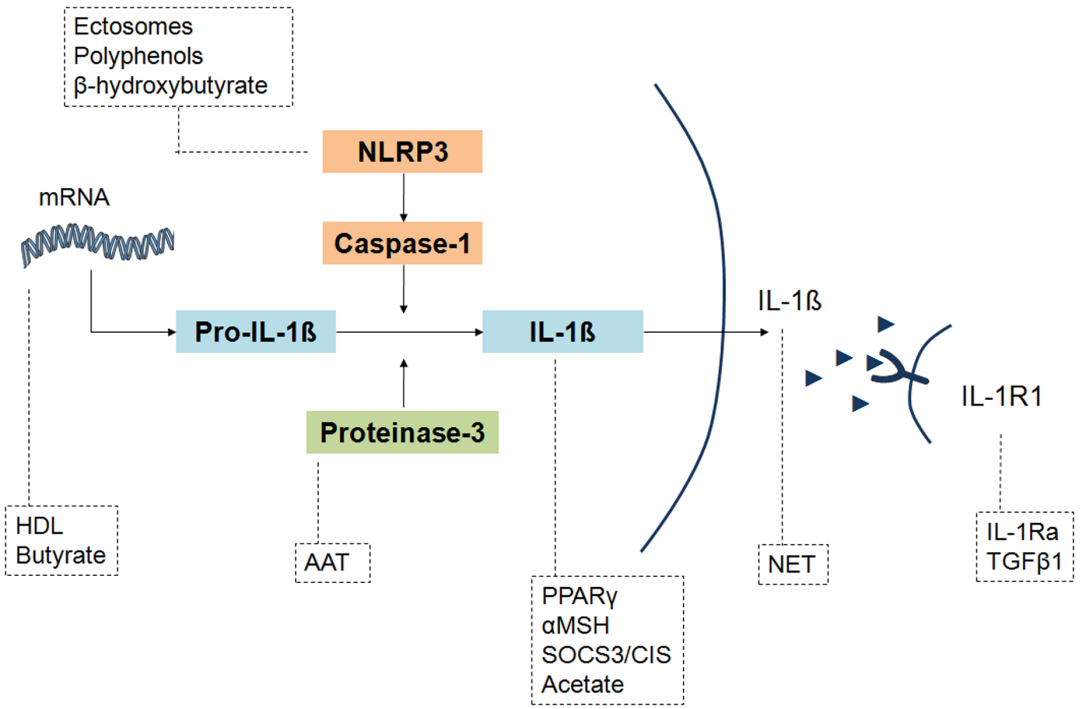

FIGURE 2 | Different levels of IL-1 inhibition by the endogenous and exogenous factors involved in the resolution of crystal-induced inflammation. IL-1β production pathway can be affected by molecules that act upstream on NLRP3 activation (ectosomes, polyphenols, β-idroxybutyrate) and IL-1β mRNA expression (HDL, butyrate), or downstream on pro-IL-1β cleavage (AAT), IL-1β formation (PPARγ, αMSH, SOCS3/CIS, acetate) and concentrations (NET), or inhibiting IL-1β bond to its receptor IL-1R1 (IL-1Ra, TGFβ1). Although the final effect of PPARγ, αMSH and acetate is an inhibition of IL-1β levels, their precise mechanism of action has not been elucidated.

in vitro, the capacity of monocytes to release IL-1β and other pro-inflammatory cytokines in response to MSU crystals, without affecting the secretion of caspase-1, the enzyme responsible for converting pro-IL-1β to cytokine's active form (Capsoni et al., 2009). By contrast, β-hydroxybutyrate (BHB), a ketone body produced in the liver, has been observed to inhibit IL-1β processing in response to MSU crystals by reducing caspase-1 activation. BHB has been shown to block NLRP3 inflammasome preventing the decline of K^+ intracellular efflux induced by NLRP3 activators (Youm et al., 2015).

Other molecules that may trigger resolution are induced during inflammatory processes promoted by crystals, even though they may play a minor role. Higher levels of IL-10 were observed in the SF from patients with acute gout with respect to OA patients (Chen et al., 2011). But although it has been demonstrated that IL-10 overexpression blocks MSU crystal-induced inflammation, including the suppression of TNF release *in vitro* and CXCL1 production *in vivo* (Murakami et al., 2002), there is no evidence of IL-10 in supernatants from differentiated macrophages incubated with MSU crystals (Yagnik et al., 2004).

Macrophage derived platelet activating factor (PAF) and related molecules could, according to one hypothesis, play a role in suppressing the inflammatory response. *In vitro* experiments demonstrated that MSU crystal-stimulated macrophages release PAF, which in turn downregulates the TNF secretion (Yagnik, 2014).

The anti-inflammatory effect of IL-10 and PAF has not been assessed in the crystal-induced IL-1β production or action/pathway.

A recently described mechanism involved in the inhibition of crystal-induced inflammation concerns alpha-1-anti-trypsin (AAT), the major, natural inhibitor of serine proteases produced by neutrophils that can be found in human serum during infections and inflammation. Serine proteases have been found to be responsible for the caspase-1-independent conversion of IL-1β precursor into the active cytokine in neutrophils (Joosten et al., 2009) which are the main inflammatory cells in crystal arthritis. It has been demonstrated that AAT not only reduces the release and the extracellular processing of IL-1β but also increases circulating levels of endogenous IL-1Ra, the natural inhibitor of IL-1 (Joosten et al., 2016).

Exogenous Factors

Although, it is well-established that nutrients or dietary metabolites possess immune-regulatory properties and can modulate the inflammatory response (Camell et al., 2015), their role in crystal-induced inflammation has only recently been taken into consideration.

Well-known for their antioxidant, anti-inflammatory and anti-cancer effects, plant polyphenols are the most studied dietary compounds. In particular, green tea epigallocatechin-3-gallate (EGCG) has been observed to reduce the inflammatory

response to CPP crystals by inhibiting IL-1β, IL-8, and CCL2 *in vitro* (Oliviero et al., 2013). An inhibitory effect exerted by EGCG was also demonstrated in urate crystal-induced peritoneal inflammation. A reduction in IL-1β levels in the peritoneal lavage fluid has been observed together with a lower NLRP3 inflammasome expression (Jhang et al., 2016).

An inhibition of NLRP3 has also been reported by investigators assessing the anti-inflammatory properties of two other dietary polyphenols, morin (Dhanasekar and Rasool, 2016) and ferulic acid (Doss et al., 2016) in a rat model of acute gout.

Other studies have investigated the beneficial effects of natural compounds in experimental gout models (Martin et al., 2009; Sabina et al., 2011; Huang et al., 2012). Although those studies did not take into full consideration their effect on IL-1β pathway, the overall result was an inhibition of the crystal-induced inflammatory process.

Some interesting findings were produced using butyrate, a short-chain fatty acid produced in the colon by the fermentation of insoluble dietary fibers (Cleophas et al., 2016). This substance has been shown to suppress urate crystal-induced IL-1β production and expression by the inhibition of the histone deacetylase (HDAC) 8, an enzyme that regulates the expression and activity of various proteins. Although the exact mechanism of action is not clear, butyrate did not increase IL-Ra or TGFβ1, classically involved in the resolution phase of the inflammatory response to pathogenic crystals.

Recently, it has been demonstrated that a high-fiber diet and acetate, a short-chain fatty acid resulting from the metabolism of fiber by gut microbiota, induce faster resolution of the inflammatory response triggered in mice after injection of MSU crystals into the knee joint. In particular, acetate promotes caspase-dependent apoptosis of neutrophils associated with reduced NF-kB activity, lower IL-1β tissue concentations, increased production of anti-inflammatory mediators, such as TGF-β, IL-10 and annexin A1, and enhanced efferocytosis (Vieira et al., 2017).

CONCLUSION

Several mechanisms seem to be involved in resolving crystal-induced inflammation. Although some are linked to exogenous dietary factors, an acute attack is limited by molecules that are activated or expressed following the attack itself through a finely tuned self-regulating mechanism.

As outlined above, these molecules can act at different molecular levels, by affecting cell-crystal interactions, gene expression and cytokine trapping. As regard to IL-1β production process, **Figure 2** evidences the substances involved upstream at the mRNA and inflammasome inhibition level, and downstream at IL-1β production, IL-1β release and IL-1R1 signaling level.

Although the precise mechanism of action of some of these factors has not been fully characterized, the final outcome is a diminished IL-1β production.

Considering the importance of IL-1 blocking agents in reducing acute attacks, firstly noted with regard to IL-1Ra anakinra, and then to the anti-IL-1β monoclonal antibody canakinumab, every molecule capable to reduce IL-1β production could represent a potential therapeutic target and have a positive impact on the clinical practice.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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C5a Regulates IL-1β Production and Leukocyte Recruitment in a Murine Model of Monosodium Urate Crystal-Induced Peritonitis

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Gouty arthritis results from the generation of monosodium urate (MSU) crystals within joints. These MSU crystals elicit acute inflammation characterized by massive infiltration of neutrophils and monocytes that are mobilized by the pro-inflammatory cytokine IL-1β. MSU crystals also activate the complement system, which regulates the inflammatory response; however, it is unclear whether or how MSU-mediated complement activation is linked to IL-1β release *in vivo*, and the various roles that might be played by individual components of the complement cascade. Here we show that exposure to MSU crystals *in vivo* triggers the complement cascade, leading to the generation of the biologically active complement proteins C3a and C5a. C5a, but not C3a, potentiated IL-1β and IL-1α release from LPS–primed MSU-exposed peritoneal macrophages and human monocytic cells *in vitro*; while *in vivo* MSU–induced C5a mediated murine neutrophil recruitment as well as IL-1β production at the site of inflammation. These effects were significantly ameliorated by treatment of mice with a C5a receptor antagonist. Mechanistic studies revealed that C5a most likely increased NLRP3 inflammasome activation via production of reactive oxygen species (ROS), and not through increased transcription of inflammasome components. Therefore we conclude that C5a generated upon MSU-induced complement activation increases neutrophil recruitment *in vivo* by promoting IL-1 production via the generation of ROS, which activate the NLRP3 inflammasome. Identification of the C5a receptor as a key determinant of IL-1-mediated recruitment of inflammatory cells provides a novel potential target for therapeutic intervention to mitigate gouty arthritis.

Keywords: C5a, gout, IL-1β, neutrophils, NLRP3 inflammasome

INTRODUCTION

Gout is the most common form of inflammatory arthritis and is associated with a substantial public health burden (Rees et al., 2014). The clinical prognosis for gout remains unsatisfactory, considering the intense pain of acute flares, loss of productivity, impairment of physical function, and long-term complications, including morbidities and mortality (Rees et al., 2014). Novel therapeutic strategies for gout are therefore needed, especially for patients who are refractory to or intolerant of conventional treatment (e.g., non-steroidal anti-inflammatory drugs, colchicine, or glucocorticoids). Emerging data have shown that aberrant expression of complex signaling pathways are involved in the inflammation of gout (Santegoets et al., 2011; Jin et al., 2014). Understanding the specific mechanisms driving the production of key cytokines involved in these pathways may offer improved prospects for the development of more efficacious and less toxic therapies (Dinarello and van der Meer, 2013; Goh et al., 2014; Perez-Ruiz et al., 2014).

Gout is caused by deposition of monosodium urate (MSU) crystals into the joints, which provokes a massive infiltration of inflammatory cells, mainly neutrophils and monocytes (Dalbeth et al., 2016). The interaction of these immune cells with the MSU crystals heightens inflammation, leading to further recruitment of increasing numbers of immune cells into the joint and escalation of the condition. IL-1 plays a pivotal role in gout-associated inflammation by instigating the release of multiple pro-inflammatory cytokines and chemokines (IL-8, IL-6, CXCL8, CXCL1), and the upregulation of adhesion molecules (selectins, integrins) on endothelial cells, which directly induces a massive infiltration of inflammatory cells (i.e., neutrophils and monocytes) at the site of crystal deposition (Brown et al., 1994; Hachicha et al., 1995). Prolonged accumulation of these cells can result in irreversible destruction of joint tissues and increases the risk of chronic inflammation. Therapies directed toward mitigating MSU-induced neutrophil infiltration and activation may therefore be beneficial for gout patients.

The exact mechanisms driving the release of IL-1β had remained elusive until the discovery that MSU crystals activate the NLRP3 inflammasome in myeloid cells of the immune system (Martinon et al., 2006; Giamarellos-Bourboulis et al., 2009; Kingsbury et al., 2011). NLRP3 belongs to the family of intracellular NOD-like receptors that, upon sensing of pathogen- or danger-associated molecular patterns (PAMPs and DAMPs, respectively), recruits the adaptor protein ASC, which aggregates and forms the inflammasome complex (Zambetti et al., 2012; Broderick et al., 2015). ASC molecules, in turn, recruit the precursor of caspase-1 (pro-casp-1), which undergoes autoproteolysis and is then able to process pro-IL-1β and pro-IL-18 to their active forms (Zambetti et al., 2012; Broderick et al., 2015).

In vitro, MSU crystals alone, much like other particulates (e.g., calcium pyrophosphate dihydrate crystals, silica, aluminum hydroxide), are not able to activate the NLRP3 inflammasome autonomously unless a "priming" pattern recognition signal, such as TLR ligands or proinflammatory cytokines, is provided alongside (Bauernfeind et al., 2009; Franchi et al., 2009): this priming signal is required for the transcription of pro-IL-1β and NLRP3 genes, whose products are activated by the second stimulus. However, *in vivo*, inflammatory responses are elicited by MSU crystals in the sterile environment of the joint space, implying that myeloid cells in this context have already received a priming signal, the source of which is currently unknown.

One of the mechanisms through which MSU crystals can induce inflammation is through activation of the complement system (Giclas et al., 1979). The complement cascade can be activated by the classical, lectin, and alternative pathways, with all pathways converging at the formation of the C3 and C5 convertases, which are required for the generation of the biologically active complement peptides (also known as anaphylatoxins), C3a and C5a, as well as the formation of the membrane attack complex (MAC) on target cells (Merle et al., 2015a). MSU crystals can activate both the classical and alternative pathways of the complement system (Doherty et al., 1983). In particular, formation of an active C5 convertase on the crystal surface was shown to trigger the cleavage of C5 proteins into C5a and C5b subunits (Russell et al., 1982). C5a is a potent chemoattractant factor for neutrophils and monocytes (Ricklin et al., 2010), and can also elicit the production of inflammatory cytokines, including IL-1β, as well as chemokines released by both endothelial cells and phagocytes (i.e., macrophages and dendritic cells; Laudes et al., 2002; Monsinjon et al., 2003).

Activated complement components have been detected in synovial fluids from gout patients during acute attacks (Doherty et al., 1988; Brodeur et al., 1991). Moreover, the MAC has been shown to promote neutrophil recruitment in a rabbit model of experimental gout arthritis (Tramontini et al., 2004). However, the chemoattractant properties of C5a and C3a have yet to be well-investigated in the context of gouty inflammation: indeed, it remains unclear whether or how these anaphylatoxins might instigate neutrophil and monocyte recruitment during crystal deposition, or contribute to IL-1β release *in vivo*.

In this study, we show that MSU crystals activate the complement system in myeloid cells, leading to generation of C3a and C5a. C5a, but not C3a, regulates IL-1β release *in vitro* and *in vivo*, and is thereby responsible for the recruitment of neutrophils and monocytes to the site of MSU-induced inflammation. Blockade of C5a receptor signaling markedly decreased leukocyte infiltration, and suggests that therapeutic targeting of C5a may prove beneficial in treating acute and chronic gouty arthritis.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) and Balb/c mice were purchased from the Biological Resource Center ((BRC), Agency for Science, Technology, and Research (A*STAR), Singapore). $C3^{-/-}$ (stock # 003641; on B6 background), $C5ar1^{-/-}$ (stock #006845) and $C3ar1^{-/-}$ mice (stock # 005712; both on Balb/c background) were from The Jackson Laboratory. Double $C3ar1^{-/-}$ and $C5ar1^{-/-}$ mice were generated by crossing $C5ar1^{-/-}$ and $C3ar1^{-/-}$ mice. All experiments were conducted with gender-

and age-matched mice, and all mutants were backcrossed to B6 or Balb/c backgrounds for at least 10 generations. Animals were bred under specific pathogen-free conditions at the BRC (A*STAR, Singapore) and experiments were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) in compliance with the Law and Guidelines for Animal Experiments of the BRC (A*STAR, Singapore).

In vitro Stimulation of Peritoneal Macrophages

Mice received an intraperitoneal (i.p.) injection of Brewer thioglycollate medium (3%) to increase macrophage yield and euthanized 3 days later. Peritoneal macrophages were collected by lavage of the peritoneal cavity with 10 ml fresh RPMI medium. Red blood cell lysis was performed using hypotonic ammonium chloride solution (0.084%) for 5 min at room temperature. Peritoneal macrophages were plated at 2×10^5/well in 96-well plates, and stimulated with *E. coli* LPS (0.1 μg/ml) for 3 h with or without MSU (250 μg/ml) for an additional 4 h, in the presence of recombinant C3a (0.25 and 1.25 μg/ml) or C5a (1–10–50 ng/ml). After 24 h, cell culture supernatants were collected.

Human monocytic (THP1) cells were plated at 1×10^5/well in 96-well plates and treated with PMA (200 nM) overnight to differentiate monocytes into macrophages. Cells were then washed and exposed to *E. coli* LPS (0.1 μg/ml) for 3 h with or without MSU (250 μg/ml) for an additional 4 h, in the presence of recombinant C5a (1–10–50 ng/ml). After 24 h, cell culture supernatants were collected.

Monosodium Urate (MSU)-Mediated Peritonitis

Eight- to ten-week-old wild-type (WT), $C3^{-/-}$, $C3ar^{-/-}$, $C5a^{-/-}$, and $C3ar^{-/-}$ $C5ar^{-/-}$ mice were injected i.p. with 3 mg MSU crystals in 0.5 ml of saline. Control mice were injected with saline alone. After 6 h, mice were euthanized with CO_2 and peritoneal exudate cells were collected by lavage with cold medium. The resulting cell suspensions were centrifuged before red blood cell lysis using hypotonic ammonium chloride solution for 5 min at room temperature, then quantification of cell number. In some experiments, mice received linear C5a receptor antagonistic peptide (1 mg/kg of mouse weight, AnaSpec Inc) by i.p. injection 5 min prior to MSU treatment.

Flow Cytometry

Total peritoneal cells were labeled with CD11b-APC (M1/70, eBioscience), Ly6C-PE-Cy7 (HK1.4, Biolegend), and Ly6G-PE (1A8, BD PharMingen) for 15 min at room temperature. Cells were then washed with PBS twice before analysis using a Fortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo (Treestar). Neutrophils were identified as $CD11b^+$ $Ly6G^+$ cells and monocytes as $CD11b^+$ $Ly6C^+$ $Ly6G^-$ cells.

ELISA

IL-1α and IL-1β were measured using DuoSet ELISA kits (R&D Systems), following the manufacturer's instructions. C3a and C5a levels in peritoneal lavage fluids were quantified by a sandwich ELISA as previously described (Kandasamy et al., 2013). The following antibody pairs were used: rat anti-mouse C3a or C5a capture antibody (2 μg/ml, overnight, 4°C), and biotinylated rat-anti mouse C3a or C5a detection antibody (2 μg/ml, 1 h, room temperature), all from BD Pharmingen. The absorbance was read at 450 nm with the reference wavelength at 570 nm, using a Tecan M200 Infinite plate reader (Tecan).

Quantitative RT-PCR

Quantitative RT-PCR was performed using the following validated SYBR Green primers: Nlrp3, 5′-CCT CTAGCTTCTGCCGTGGTCTCT-3′ and 5′-CGAAGCAGCAT TGATGGGACA-3′; Asc, 5′-CTGAGCAGCTGCAAACGACTA AA-3′ and 5′-CTTCTGTGACCCTGGCAATGAGT-3′; Casp1, 5′-CTGTCAGGGGCTCACTTTTCATTG-3′ and 5′-AATGTCC CGGGAAGAGGTAGAAAC-3′; Il1a, 5′-TCAGCACCACTTGG TAAATGAC-3′ and 5′-GTGTTTCTGGCAACTCCTTCAGC-3′; Il1b, 5′-GGTCAAAGGTTTGGAAGCAG-3′ and 5′-TGTGAAA TGCCACCTTTTGA-3′; Gapdh, 5′-TCGTCCCGTAGACAAAA TGG-3′ and 5′-TTGAGGTCAATGAAGGGGTC-3′. Amplification was performed using an Applied Biosystems 7500 Real-Time PCR System. The relative expression level of each gene was evaluated using the $\Delta\Delta C_t$ method. The difference between the C_t of the target gene and the C_t of the Gapdh housekeeping gene was normalized to the ΔC_t of the untreated condition.

ROS Production

Intracellular production of reactive oxygen species (ROS) was quantified using the H_2DCFDA fluorometric method: briefly, peritoneal macrophages were labeled with H_2DCF-DA (20 μM; BioChemika Fluka) for 30 min and then washed with PBS before 2×10^5 cells per well were seeded into black 96-well plates. Cells were incubated with C5a (50 ng/ml) or without, for 5 h before fluorescence was measured at 10-min intervals for 100 min using the Infinite M200 plate reader (Tecan; excitation 485 nm, emission 538 nm). ROS levels are displayed as the percentage increase in ROS relative to untreated controls.

Statistical Analysis

Statistical significance was assessed using unpaired two-tailed *t* tests. Data were analyzed using Prism 7 (GraphPad).

RESULTS

C5a, But Not C3a, Boosts IL-1α/β Release from Peritoneal Macrophages

We first examined the interaction of the complement components C3a and C5a with macrophages *in vitro*. Mouse peritoneal macrophages were primed with LPS, and incubated with MSU crystals or not in the presence or absence of increasing concentrations of recombinant C3a or C5a. In the absence of MSU crystals, LPS alone, LPS and C3a, LPS and C5a did not induce the release of IL-1β and IL-1α (**Figures 1A,B**); while the addition of MSU crystals triggered the release of both IL-1α and IL-1β from macrophages (**Figures 1A,B**). This mechanism

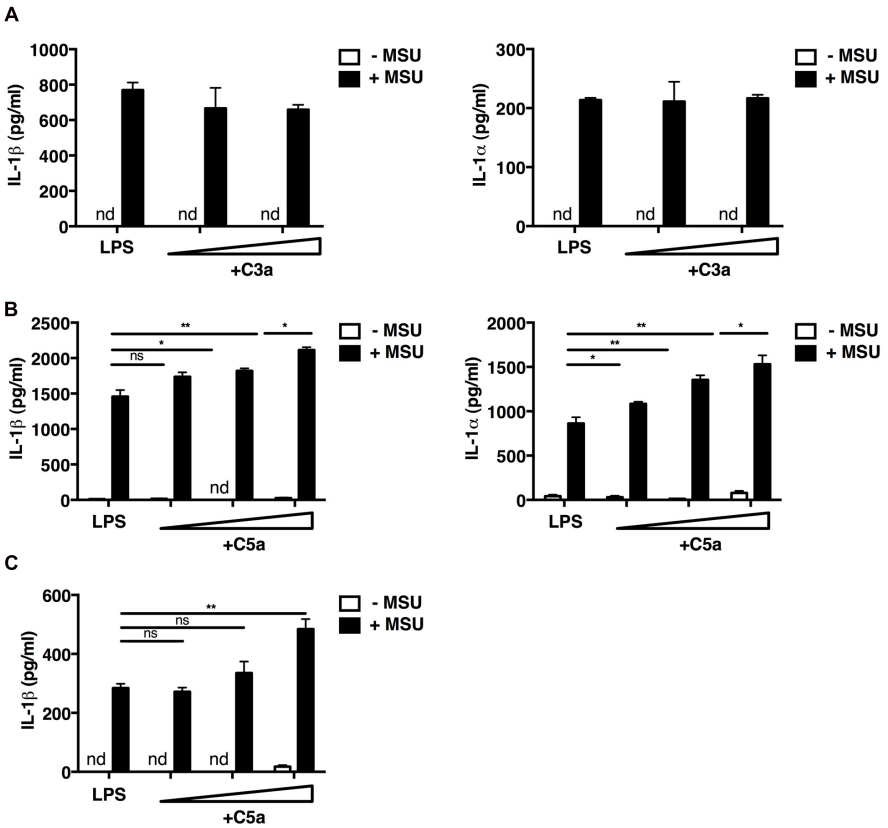

FIGURE 1 | C5a enhances IL-1α and IL-1β release from peritoneal macrophages and a human monocytic cell line. LPS-primed mouse peritoneal macrophages **(A,B)** were incubated or not with C3a alone (0.25 and 1.25 μg/ml; **A**), C5a alone (1–10–50 ng/ml; **B**), or with C3a or C5a in combination with MSU crystals (250 μg/ml). **(C)** LPS-primed human THP1 monocytic cells were left untreated or treated with C5a alone (1–10–50 ng/ml) or in the presence of MSU crystals (250 μg/ml). Culture supernatants were collected after 24 h and IL-1α/β levels were measured by ELISA. Values are shown as means ± standard error; $n = 3$. $^{**}p < 0.01$; $^{*}p < 0.05$. nd, none detected.

is consistent with the established dual activation model of the NLRP3 inflammasome, in that LPS is required to drive expression of the IL-1β precursor and NLRP3, while a second signal, such as MSU crystals, controls inflammasome formation and activation of caspase-1.

Macrophages co-stimulated with LPS/MSU crystals and increasing concentrations of C5a boosted IL-1β and IL-1α production in a dose-dependent manner (**Figure 1B**), whereas C3a had no such effect (**Figure 1A**). Similar results were obtained when the human monocytic THP1 cell line was used (**Figure 1C**). These data indicate that IL-1α/β release from peritoneal macrophages and human monocytic cells requires both LPS and MSU crystals, and that C5a, but not C3a, boosts this inflammasome-mediated release.

MSU-Induced Complement Activation Elicits Neutrophil and Monocyte Recruitment in a Mouse Model of Gout

The mechanisms underlying complement-mediated recruitment of inflammatory leukocytes by MSU crystals *in vivo* are largely unknown. Therefore we first evaluated the ability of MSU crystals to activate the complement cascade *in vivo* by measuring amounts of C3a and C5a in the peritoneal fluid of mice following injection of MSU crystals, or vehicle only, into the peritoneal cavity of two commonly used mouse strains, C57Bl/6 and Balb/c. Two hours after MSU crystal injection C3a and C5a levels significantly increased in the lavage fluids from both C57Bl/6 (**Figure 2A**) and Balb/c mice (**Figure 2B**), relative to control animals, confirming that MSU crystals have the capacity to activate the complement cascade *in vivo*.

To evaluate the importance of the complement cascade in sterile inflammation induced by MSU crystals *in vivo*, we next assessed the infiltration of neutrophils and inflammatory monocytes into the peritoneal cavity of $C3^{-/-}$ mice, which are unable to activate the complement cascade beyond the formation of the C3-convertase. $C3^{-/-}$ and wild-type (WT) control mice were injected i.p. with MSU crystals or vehicle; after 6 h, cells were collected by peritoneal lavage, labeled for surface molecule expression and characterized by flow cytometry. WT animals receiving MSU crystals exhibited a robust inflammatory response, characterized by massive peritoneal infiltration of neutrophils and monocytes, compared to mice receiving vehicle (**Figures 3A,B**). MSU-elicited mobilization of neutrophils and monocytes in the peritoneal cavity of

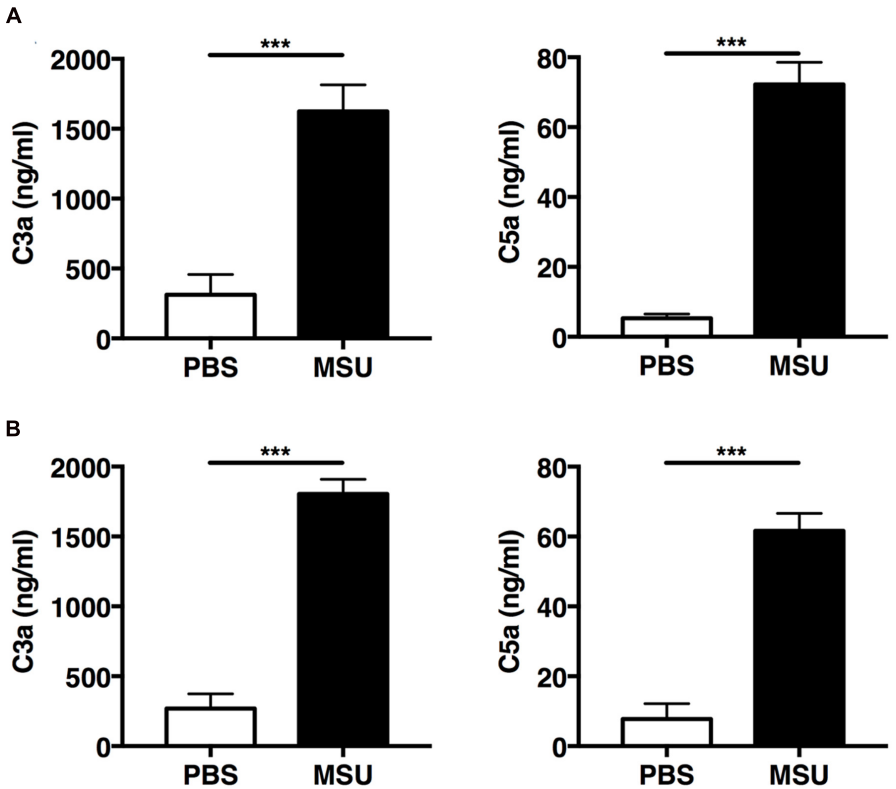

FIGURE 2 | Monosodium urate (MSU) crystals induce rapid activation of complement *in vivo*. C57BL6 ($n = 7$; **A**) and Balb/c mice ($n = 8$; **B**) were injected with 3 mg of MSU crystals or PBS and peritoneal lavage was performed 2 h later. C3a and C5a levels in the peritoneal lavage were measured by ELISA. Values are shown as means ± standard error. ***$p < 0.001$.

$C3^{-/-}$ mice was significantly suppressed (**Figures 3A,B**), indicating that complement proteins play an important role in regulating neutrophil and monocyte recruitment in mice, thereby promoting gouty inflammation.

C5a is the Major Contributor to Leukocyte Infiltration during MSU-Induced Peritonitis

Active C3a and C5a are potent inflammatory mediators and chemoattractants for phagocytic cells, including neutrophils and monocytes. Early case reports found increased levels of C3a/C3adesArg in two patients with gout (Moxley and Ruddy, 1985; Jose et al., 1990); we therefore investigated whether C3a and C5a generated by MSU crystals *in vivo* are responsible for leukocyte infiltration. First, we induced MSU-elicited peritonitis in mice deficient in both C3a and C5a receptors ($C3ar1^{-/-}C5ar1^{-/-}$): significantly fewer neutrophils and monocytes were present in peritoneal lavage fluid from $C3ar1^{-/-}C5ar1^{-/-}$ mice compared with WT controls (**Figure 4A**), indicating that anaphylatoxin-mediated signaling regulates leukocyte infiltration following MSU administration *in vivo*.

To refine our understanding of the roles played by C3a and C5a in MSU-elicited peritoneal neutrophil recruitment *in vivo*, mice deficient in C3aR alone, C5aR alone, and WT controls were injected i.p. with MSU crystals. Significantly fewer neutrophils were present in the peritoneal lavage fluid of $C5ar1^{-/-}$ mice (**Figure 4C**), but not $C3ar1^{-/-}$ mice (**Figure 4B**), compared to WT controls, indicating that C5aR, but not C3aR, signaling is crucial for neutrophil infiltration. We also observed a tendency toward a reduction of monocyte recruitment in $C5ar^{-/-}$ mice compered to WT controls, but this difference does not reach statistical significance.

Human C5a receptor cyclic/linear antagonistic peptides have been used to suppress inflammation in experimental rodent models of inflammatory bowel disease, ischemia/reperfusion injury, and immune complex glomerulonephritis (Paczkowski et al., 1999; Short et al., 1999; March et al., 2004; Alexander et al., 2015). Therefore, we sought to determine whether a C5aR antagonist could also suppress C5a-mediated recruitment of neutrophils and monocytes in mice after MSU challenge. WT mice were treated i.p. with the C5aR antagonist or vehicle prior to MSU challenge; peritoneal lavage after 6 h revealed that mice pretreated with the C5aR antagonist peptide had significantly fewer neutrophils and monocytes in their peritoneal cavity compared to mice receiving vehicle alone (**Figure 4D**). Our results support a central role for C5a-mediated signaling in the MSU-elicited pathology caused largely by neutrophil infiltration in mice.

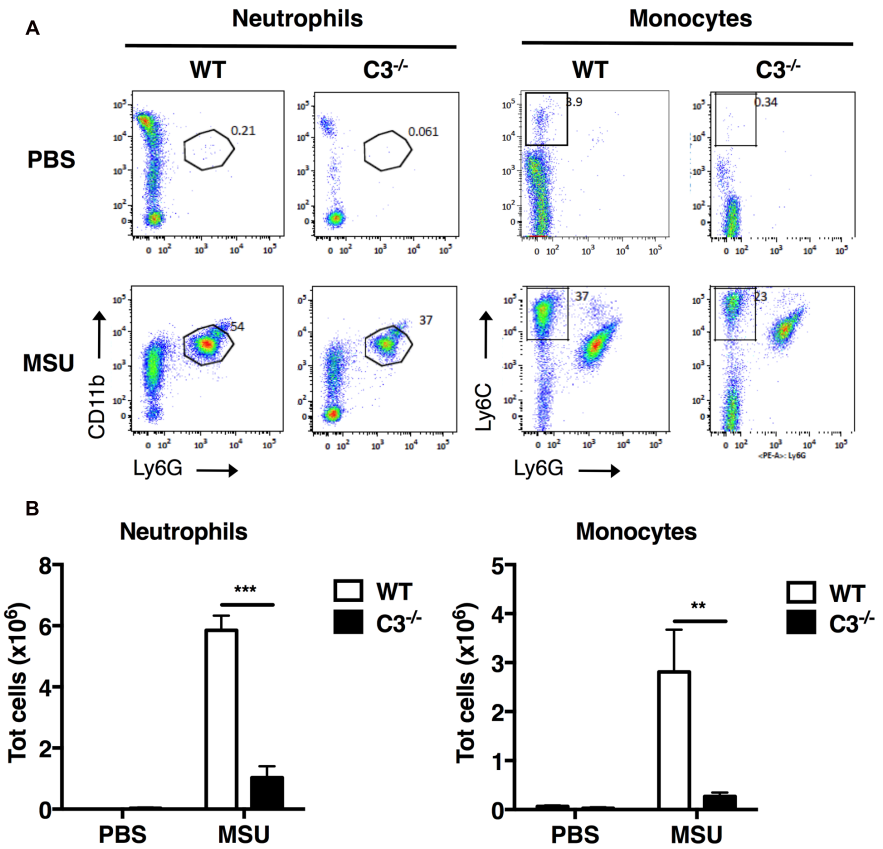

FIGURE 3 | C3 regulates recruitment of neutrophils and monocytes *in vivo* upon MSU challenge. MSU crystals (3 mg) were injected i.p. into WT or C3$^{-/-}$ mice. After 6 h, peritoneal cells were collected by lavage, and the numbers of neutrophils (CD11b^{+}Ly6G^{+}) and monocytes (CD11b^{+}Ly6C^{+}Ly6G^{-}) counted by flow cytometry. Representative dot plots are shown in **(A)**, the mean total number of cells for each condition is shown in **(B)** as means ± standard error ($n = 7$ per condition). ***$p < 0.001$; **$p < 0.01$.

C5a Regulates IL-1β Production *In vivo* Induced by MSU Crystals

The importance of neutrophils and proinflammatory cytokines in MSU-induced inflammation led us to analyze, in more detail, the mechanisms underlying neutrophil recruitment induced by C5a. Recently, basic and clinical research studies have implicated IL-1β and its maturation by the NLRP3 inflammasome in the pathogenesis of gout (Martinon et al., 2006; So et al., 2007; Kono et al., 2010). IL-1β released from macrophages activates IL-1 receptors on epithelial cells and resident macrophages, leading to the release of pro-inflammatory cytokines and chemokines, which, in turn, recruit and activate leukocytes, amplifying the inflammatory positive-feedback loop (Kono et al., 2010). This inflammatory cascade is the major cause of gout. IL-1 receptor antagonists, such as IL-1RA or Anakinra, or anti-human IL-1β antibodies, suppress the acute neutrophil response to MSU challenge in mice (So et al., 2007; Schlesinger, 2012; Ottaviani et al., 2013; Goh et al., 2014), providing robust evidence that this pathway is major target for beneficial therapeutic interventions.

Because we previously demonstrated that C5a is crucial for leukocyte recruitment upon MSU challenge *in vivo*, we next sought to determine whether C5a regulates IL-1β production. IL-1β levels were measured in the peritoneal lavage fluid collected from WT and C3$^{-/-}$ mice: IL-1β levels were significantly lower in C3$^{-/-}$ mice compared with WT mice (**Figure 5A**). To assess the contribution of C3a and C5a signaling in IL-1β production induced by MSU crystals *in vivo*, IL-1β levels were also measured in peritoneal lavage of C3ar1$^{-/-}$ (C3aR$^{-/-}$) or C5ar1$^{-/-}$ (C5aR$^{-/-}$) mice: MSU administration induced abundant and comparable IL-1β production in WT and C3ar1$^{-/-}$ mice, but far less so in C5ar1$^{-/-}$ mice (**Figure 5B**). These results indicate that C5a generated by complement activation induced by MSU triggers IL-1β production *in vivo*, which in turn regulates neutrophil recruitment.

C5a Triggers ROS Production in Macrophages

To gain insight into the molecular mechanisms by which C5a induces IL-1β in peritoneal macrophages, we first examined the possibility that C5aR signaling primes and/or activates the NLRP3 inflammasome. Peritoneal macrophages were exposed to LPS alone (a classical NLRP3 priming signal), C5a alone, or LPS in combination with C5a, for 4 h before

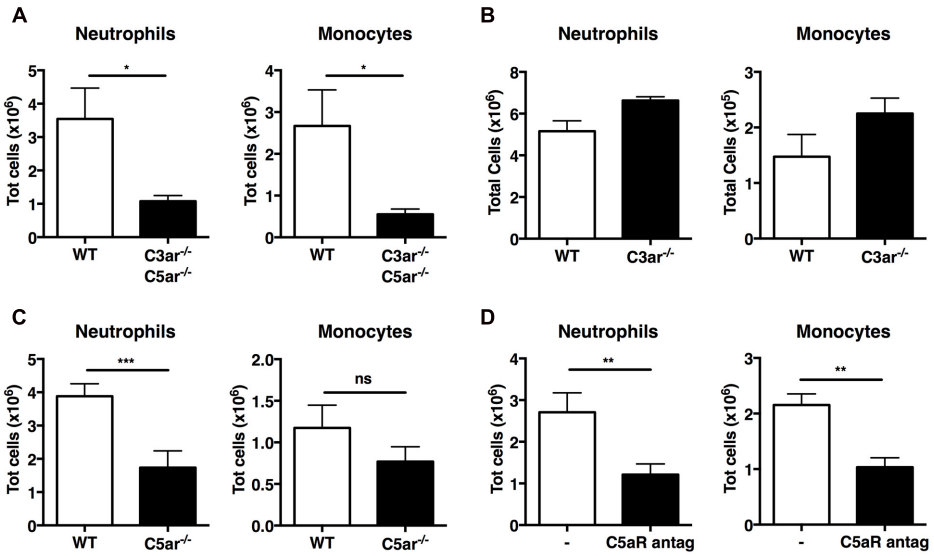

FIGURE 4 | C5a, but not C3a, regulates recruitment of neutrophils *in vivo* upon MSU challenge. Neutrophil and monocyte recruitment was measured in the peritoneal cavity of WT ($n = 6$), C3ar1$^{-/-}$C5ar1$^{-/-}$ ($n = 4$; **A**), C3ar1$^{-/-}$ ($n = 4$; **B**) and C5ar1$^{-/-}$ ($n = 6$; **C**) mice 6 h after MSU crystal injection. **(D)** Mice ($n = 6$) received an i.p. injection of a linear C5aR antagonistic peptide prior to receiving MSU crystals. Leukocyte influx was then assessed after 6 h by flow cytometry. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. nd, none detected.

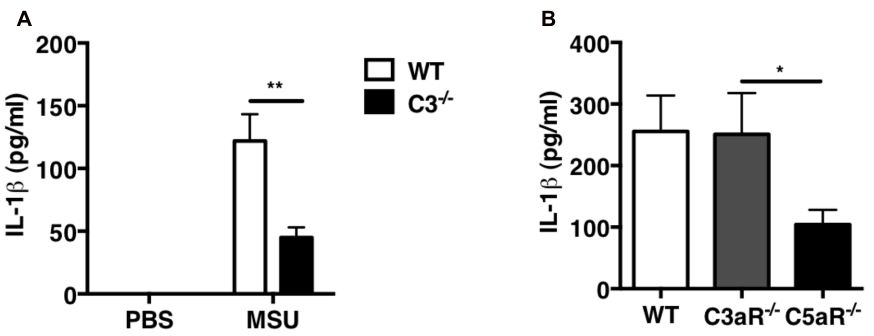

FIGURE 5 | IL-1β production is impaired in C3$^{-/-}$ and C5ar$^{-/-}$ mice after MSU challenge *in vivo*. IL-1β levels were measured in peritoneal lavage fluids from WT ($n = 6$), C3$^{-/-}$ ($n = 6$; **A**), C3ar1$^{-/-}$ (C3aR$^{-/-}$; $n = 5$), and C5ar1$^{-/-}$ (C5aR$^{-/-}$; $n = 13$) mice **(B)** 2 h after i.p. injection of MSU crystals. Values are shown as means ± standard error. **$p < 0.01$; *$p < 0.05$.

quantification of the abundance of mRNA transcripts of the inflammasome components Nlrp3, Asc and Casp1, as well as Il1b and Il1a. While LPS robustly triggered Nlrp3, Il1b and Il1a transcription, C5a alone failed to do so (**Figure 6A**), and can therefore not be considered a priming stimulus.

Next we sought to investigate alternate mechanisms by which C5a boosts NLRP3 inflammasome activation. Since ROS have been shown to act as a primary inducer of NLRP3 inflammasome activity, we asked whether C5a, alike to MSU, triggers ROS production. We found that C5a exposure robustly increased ROS production in peritoneal macrophages (**Figure 6B**), suggesting that C5a is likely to increase NLRP3 activation at least in part via ROS production.

DISCUSSION

The anaphylatoxin C5a is generated upon complement activation and contributes to the development of many inflammatory disorders, including glomerulonephritis (Welch, 2002), pulmonary hypersensitivity (Shushakova et al., 2002), and contact hypersensitivity (Tsuji et al., 2000). C5a is a potent mediator of the inflammatory response as a result of its ability, upon engagement of C5a receptors, to regulate vasodilation and to mediate chemotaxis and activation of inflammatory leukocytes (granulocytes, mast cells, and macrophages; Merle et al., 2015b). However, whether C5a is crucial *in vivo* for gouty inflammation was unclear. In the present study, we identify C5a generated by MSU crystals *in vivo* as one of the key regulators of IL-1β release, which in turn triggers the recruitment of neutrophils, a hallmark

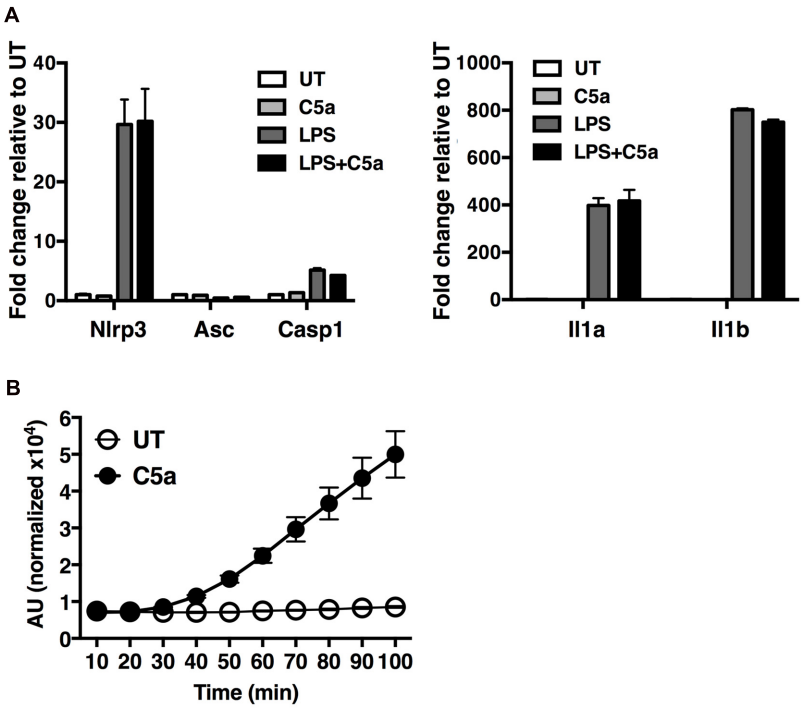

FIGURE 6 | C5a robustly elicits ROS production in peritoneal macrophages. (A) Expression of Nlrp3, Asc, Casp1, Il1b and Il1a genes was measured in macrophages stimulated with LPS alone (0.1 μg/ml), C5a alone (50 ng/ml) or LPS in combination with C5a, by real-time quantitative RT-PCR. Results were calculated using the ΔΔCt method and data presented as mean +/− standard deviations of three biological replicates. **(B)** ROS production evaluated as H_2DCF-DA incorporation into macrophages stimulated with C5a (50 ng/ml) or medium. ROS levels are represented as the percentage increase in treated samples relative to untreated samples (UT) and the error is represented as the standard deviation. AU, arbitrary units.

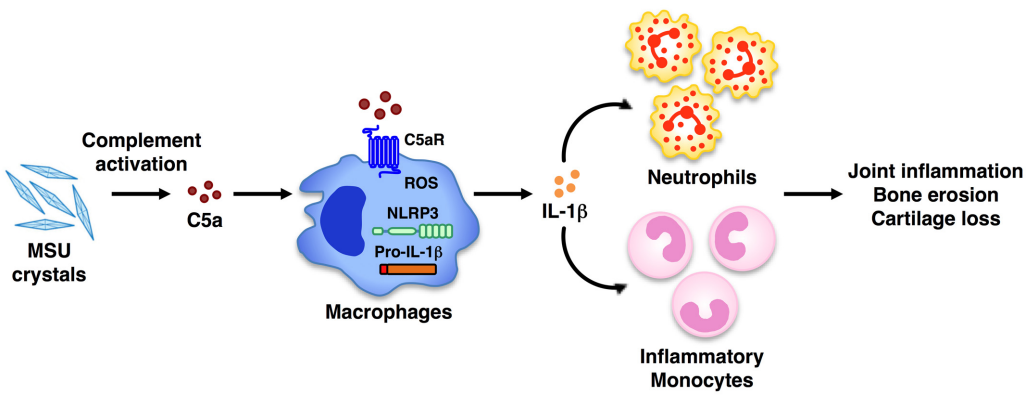

FIGURE 7 | Model of C5a-induced IL-1β release and leukocyte infiltration upon MSU crystal challenge *in vivo*. Peritoneal administration of MSU crystals causes activation of the complement system, which leads to the generation of the anaphylatoxin C5a. Upon engagement of its own receptor, C5a in turn elicits the activation of the NLRP3 inflammasome, leading to IL-1β release. This triggers the release of chemokines from resident cells, which recruit inflammatory cells (i.e., neutrophils, monocytes) to the tissue site. Activated inflammatory leukocytes, cytokines, and chemokines contribute to the chronic inflammation that can lead to bone erosion and cartilage loss. Therapeutic blockade of C5aR signaling in humans may be an alternative new approach to ameliorate the inflammatory attack in gouty arthritis.

of gouty inflammation (**Figure 7**). Specifically, C5aR deficiency was associated with less neutrophil infiltration induced by MSU crystals *in vivo*, and lower IL-1β levels.

Early studies suggested that MSU crystals can promote inflammation through complement activation: complement proteins can deposit on the MSU crystal surface leading to the activation of the classical and alternative complement pathways (Russell et al., 1982; Doherty et al., 1983). Rabbits deficient in the complement component C6, critical for MAC formation, have diminished joint swelling in a model of experimental gout

arthritis, associated with decreased recruitment of inflammatory leukocytes, particularly neutrophils, and lower IL-8 levels in the joint (Tramontini et al., 2004). Our data demonstrate that C5a-mediated signaling is also crucial for the ingress of neutrophils into the peritoneal cavity in response to MSU crystals *in vivo*. Since C5ar$^{-/-}$ mice are able to assemble MAC, our results indicate that C5a is likely to play a role that supersedes that of the MAC in sterile crystal-induced inflammation in mice.

What are the mechanisms triggering C5a-mediated IL-1 release in response to MSU crystals *in vivo*? Recent data from our and other groups have shown the critical role of the complement system in promoting NLRP3 inflammasome activation leading to IL-1β release (Laudisi et al., 2013; Triantafilou et al., 2013; An et al., 2014; Cumpelik et al., 2015). It was reported that C5a stimulation induced pro-IL-1β expression and IL-1β release in human primary monocytes in a dose-dependent manner following a mechanism that requires Ca^{2+} and K^{+} fluxes, and cathepsin B activity (An et al., 2014). C5aR blocking using an anti-C5aR antibody abolished the expression of pro-IL-1β and caspase-1 activation *in vitro* (An et al., 2014). We found that *in vitro* C5a exposure of mouse peritoneal macrophages did not trigger increased transcription of the inflammasome proteins Nlrp3, Asc and Casp1, or of Il1β and Il1α, but instead boosted ROS production. These findings highlight the fact that although the mechanism may diverge in different cell types (An et al., 2014; Cumpelik et al., 2015), C5a/C5aR interactions do provoke inflammasome activation and thereby IL-1 release. Beside IL-1, other mechanisms elicited by MSU-induced C5a *in vivo* may exist. C5a shows chemoattractant properties for neutrophils and monocytes in other model of inflammation. Thus, on one side, MSU-elicited C5a can induce production of IL-1 and on the other side, lead to chemotaxis of inflammatory leukocytes.

We have shown that MSU crystals also elicit the generation of the anaphylatoxin, C3a. The possible contribution of C3a to the mobilization of leukocytes during sterile inflammation has not been reported: we found that C3a does not synergize with the priming signal LPS in boosting pro-inflammatory cytokine production, such as IL-1α and IL-1β, from mouse peritoneal macrophages stimulated *in vitro* with LPS/MSU crystals, and that C3aR deficiency did not diminish leukocyte influx or IL-1β levels induced by MSU crystals *in vivo*. These data indicate that C3aR signaling is dispensable for acute MSU crystal-induced inflammation and highlight the distinct roles of C3a and C5a. Although C3a and C5a receptor signaling pathways share a high degree of functional overlap (e.g., cAMP, MAPK/ERK, NF-κB pathways), our data indicate that C3a is not directly involved in NLRP3 inflammasome activation and induction of IL-1β release in MSU-induced inflammation both *in vitro* and *in vivo*. It would be of particular interest in future studies to identify C5a-specific mechanisms that regulate inflammasome activation in the context of sterile inflammation, as well as to define the pro- and anti-inflammatory effector functions elicited by C5a. A recent study has shown that neutrophil-derived microvesicles isolated from human synovial fluid within 1 day of an acute gout attack exhibit anti-inflammatory effects through inhibition of C5a signaling and NLRP3 inflammasome activation (Cumpelik et al., 2015).

Studies assessing the efficacy of antibodies or antagonistic peptides blocking C5a function in gouty arthritis are lacking. However, C5 blockers are currently in use in clinical and pre-clinical studies for a number of other diseases. Eculizumab (Soliris®), a humanized anti-C5 monoclonal antibody, prevents C5 from being cleaved into C5a and C5b by C5 convertase, and is currently in trials for paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS), with plans to extend its use to age-related macular degeneration, myasthenia gravis, optic neuritis, early septic organ dysfunction, and prevention of rejection of kidney transplants (Melis et al., 2015; Risitano, 2015; Horiuchi and Tsukamoto, 2016). In addition to C5 antibodies, peptidic and non-peptidic C5aR antagonists are in clinical trials, and some have been proven efficacious (Morgan and Harris, 2015). Anti-complement agents have received attention as a new treatment strategy for refractory inflammatory diseases: considering the close link between complement and rheumatic diseases it would also be important to test anti-complement agents in these diseases. Our findings suggest the possibility that C5 inhibitors may be an alternative approach to diminish IL-1-mediated neutrophil infiltration associated with crystal-induced acute inflammation, with the potential to lead to amelioration of acute and chronic gouty arthritis.

AUTHOR CONTRIBUTIONS

Study concept and design: AH, FL, and AM. Study supervision: AM. Acquisition of data: HK, AH, FL, HD, and MK. Analysis and interpretation of data: HK, AH, FL, MK, BS, and AM. Technical support: HD. Writing of the manuscript: AM. Critical revision of the manuscript: HK, AH, FL, BS, and GGT.

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Piperine Suppresses Pyroptosis and Interleukin-1β Release upon ATP Triggering and Bacterial Infection

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Piperine is a phytochemical present in black pepper (*Piper nigrum* Linn) and other related herbs, possessing a wide array of pharmacological activities including anti-inflammatory effects. Previously, we demonstrated that piperine has therapeutic effects on bacterial sepsis in mice, but the underlying mechanism has not been fully elucidated. In this study, we aimed to investigate the influences of piperine on pyroptosis in murine macrophages. The results showed that piperine dose-dependently inhibited ATP-induced pyroptosis, thereby suppressing interleukin-1β (IL-1β) or high mobility group box-1 protein (HMGB1) release in LPS-primed bone marrow-derived macrophages and J774A.1 cells. Accompanying this, ATP-induced AMP-activated protein kinase (AMPK) activation was greatly suppressed by piperine, whereas AMPK agonist metformin counteracted piperine's inhibitory effects on pyroptosis. Moreover, piperine administration greatly reduced both peritoneal and serum IL-1β levels in the mouse model intraperitoneally infected with *Escherichia coli*, suggestive of suppressing systemic inflammation and pyroptosis. Our data indicated that piperine could protect macrophages from pyroptosis and reduced IL-1β and HMGB1 release by suppressing ATP-induced AMPK activation, suggesting that piperine may become a potential therapeutic agent against bacterial sepsis.

Keywords: piperine, inflammasome activation, interleukin-1β, pyroptosis, AMP-activated protein kinase

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INTRODUCTION

Piperine is an alkaloid present in black pepper (*Piper nigrum* Linn) and other related herbs (Wattanathorn et al., 2008). This alkaloid has been reported to possess a broad spectrum of pharmacological activities. It is well known for its anti-depressive and anti-epileptic activities (Pal et al., 2011; Mao et al., 2014). It is also known as a booster for promoting bioavailability of other drugs thus enhancing their pharmacological effects (Johnson et al., 2011; Di et al., 2015). Interestingly, piperine has been demonstrated to be a potential agent with anti-obesity (BrahmaNaidu et al., 2014), anti-gastric ulcer (Bai and Xu, 2000), anti-acute pancreatitis (Bae G. S. et al., 2011), and anti-arthritis (Murunikkara et al., 2012; Ying et al., 2013) properties. Moreover, piperine is also effective for the treatment of diarrhea (Mehmood and Gilani, 2010) and endotoxin-induced septic shock in mice (Bae et al., 2010). Therefore, piperine may be generally regarded as an anti-inflammatory agent against various inflammatory disorders as a consequence of bacterial infections or autoimmune responses.

Recently, we have demonstrated that piperine administration reduces mouse mortality, and alleviates their internal organ damages upon bacterial infection (Pan et al., 2015). One potential mechanism is that piperine treatment promotes amino acid metabolism and thus enhances mTORC1 signaling in peritoneal resident macrophages. The functions of the peritoneal macrophages are greatly enhanced in terms of their bacterial phagocytic ability and their cytokine secretion ability upon inflammatory stimulation (Pan et al., 2015). However, it is still unclear how piperine prevents internal organs from injury under the circumstance of systemic inflammatory responses during bacterial sepsis.

One consequence of bacterial infection is inflammasome activation. The inflammasome is a multiple protein complex and its activation represents the first line of innate defense against bacterial infection (Lamkanfi and Dixit, 2014; Wegiel et al., 2014). The activation of inflammasome requires two signals. First, the innate immune cells is primed by recognizing the pathogen-associated molecular patterns (PAMPs) expressed on the pathogen through their pattern recognition receptors (PRRs), resulting in the expression of critical components of inflammasome, such as nucleotide and oligomerization domain, leucine-rich repeat containing protein family, pyrin containing domain 3 (NLRP3) and pro-interleukin-1β (pro-IL-1β). Second, the inflammasome is assembled in the PAMP-primed cells upon further stimulation by damage-associated molecular patterns (DAMPs) such as ATP, culminating in recruitment of the apoptosis-associated speck-like protein containing CARD (ASC) adaptor protein. Consequently, pro-caspase-1 is activated by the inflammasome to produce the active caspase-1, which further converts pro-IL-1β into mature form IL-1β (Lamkanfi and Dixit, 2014). The latter is a potent endogenous pyrogen that promotes an increase in body temperature as well as mediating inflammatory responses. Beyond the release of mature IL-1β, one prominent consequence of inflammasome activation is pyroptosis—an inflammatory programmed cell death, which is dependent on the activation of inflammatory caspase-1 or caspase-11. Activated caspase-1 or caspase-11 can cleave the gasdermin D to release its N-terminal fragment which is critical for pyroptosis (Shi et al., 2014; Kayagaki et al., 2015). Therefore, induction of pyroptosis requires both PAMP and DAMP stimulation, as having been elegantly evaluated recently (Cullen et al., 2015), constituting the canonical inflammasome signaling. In non-canonical inflammasome signaling, lipopolysaccharide (LPS), upon penetrating into the cell, directly binds caspase-11 and activates it, leading to caspase-1 activation and pyroptosis (Shi et al., 2014; Kayagaki et al., 2015).

Many studies have indicated that inflammasome activation and pyroptosis provide protection against bacterial infection (Ceballos-Olvera et al., 2011) and experimental colitis (Zaki et al., 2010; Demon et al., 2014; Oficjalska et al., 2015). Without the protection of inflammasome mechanism due to lack of caspase-1 and caspase-11 genes, mice are vulnerable to intracellular bacterial infection (Maltez et al., 2015). However, increasing evidence has indicated that pyroptosis may be a major cause that leads to multiple organ failure and septic death (Masters et al., 2012; Wree et al., 2014). In support of this notion, mice are resistant to bacterial-induced death when the pyroptotic mechanism is lost due to caspase-11 and gasdermin D deficiency (Kayagaki et al., 2015). Although it has once believed that 'cytokine storm' is the main cause of sepsis, recent studies have proved that septic death can still take place in mice lacking caspase-1 activation and IL-1β production (due to caspase-1 gene deletion but retaining of caspase-11 gene), reinforcing the idea that caspase-11-mediated pyroptosis is critical for septic shock (Kayagaki et al., 2013). However, the release of IL-1β may still be regarded as a marker of pyroptosis, as one recent study has proved that it can only be released by pyroptotic cells instead of viable ones (Cullen et al., 2015).

In this study, we aimed to investigate whether piperine could suppress pyroptosis in macrophages. By using *in vitro* cell models where mouse bone marrow-derived macrophages (BMDMs) and J774A.1 cells were primed with LPS (a Gram-negative bacterial PAMP) followed by triggering with extracellular ATP [a DAMP released by hosts or bacteria (Mempin et al., 2013; Ren et al., 2014; Wegiel et al., 2014)], we found that piperine treatment significantly suppressed ATP-induced pyroptosis, which was associated with suppression of AMPK activity. Piperine administration markedly reduced IL-1β levels in the peritoneal lavage fluids and serum of mice with bacterial sepsis as compared with vehicle, indicating attenuation of systemic inflammation in the circumstance of bacterial sepsis. Our data suggest that piperine may be used to prevent bacterial sepsis by suppression of pyroptosis.

MATERIALS AND METHODS

Reagents and Animals

Propidium iodide (PI), dimethyl sulfoxide (DMSO), Hoechst 33342, adenosine triphosphate (ATP) (P8232) and LPS (from *Escherichia coli* O111:B4) (L4391) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Piperine was purchased from Guangdong Institute for Drug Control (Guangzhou, China), dissolved in DMSO and stored at −20°C. DMEM, fetal bovine serum (FBS), penicillin and streptomycin were products of ThermoFisher/Gibco (Carlsbad, CA, USA). Metformin was obtained from MedChem Express (Princeton, NJ, USA), dissolved in DMEM at 300 mM and stored at −20°C. The antibody to NLRP3 (AG-20B-0014) was purchased from Adipogen AG (Liestal, Switzerland). Antibodies against caspase-1p10 (sc-514) and actin (sc-1616-R) were obtained from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA). Antibodies against IL-1β (#12242), HMGB1 (#3935), AMPKα (#5832), phospho(p)-AMPKα(Thr172) (#2535), p70S6K (#2708), p-p70S6K(Thr389) (#9234), caspase-3 (#9662), β-tubulin (#2128) and horseradish peroxidase (HRP)-conjugated goat anti-mouse/-rabbit/-rat IgG were bought from Cell Signaling Technology (Danvers, MA, USA).

Female C57BL/6 mice were bought from the Experimental Animal Center of Southern Medical University (Guangzhou, China). Animal experiments were designed following the National Institutes of Health guidelines and were approved by

the Committee on the Ethics of Animal Experiments of Jinan University.

Cell Line and Cell Culture

The J774A.1 cells was obtained from the Kunming Cell Bank of Type Culture Collection, Chinese Academy of Sciences (Kunming, China) and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM $_{L}$-glutamine (DMEM complete medium) at 37°C in a humidified incubator of 5% CO_2. The cells were sub-cultured every 2–3 days.

Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow was collected from femora of C57BL/6 mice. BMDMs were differentiated as reported previously (Kayagaki et al., 2013). In brief, bone marrow cells were differentiated in DMEM supplemented with 10% FBS and 20% (v/v) M-CSF-conditioned medium from L-929 fibroblasts for 6 days. BMDMs were then cultured in fresh DMEM complete medium overnight in 24-well plates at 1.5×10^5 cells/well in 0.5 ml.

Pyroptotic Assay

Cell death was measured by PI incorporation as described previously (Py et al., 2014). Cells were pre-treated with indicated concentration of piperine for 4 h in DMEM complete medium, and then primed with 500 ng/ml LPS for 4 h. Subsequently, the culture medium was replaced with Opti-MEM and indicated concentrations of ATP. Cell nuclei were revealed by Hoechst 33342 staining (5 μg/ml; staining for all cells) and PI (2 μg/ml; staining for pyroptotic cells) for 10 min. The cells were observed using a Zeiss Axio Observer D1 microscope equipped with a Zeiss LD Plan-Neofluar 20×/0.4 Korr M27 objective lens. Fluorescence images were captured with a Zeiss AxioCam MR R3 cooled CCD camera controlled with ZEN software (Carl Zeiss).

Cytometric Bead Array

Soluble IL-1β was determined by cytometric bead array (CBA) using the Mouse IL-1β Flex Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Data were acquired using CELLQuest software on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA, USA).

Precipitation of Soluble Proteins in Supernatants

Proteins in culture supernatants (equal volume for each sample) were precipitated overnight with 7.2% trichloroacetic acid plus 0.15% sodium deoxycholate as previously described (Lin et al., 2016). The precipitates are lysed in equal volume of 1x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer.

Western Blot Analysis

Western blotting was performed as described previously (Lin et al., 2016) to detect proteins in the supernatants and cell lysates, respectively. In brief, total proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Amersham, RPN303F). After being blocked, the membranes were probed with indicated primary antibodies, followed by a HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Bands were revealed by a BeyoECL Plus kit (Beyotime, P0018) and recorded on X-ray films (Kodak, 6535873). The densitometry of each band was quantified by FluorChem 8000 (Alpha Innotech; San Leandro, CA, USA).

Bacterial Infection

C57BL/6 mice were acclimated for 1 week and intragastrically administered with piperine solution or vehicle (2% Tween-80 in PBS) once a day for 5 consecutive days. *E. coli* strain DH5α was grown in Luria Broth (LB) media and the bacterial cell density was determined using an ultraviolet-visible spectrophotometer (NanoDrop2000, Thermo Scientific). The colony-forming units (CFUs) corresponding to known cell densities were determined on LB agar plates. The mice were intraperitoneally (i.p.) injected with 2×10^9 CFU/mouse of viable *E. coli* cells in 0.5 ml of PBS. After bacterial infection for indicated time lengths, the mice were sacrificed and peritoneal lavage fluids were collected with 1.5 ml PBS for IL-1β determination by CBA. The colon tissues were also collected for western blotting analysis. In a separate experiment, mouse sera were collected from retro-orbital venous blood and IL-1β in serum was assayed by CBA method.

Statistical Analysis

All experiments were performed independently at least three times, with one representative experiment shown. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism 4.0 (GraphPad; San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey *post hoc* test and unpaired Student's *t*-test were used to analyze the statistical significance among multiple groups and between two groups, respectively. *P*-values < 0.05 were considered statistically significant.

RESULTS

Piperine Inhibits ATP-Induced Pyroptosis in BMDMs

Our previous work demonstrated that piperine administration significantly alleviated injury of internal organs and reduced mouse mortality in bacterial sepsis (Pan et al., 2015). As pyroptosis has been reported being causative of sepsis-induced organ damages, we explored whether piperine could suppress pyroptosis in LPS-primed macrophages upon ATP triggering. We firstly evaluated the effects of piperine on ATP-induced pyroptosis, which can be revealed by PI staining (Py et al., 2014). BMDMs were pretreated with piperine followed by LPS priming (in the presence of piperine) and were then triggered by ATP treatment (without LPS and piperine). The results showed that LPS priming did not induce pyroptosis in BMDMs, and

piperine at a dose up to 160 μM was non-toxic to the cells during short periods of incubation (**Figures 1A,B**). Upon ATP triggering, however, the LPS-primed BMDMs rapidly underwent pyroptosis. Interestingly, piperine pretreatment significantly attenuated ATP-induced pyroptosis in a dose-dependent manner (**Figures 1A,B**).

It has been demonstrated that in LPS-primed macrophages ATP treatment activates NLRP3 inflammasome leading to pro-caspase-1 processing into active caspase-1p10; the latter in turn cleaves pro-IL-1β (31 kDa) to produce a 17-kDa IL-1β (mature form) while pyroptosis is induced and mature IL-1β is released from the pyroptotic cells rather than the living ones (Cullen et al., 2015). Therefore, the levels of mature IL-1β and active caspase-1p10 in cell culture supernatants are markers of inflammasome activation and pyroptosis. Consistent with these reports (Latz et al., 2013; Cullen et al., 2015), western blotting results showed that LPS priming greatly induced the expression of pro-IL-1β in the cells, whereas pro-caspase-1 was constitutively expressed in BMDM cells (**Figure 1C**). Without ATP treatment, both active caspase-1p10 and mature IL-1β were undetectable in the supernatants either treated with piperine or not; upon ATP triggering, they were significantly released into the supernatants, reflective of inflammasome activation and pyroptosis. Notably, piperine pretreatment dose-dependently suppressed the release of both active caspase-1p10 and mature IL-1β into the supernatants (**Figures 1C–E**). Together, these results indicated that piperine pretreatment could significantly attenuated ATP-induced pyroptosis, thus reducing IL-1β release, in LPS-primed BMDMs.

Piperine Suppresses ATP-Induced Pyroptosis in J774A.1 Cells

Next, we examined the effects of piperine on ATP-induced pyroptosis in LPS-primed murine J774A.1 macrophage cell line. Piperine pretreatment dose-dependently suppressed ATP-induced pyroptosis as in BMDMs (**Figures 2A,B**). Consistently, ATP treatment induced the release of HMGB1, another danger signal molecule that is associated with inflammatory cell death (de Souza et al., 2012; Gdynia et al., 2016). Piperine pretreatment once again suppressed the effect of ATP on HMGB1 release (**Figures 2C,D**). As IL-1β release in the supernatants was hardly detectable by western blotting, we used a more sensitive CBA method (with a detection limit of ∼5 pg/ml) to measure soluble IL-1β levels. The result showed that piperine pretreatment significantly inhibited ATP-induced IL-1β release (**Figure 2E**). This is likely because piperine had directly suppressed the expression of pro-IL-1β in the cells treated with LPS plus ATP (**Figure 2C**, cell lysate). Apoptosis had not been involved in ATP-induced cell death, as caspase-3 cleavage (activation) was not observed in all groups (**Figure 2C**). Together, these results indicated that piperine treatment could suppress pyroptosis and reduced IL-1β and HMGB1 release in J774A.1 cells in response to ATP triggering.

Based on the above-mentioned results from both BMDMs and J774A.1 cells, piperine treatment could inhibit the pyroptosis in LPS-primed macrophages in response to ATP triggering.

ATP-Induced AMPK Activation Is Suppressed by Piperine Treatment

It has been shown that AMPK activity is suppressed by LPS, free fatty acid, and other inflammatory stimulators (Yang et al., 2010; Wen et al., 2011; Wang et al., 2016). However, AMPK signaling can be activated by bacterial infection (Bae H. B. et al., 2011) while the suppressed AMPK activity in LPS-primed macrophages can be dramatically activated (as reflected by its phosphorylation at Thr172) in response to ATP treatment (Bae H. B. et al., 2011; Moon et al., 2015). Consistent with these reports, we also observed that AMPK activity was suppressed by LPS but greatly increased in both LPS-primed BMDMs (**Figures 3A,B**) and J774A.1 cells (**Figures 3C,D**) upon ATP stimulation. Interestingly, ATP-induced AMPK activation could be markedly suppressed by piperine pretreatment (**Figures 3A–D**). Consistent with our previous study (Pan et al., 2015), piperine could increase the phosphorylation of p70S6K, indicative of enhanced mTORC1 activity, in macrophages (**Figure 1A**). In line with the activation of AMPK upon ATP treatment, the mTORC1 activity was sharply suppressed by ATP. These results in conjunction with previous studies suggested that AMPK signaling may regulate cell survival and death under inflammatory stresses. Our results also suggested that piperine might inhibit ATP-induced pyroptosis by suppressing AMPK activation.

Piperine Attenuates Pyroptosis by Suppressing AMPK Signaling

Having found that AMPK activity was associated with piperine-mediated suppression of pyroptosis in macrophages in response to ATP stimulation, we next investigated whether such suppressive effects of piperine could be counteracted by boosting AMPK signaling with metformin, a well-known AMPK agonist (Su and Dai, 2016). As expected, metformin could reverse piperine-mediated suppression of AMPK activity in macrophages in response to LPS and ATP stimulation (**Figures 4A,B**). Notably, metformin treatment counteracted piperine-mediated suppression of HMGB1 release from J774A.1 cells (**Figure 4C**, supernatant). Metformin treatment also counteracted the inhibitory effect of piperine on ATP-induced active caspase-1p10 release from BMDMs, but the reduced IL-1β release induced by ATP in the presence of piperine was not completely restored by metformin. This was likely due to that the expression of pro-IL-1β was greatly suppressed by piperine of 160 μM in LPS+ATP-treated BMDMs (**Figure 4D**, cell lysate). Consistent with these results, metformin fully reversed the effect of 80 μM piperine on suppressing ATP-induced pyroptosis in BMDMs (**Figures 5A,B**) and J774A.1 cells (**Figures 5C,D**), while partially reversing the inhibitory effect of 160 μM piperine on ATP-induced pyroptosis in BMDMs (**Figure 5B**) probably due to the robust inhibitory effect of this high-dose piperine. These results demonstrated that piperine pretreatment protected LPS-primed macrophages from ATP-induced pyroptosis by at least partly suppressing AMPK activation.

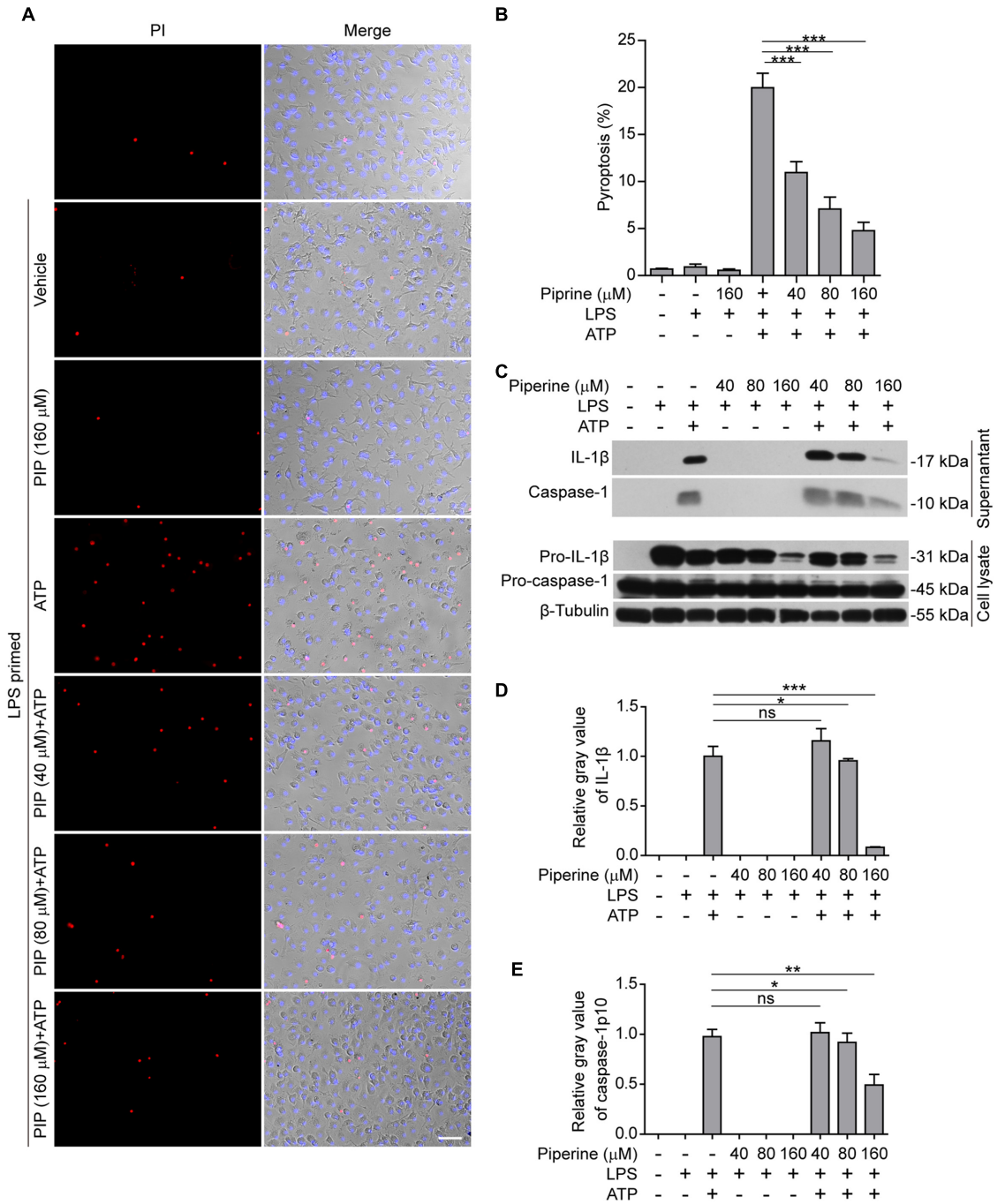

FIGURE 1 | Piperine inhibited ATP-induced pyroptosis in bone marrow-derived macrophages (BMDMs). (A) Cells were pre-treated with graded concentrations of piperine for 4 h, followed by LPS (500 ng/ml) priming for 4 h (in the presence of piperine). Then the cells were stimulated with ATP (3 mM) for 30 min (in the absence of piperine and LPS). After these treatments, the cells were stained with 2 μg/ml propidium iodide (PI; red, staining dead cells) plus 5 μg/ml Hoechst 33342 (blue, staining all cells) for 10 min, and observed with fluorescent microscopy. One set of representative images of three independent experiments are shown. Bright-field images are also shown in merge. PIP, piperine. Scale bar, 50 μm. **(B)** PI-positive cells in five random fields (around 100 cells per field) were calculated and statistically analyzed. Data are shown as mean ± SD ($n = 5$). **(C)** Cells were treated as in **(A)**. The equal volumes of culture supernatants were collected and the proteins in the supernatants were precipitated overnight with 7.2% trichloroacetic acid plus 0.15% sodium deoxycholate. After centrifugation at 13,000 × *g* for 30 min, the pellets were lysed in equal volume of 1x SDS-PAGE loading buffer. Total proteins precipitated from equal volumes of supernatants were loaded for western blotting. The cells were lysed with 1× SDS-PAGE loading buffer. Equal amounts of proteins in the cell lysates were loaded for western blotting. β-Tubulin was recruited as a loading control in cell lysates. **(D,E)** Relative gray values of IL-1β **(D)** and caspase-1p10 **(E)** blots from respective supernatants were quantified. Data are shown as mean ± SD ($n = 3$). Statistical significance was analyzed by one-way ANOVA with Tukey *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant.

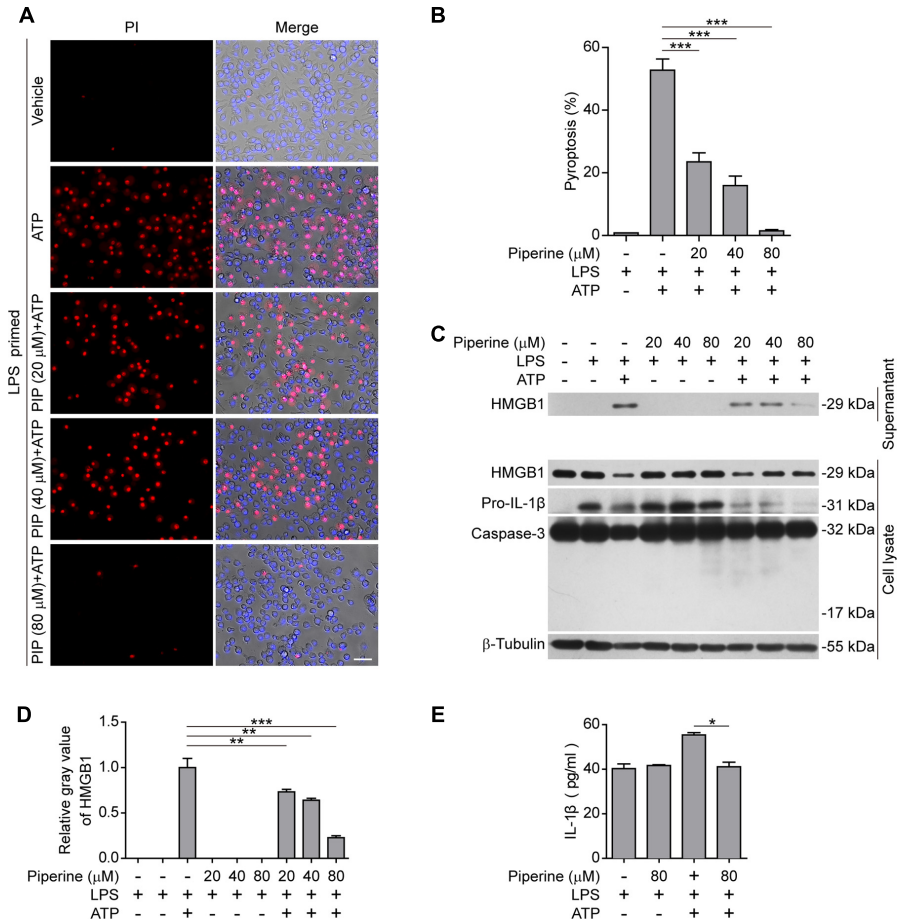

FIGURE 2 | Piperine suppressed ATP-induced pyroptosis in J774A.1 cells. (A) Cells were pre-treated with graded doses of piperine for 4 h, followed by LPS (500 ng/ml) priming for 4 h (in the presence of piperine). Then the cells were stimulated with ATP (4 mM) for 1 h (in the absence of piperine and LPS). Pyroptotic cells were stained with 2 μg/ml propidium iodide (PI) plus 5 μg/ml Hoechst 33342 for 10 min, and observed by fluorescent microscopy. Bright-field images are also shown in merge. PIP, piperine. Scale bar, 50 μm. **(B)** PI-positive cells in 5 random fields (around 150 cells per field) were calculated for each group. Data are shown as mean ± SD ($n = 5$). **(C)** Cells were treated as in **(A)**. Proteins in cell lysates and culture supernatants were evaluated by western blotting. β-Tubulin was used as a loading control for cell lysates. **(D)** Relative gray values of HMGB1 blots from respective supernatants are shown as mean ± SD ($n = 3$). **(E)** IL-1β levels in the cell culture supernatants were evaluated by cytometric bead array (CBA) assay according to the manufacturer's instructions. Data are shown as mean ± SD ($n = 3$). Statistical significance was analyzed by one-way ANOVA with Tukey *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Piperine Administration Reduces Systemic IL-1β Release in Mouse Bacterial Sepsis

Previously, we have demonstrated that piperine administration *in vivo* can protect mice from bacterial infection and that the mice having received piperine administration showed less mortality and histological injuries in their liver and colon as compared to the vehicle group (Pan et al., 2015). In this study, we further investigated whether piperine administration reduced IL-1β release in C57BL/6 mice upon bacterial infection. The results showed that bacterial infection induced IL-1β release into the peritoneal lavage fluids and blood in a time-dependent manner (**Figures 6A,B**). Piperine administration significantly reduced IL-1β levels both in the peritoneal lavage fluids and serum (**Figures 6A,B**), suggesting that bacterial-induced systemic inflammation and pyroptosis was suppressed by piperine. Western blotting also demonstrated that bacterial infection increased both pro-IL-1β and mature IL-1β (17 kDa) expression in the colonic tissues, suggesting inflammasome activation in the colon, which might be infected by bacteria injection in the peritoneal cavity. Piperine administration greatly suppressed the expression of pro-IL-1β and IL-1β in the colonic tissues (**Figures 6C–E**), which was likely to reduce the secretion of IL-1β from the colon. Altogether, these results suggested that piperine administration could inhibit systemic inflammatory responses during bacterial sepsis, probably through suppressing pyroptosis of activated immune cells including macrophages.

DISCUSSION

It has been shown that piperine exhibits anti-septic effects in a mouse model of bacterial sepsis probably by boosting

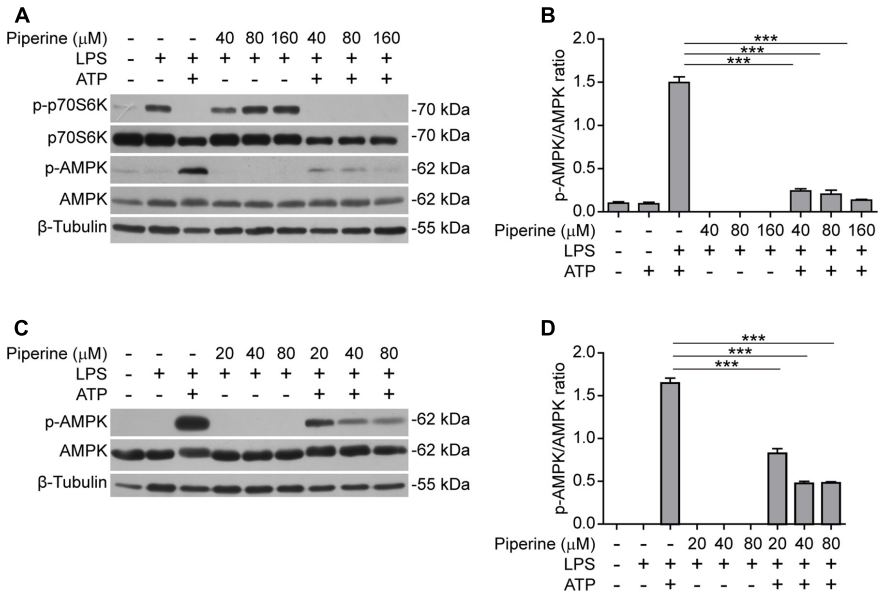

FIGURE 3 | Piperine attenuated ATP-induced AMPK activation in macrophages. BMDMs **(A,B)** or J774A.1 cells **(C,D)** were pre-treated with graded concentrations of piperine for 4 h, followed by LPS (500 ng/ml) priming for 4 h before ATP (3 mM) stimulation for 30 min (BMDMs) or ATP (4 mM) stimulation for 1 h (J774A.1 cells), respectively, as mentioned in **Figure 1**. **(A,C)** The cells were lysed by 1x SDS-PAGE loading buffer and equal amounts of the total proteins in each sample were analyzed by western blotting with indicated antibodies. β-Tubulin was recruited as a loading control. **(B,D)** The densitometry ratios of p-AMPK relative to AMPK of BMDMs **(B)** or J774A.1 cells **(D)** were analyzed. Data are presented as mean ± SD ($n = 3$). Statistical significance was analyzed by one-way ANOVA with Tukey *post hoc* test. ***$P < 0.001$.

the functions of peritoneal resident macrophages through the upregulation of mTORC1 signaling (Pan et al., 2015). However, this may not thoroughly explain why piperine has alleviated the damages to internal organs upon bacterial infection. In this study, we further demonstrated that piperine treatment decreased ATP-induced pyroptosis and IL-1β release in LPS-primed macrophages *in vitro*, and suppressed IL-1β expression and release *in vivo* in the same murine septic model. These data strongly suggest that piperine may have suppressed pyroptosis *in vivo* in the circumstance of bacterial infection, thereby protecting mice from septic death by reducing systemic inflammatory responses as well as organ damages.

Sepsis is a worldwide medical problem, since the pathological progression from systemic infection to septic shock or death can be very fast and there are at present no therapeutic drugs to prevent it (Dellinger et al., 2013). One consequence of sepsis is multiple organ failure in the patients. Several cell death types, including apoptosis, necrosis and pyroptosis, have been implicated in this process (Aziz et al., 2014). However, recent studies tend to agree that pyroptosis is the major cause of organ injury from sepsis (Wree et al., 2014; Kayagaki et al., 2015). Indeed, hepatic pyroptosis due to hyperactive NLRP3 inflammasome has been found to contribute to liver damage (Wree et al., 2014). Consistent with this concept, mice with *caspase-1/-11* or *gasdermin D* gene deletion, thus lacking inflammasome activation and pyroptosis, are resistant to endotoxin-induced sepsis (Kayagaki et al., 2011, 2015). In further support of this, inhibition pyroptosis by neutralization of LPS with antimicrobial peptide LL-37 significantly protects mice from cecal ligation and puncture (CLP)-induced death (another commonly used septic mouse model) (Hu et al., 2016). Therefore, preventing pyroptosis during bacterial infection should alleviate sepsis-associated multiple organ failure and pathological process.

Pyroptosis can be induced by ATP treatment in LPS-primed macrophages *in vitro*. Such a cellular pyroptotic model may represent a severe circumstance of *in vivo* infection, since ATP can be released by both bacteria and host cells during bacterial infection (Mempin et al., 2013; Ren et al., 2014). In this study, we demonstrated that piperine pretreatment suppressed ATP-induced pyroptosis in LPS-primed macrophages *in vitro*, which was evidenced by the reduction of soluble mature IL-1β, active caspase-1p10 and HMGB1 in the supernatants of piperine-pretreated macrophages. One reason for the reduced soluble mature IL-1β levels might be due to piperine-mediated suppression of pyroptosis. A second reason was likely due to that high doses of piperine significantly reduced the pro-IL-1β level in LPS-primed BMDMs irrespective of ATP treatment (**Figure 1C**) as well as in LPS-activated J774A.1 cells in the presence of ATP (**Figure 2C**). The precise mechanism underlying reduced pro-IL-1β levels is unknown but it is probably that a high dose of piperine may influence the expression of pro-IL-1β at transcriptional and translational levels or at post-translational levels. Importantly, piperine may also suppressed *in vivo* pyroptosis in the murine bacterial septic model, as reflected by reduced mature IL-1β levels in serum and infected sites. Consistent with the *in vitro* observation, piperine administration also down-regulated the pro-IL-1β levels in the colon, which might also contribute to reduced mature IL-1β levels in this tissue (**Figures 6C,D**). As

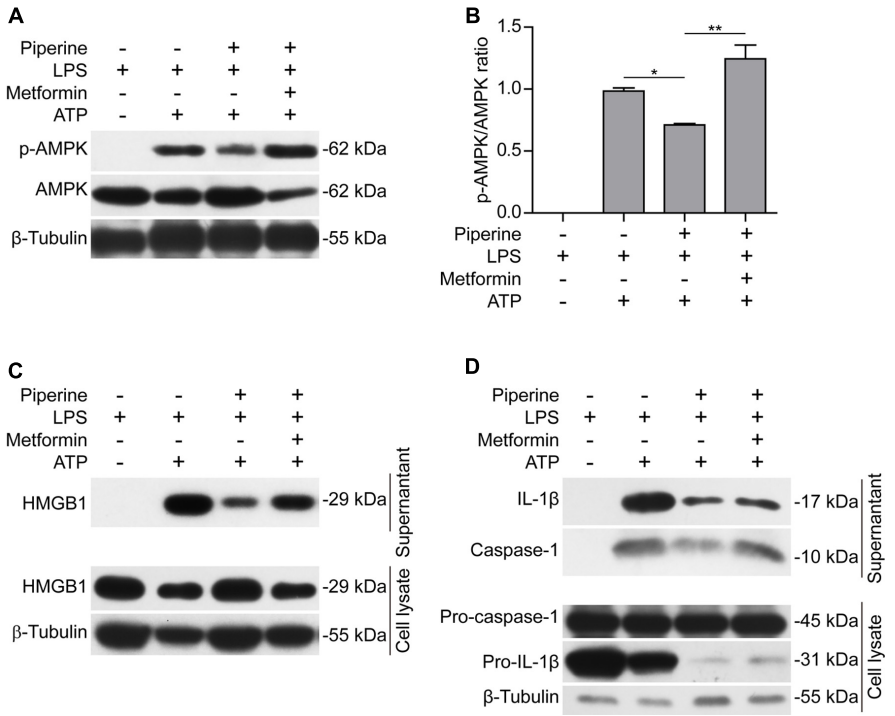

FIGURE 4 | Metformin, an AMPK agonist, counteracted the effect of piperine on suppressing ATP-induced AMPK activation and inflammatory mediator release. J774A.1 cells **(A–C)** were pre-treated with 80 μM piperine and BMDMs **(D)** were pre-treated with 160 μM piperine for 4 h. Without being washed out of piperine, these cells were primed with LPS (500 ng/ml) for 4 h. Next, the cells were treated with metformin (1 mM) for 1 h (in the absence of piperine and LPS). Finally, in the presence of metformin, the BMDMs were stimulated with 3 mM ATP (final concentration) for 30 min while the J774A.1 cells were treated with 4 mM ATP (final concentration) for 1 h. After the cells were lysed with 1× SDS-PAGE loading buffer, protein levels were evaluated by western blotting **(A,C,D)**. β-Tubulin was used as a loading control for cell lysates. **(B)** The densitometry ratios of p-AMPK relative to AMPK in the blots of **(A)** were analyzed by FluorChem 8000 (Alpha Innotech). Data are presented as mean ± SD ($n = 3$). Statistical significance was analyzed by one-way ANOVA with Tukey *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$.

pyroptosis has a critical role in septic shock (Wree et al., 2014; Kayagaki et al., 2015), reduced pyroptosis by piperine may diminish the injury of organs. Indeed, piperine administration *in vivo* alleviated injuries of internal organs and reduced mouse mortality upon bacterial infection (Pan et al., 2015). This is consistent with a previous study showing that piperine can inhibit LPS-induced endotoxin shock, although there was no reduction in IL-1β levels in serum (detected at 3 h post LPS injection) of mice treated with piperine intraperitoneally (Bae et al., 2010). The discrepancy between their results and ours may be due to the differences of animal models and drug administration routes. In our study, the mice were directly infected with viable bacteria and piperine was given intragastrically. Moreover, the release of IL-1β in our experimental model was time-dependent and we observed varied serum IL-1β levels in the vehicle and piperine groups at 6 and 12 h. It is unclear whether piperine also suppressed serum IL-1β levels in LPS-inoculated mice in a time-dependent manner.

Chronic inflammations including autoimmune diseases are associated with inflammasome activation (Yang and Chiang, 2015; Zhang et al., 2016). Negative regulation of the inflammasome activation is believed to ameliorate these diseases (Vande Walle et al., 2014), whereas release of IL-1β, IL-18, HMGB1 or uric acid may induce or aggravate disease symptoms (Lamkanfi et al., 2011; Yang and Chiang, 2015). Piperine has been reported to prevent chronic inflammatory diseases, including epilepsy (Pal et al., 2011) and arthritis (Umar et al., 2013). Some studies have indicated or implied that piperine alleviates chronic inflammatory diseases (such as diabetic nephropathy (Samra et al., 2016) and arthritis (Ying et al., 2013)) by suppressing inflammasome activation and thus inhibiting IL-1β release. Consistent with our study, one recent report indicated that piperine inhibits periodontitis in a rat model at a dose of 100 mg/kg (Dong et al., 2015). This is likely due to its inhibitory effect on the expression of IL-1β (or pro-IL-1β), matrix metallopeptidase (MMP)-8 and MMP-13 in the local gingiva tissues. However, it is unclear whether piperine suppresses pyroptosis and IL-1β release in the gingiva tissues of the periodontitis model.

It is of importance to uncover the underlying mechanism by which piperine suppressed pyroptosis and IL-1β release. Our previous study has demonstrated that piperine treatment promotes amino acid metabolism to enhance mTORC1 signaling (Pan et al., 2015). Therefore, we investigated whether piperine influenced the mTORC1 signaling in LPS-primed macrophages upon ATP stimulation. Our results demonstrated that the mTORC1-p70S6K pathway was greatly activated in macrophages by LPS priming, but was completely suppressed by ATP treatment

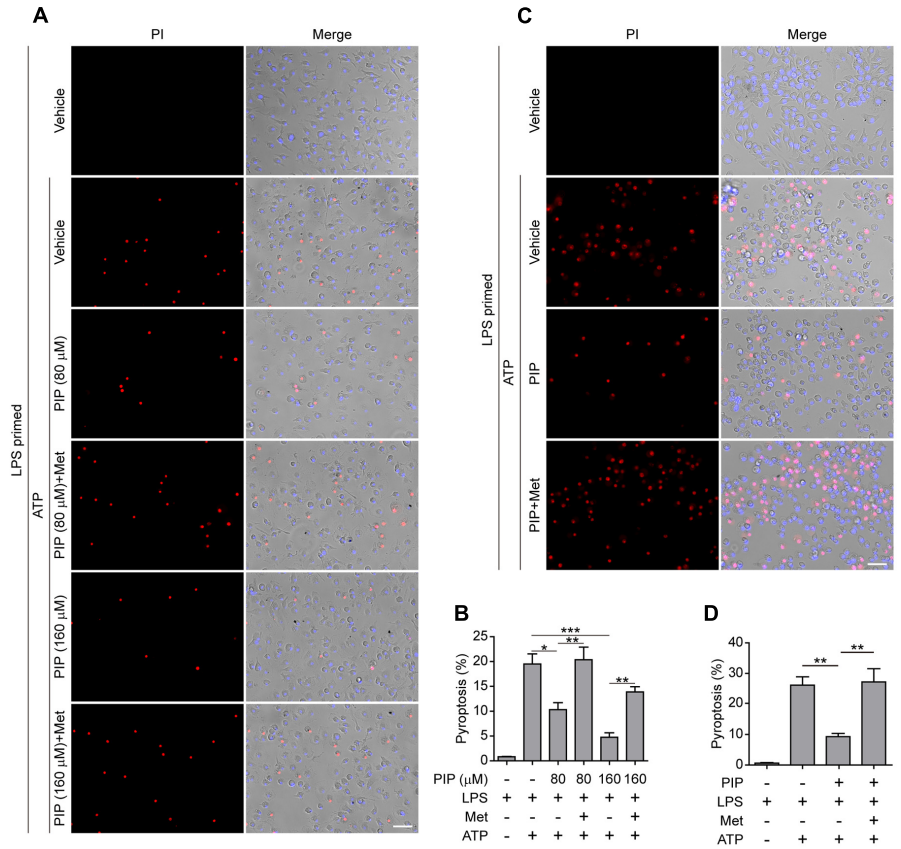

FIGURE 5 | Metformin reversed piperine's effect on suppression of pyroptosis in macrophages treated with ATP. BMDMs **(A,B)** or J774A.1 cells **(C,D)** were pre-treated with indicated concentrations of piperine (BMDMs) or 80 μM piperine (J774A.1) for 4 h, and then primed with LPS (500 ng/ml) for 4 h in the presence of piperine. After washing out of piperine and LPS, the cells were then treated with metformin (1 mM) for 1 h followed by 3 mM ATP for 30 min (BMDMs) or 4 mM ATP for 1 h (J774A.1 cells) in the presence of metformin. The pyroptotic cell ratios in BMDMs **(B)** or J774A.1 cells **(D)** were evaluated as described in **Figure 1**. Data are presented as mean ± SD ($n = 5$). Statistical significance was analyzed by one-way ANOVA with Tukey *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PIP, piperine; Met, metformin.

(**Figure 3A**). Consistent with a previous observation (Moon et al., 2015), our study also demonstrated that ATP treatment greatly induced the activation of AMPK. Both mTORC1 and AMPK are key regulators of energy metabolism. Under nutrition or other stresses, AMPK is activated (Hardie, 2011b). Multiple cellular activities can be regulated by AMPK activation (Hardie, 2011a). For example, AMPK phosphorylates and inhibits the activity of acetyl-CoA carboxylase (ACC), an enzyme responsible for the catalysis of acetyl-CoA into malonyl-CoA during the β-oxidation of fatty acids (Ha et al., 1994; Hardie and Pan, 2002). It also inhibits the activity of mTORC1 by phosphorylation of TSC2 (a suppressor of mTORC1) and Raptor [one member of mTORC1 (Inoki et al., 2003; Gwinn et al., 2008)]. This leads to a general inhibition of protein translation (Hardie, 2011a; Thoreen et al., 2012). In the innate immunity, AMPK activation in macrophages and neutrophils enhances their phagocytosis ability against pathogens (Bae H. B. et al., 2011). Interestingly, AMPK signaling can be suppressed by LPS, fatty acid and other inflammatory stimulators (Yang et al., 2010). However, it can be dramatically re-activated when a second DAMP signal (such as ATP) is added, accompanied by inflammasome assembly (Moon et al., 2015). Under this circumstance, the cells resort to HK1-dependent glycolysis upon inflammasome activation (Moon et al., 2015). This means that there is a sharp switch of energy metabolism from oxidative phosphorylation to glycolysis during the process of inflammasome activation (including the progression phases from LPS priming to ATP stimulation). As mTORC1 can stabilize the hypoxia-induced factor-1α (HIF-1α), it favors cell survival in LPS-primed macrophages (Gilroy and Yona, 2015). However, AMPK activation, through suppressing mTORC1 activity, decreases the level of HIF-1α and makes the cells undergo death processes (Jiang et al., 2001; McGettrick and O'Neill, 2013). Although piperine treatment seemed unable to reverse the suppression of mTORC1 activity (as reflected by p70S6K phosphorylation) by ATP, it significantly suppressed ATP-induced AMPK activation (**Figure 3A**). This implies that piperine attenuated the inhibitory activity of AMPK on mTORC1 signaling upon ATP treatment. In addition, metformin (acting as an AMPK agonist) could counteract the effect of piperine on reducing ATP-induced

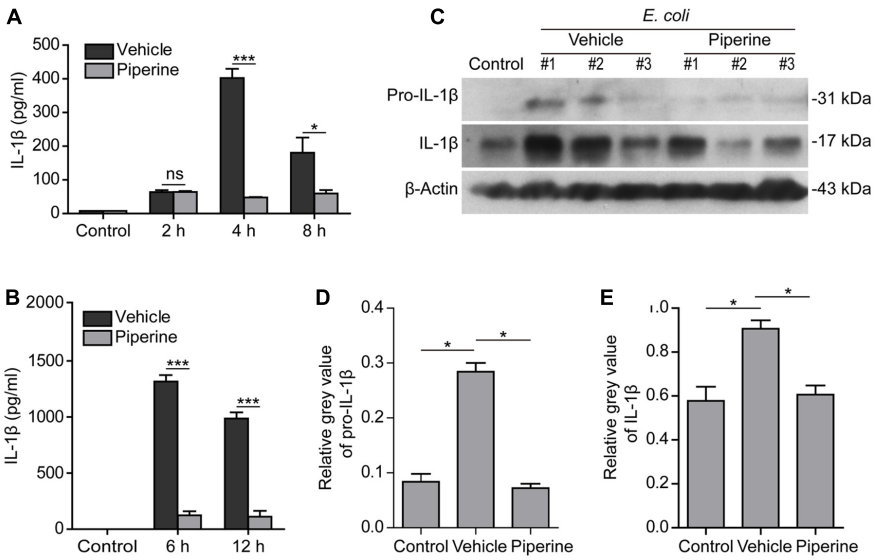

FIGURE 6 | Piperine administration reduced systemic IL-1β release in mouse bacterial sepsis. Piperine was suspended in 2% Tween-80 in PBS at a concentration of 1 mg/ml. C57BL/6 mice (5 per group for each time point) were administered with piperine (20 mg/kg) or vehicle via gavage once a day for five consecutive days and injected intraperitoneally (i.p.) with viable *E. coli* (2×10^9 CFU/mouse, in 0.5 ml PBS) 1 h after the last gavage. Peritoneal lavage fluids were collected with 1 ml of PBS. Serum was isolated from retro-orbital blood sample. Soluble IL-1β was evaluated by CBA (cytometric bead array) according the manufacturer's instruction. **(A)** The levels of soluble IL-1β in peritoneal lavage fluids at 2, 4, and 8 h post bacterial infection. Data are presented as mean ± SD ($n = 5$). **(B)** The serum levels of IL-1β at 6 and 12 h post bacterial infection. Data are presented as mean ± SD ($n = 5$). The significance was analyzed with unpaired Student's *t*-test. **(C)** A middle colon section with a length of 1 centimeter was cut from each mouse and immediately lysed by grinding with 500 μl 2x SDS-PAGE loading buffer. Western blotting was used to detect the expression of pro-IL-1β and IL-1β in the colonic cell lysates. β-Actin was used as a loading control. Blots of three mice from each group are shown. **(D,E)** Relative gray values of pro-IL-1β **(D)** and IL-1β in **(E)** were analyzed by one-way ANOVA with Tukey *post hoc* test. Data are shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{***}P < 0.001$.

pyroptosis. These results suggested that suppressing AMPK activation by piperine (or by other AMPK inhibitors) during systemic bacterial infection may be helpful for preventing the pathological development of sepsis. However, more research is warranted to reveal whether piperine ameliorates other inflammatory diseases, including epilepsy, obesity, arthritis and ulcer by the common mechanism of inflammasome suppression as in bacterial sepsis.

Piperine is the major plant alkaloid in pepper, a daily used food seasoning. Therefore, it is believed to be low toxic to mammalian cells and human body. Indeed, our preliminary experiments showed that piperine had no overt toxicity to mouse BMDMs at a dose up to 160 μM *ex vivo* and to mice at a dose up to 40 mg/kg/day *in vivo*. Supporting this, a previous study showed that piperine has no oral acute toxicity on mice at a dose up to 5000 mg/kg (Gupta et al., 2015). This means that piperine may be safe for humans at doses above tens of milligrams based on translating dosages from animal models to human clinical trials (Blanchard and Smoliga, 2015). Although there are currently no clinical trials testing piperine for bacterial sepsis, other clinical trials have recruited piperine at doses of 20–25 mg each time or per day[1]. Another clinical trial had used piperine locally at 1 and 0.15 mM to evaluate the effect of piperine in patients with oropharyngeal dysphagia[2]. Thus, the *in vitro* and *in vivo* doses used in this study have relevance for translating into human study.

In summary, we revealed that piperine treatment could significantly reduce ATP-induced pyroptosis in macrophages probably through the suppression of AMPK signaling. In a murine bacterial sepsis model, piperine administration sharply decreased systemic IL-1β levels, suggestive of suppression of systemic inflammation and pyroptosis. Our data highlight that piperine may act as a suppressant of pyroptosis to exhibit its therapeutic effects on bacterial sepsis and other inflammatory disorders, which deserves further clinical investigation.

AUTHOR CONTRIBUTIONS

Y-DL, W-JB, C-GL, L-HX, and H-XW performed the experiments. D-YO and X-HH designed the research. Y-DL, C-GL, and HP analyzed the data. D-YO, X-HH, and Y-DL wrote the paper. All authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

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[1]http://clinicaltrialsfeeds.org/clinical-trials/results/term=Piperine

[2]https://clinicaltrials.gov/show/NCT01383694

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Curcumin Represses NLRP3 Inflammasome Activation via TLR4/MyD88/NF-κB and P2X7R Signaling in PMA-Induced Macrophages

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Aims: In the NOD-like receptor (NLR) family, the pyrin domain containing 3 (NLRP3) inflammasome is closely related to the progression of atherosclerosis. This study aimed to assess the effects of curcumin on NLRP3 inflammasome in phorbol 12-myristate 13-acetate (PMA)-induced macrophages and explore its underlying mechanism.

Methods: Human monocytic THP-1 cells were pretreated with curcumin for 1 h and subsequently induced with PMA for 48 h. Total protein was collected for Western blot analysis. Cytokine interleukin (IL)-1β release and nuclear factor kappa B (NF-κB) p65 translocation were detected by ELISA assay and cellular NF-κB translocation kit, respectively.

Results: Curcumin significantly reduced the expression of NLRP3 and cleavage of caspase-1 and IL-1β secretion in PMA-induced macrophages. Moreover, Bay (a NF-κB inhibitor) treatment considerably suppressed the expression of NLRP3 inflammasome in PMA-induced THP-1 cells. Curcumin also markedly inhibited the upregulation of toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), phosphorylation level of IκB-α, and activation of NF-κB in PMA-induced macrophages. In addition, purinergic 2X7 receptor (P2X7R) siRNA was administered, and it significantly decreased NLRP3 inflammasome expression in PMA-induced macrophages. Furthermore, curcumin reversed PMA-stimulated P2X7R activation, which further reduced the expression of NLRP3 and cleavage of caspase-1 and IL-1β secretion. Silencing of P2X7R using siRNA also suppressed the activation of NF-κB pathway in PMA-induced macrophages, but P2X7R-silenced cells did not significantly decrease the expression of TLR4 and MyD88.

Conclusion: Curcumin inhibited NLRP3 inflammasome through suppressing TLR4/MyD88/NF-κB and P2X7R pathways in PMA-induced macrophages.

Keywords: Curcumin, NLRP3 inflammasome, TLR4/MyD88/NF-κB, P2X7R, macrophages

INTRODUCTION

Atherosclerosis is a chronic and progressive immunoinflammatory disease. Monocytes are one of major factors in the development of this disease. Focal recruitment of circulating monocytes is one of the earliest cellular responses, which underlie disease progression. Moreover, inflammatory factors, which are released by the newly differentiated macrophages, play key roles in the pathophysiology of atherosclerosis (Glass and Witztum, 2001; Libby, 2002; Hansson, 2005).

For the NOD-like receptor (NLR) family, the pyrin domain containing 3 (NLRP3) inflammasome plays a crucial role in the inflammatory response (Schroder and Tschopp, 2010). NLRP3 inflammasome is a multiprotein complex that consists of NLRP3, an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1 (Latz et al., 2013). Upon stimulation, NLRP3 recruits its adaptor ASC and pro-caspase-1 to form an inflammasome complex; consequently, caspase-1 is activated, which causes the cleavage of the pro-forms of interleukin (IL)-1β and IL-18 to their mature forms (Martinon et al., 2009). Moreover, cholesterol crystals induce NLRP3 inflammasome activation and IL-1β secretion in human macrophages (Duewell et al., 2010; Rajamaki et al., 2010). IL-1β is a fundamental pro-inflammatory cytokine in mediating atherosclerosis progression (Elhage et al., 1998; Duewell et al., 2010).

Curcumin, a natural polyphenolic compound in *Curcuma longa*, exhibits various biological properties, including anti-inflammatory, antioxidant, and anti-infection (Shishodia, 2013). Briefly, curcumin administration has been previously associated to regulate different inflammatory cytokines, such as IL-1β (Sun et al., 2013), extracellular matrix metalloproteinase inducer, and matrix metalloproteinase-9 expression (Cao et al., 2014). In addition, the effect of curcumin is associated with the inhibition of different signaling pathway activations, including the activation of toll-like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), and mitogen-activated protein kinase pathways (Min et al., 2013; Zhou et al., 2015). However, whether the anti-atherogenic effects of curcumin involve in suppressing NLRP3 inflammasome activation has never been indicated.

Hence, the present study aims to: (i) identify the expression of NLRP3 inflammasomes under curcumin treatment in monocytes/macrophages; and (ii) elucidate the relative mechanism of curcumin treatment on the inflammatory activity of monocytes/macrophages.

MATERIALS AND METHODS

Cell Culture

Human monocytic cell line (THP-1) was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained at a density of 10^6/ml in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma–Aldrich, USA), and 1% pen/strep solution at 37°C in a 5% CO_2 incubator. The cells were cultured in 6-well plates for 48 h in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA), which allowed them to differentiate into adherent macrophages. Cells were stimulated for 1 h with curcumin (0–50 μM, Sigma–Aldrich, USA) or 5 μM Bay 11-7082 (NF-κB-specific inhibitor; Beyotime Biotech, China) and subsequently treated with PMA for another 48 h.

Determination of Cell Viability (CCK8 Assay)

CCK8 assay (WST-8, Dojindo, Kumamoto, Japan) was used to evaluate the cytotoxicity of curcumin on PMA-induced macrophages, according to the manufacturer's recommendation. PMA-induced macrophages were seeded in 96-well plates at 6×10^3 cells/well. At 24 h later, cells were incubated with curcumin (0–100 μM) for 48 h.

siRNA Transfection

Cells were transfected with 20 nM of siRNA for 8 h with siRNA transfection reagent (RiboBio, Guangzhou, China) to knockdown purinergic 2X7 receptor (P2X7R). Briefly, cells were treated with 100 nM of PMA for 48 h and washed in fresh medium without antibiotics. Afterward, the cells were treated with siRNA duplex solution for 8 h. The medium was subsequently replaced with normal culture medium. Control cells were transfected with scrambled sequence siRNA control (RiboBio, Guangzhou, China). The cell lysates were utilized for Western blot analysis to verify the efficacy of protein knockdown by siRNA.

Western Blotting

Protein isolation and Western blot analysis were performed comparably, as described in literature (Huang et al., 2011). Briefly, membrane protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% milk at room temperature and incubated at 4°C overnight with NLRP3, IL-1β, P65, phospho-P65, P-IκB-α, IκB-α, GAPDH, P2X7R, Bax, Bcl-2 (Cell Signaling Technology, Boston, MA, USA) (1:1000 dilution in TBST) or TLR4, and MyD88 (Abcam, Cambridge, MA, UK) (1:500 dilution in TBST) or caspase-1 (Santa Cruz, CA, USA) (1:500 dilution in TBST). Proteins were visualized with ECL procedure (Bio-Rad, USA). The results were analyzed using Quantity One (Bio-Rad) software.

ELISA for Cytokine Measurements

Cytokines were measured by ELISA in 48-h culture supernatants. On this basis, cells were preincubated in the presence or absence of curcumin (6.25–25 μM) for 1 h or 5 μM Bay 11-7082 (NF-κB-specific inhibitor) for 30 min. PMA was added to the cells at a final concentration of 100 nM, and the cells were further incubated for 48 h. Culture supernatants were analyzed to determine IL-β concentrations using sandwich enzyme immunoassay kits (R&D Systems Europe Ltd, Abingdon, UK), according to the manufacturer's instructions.

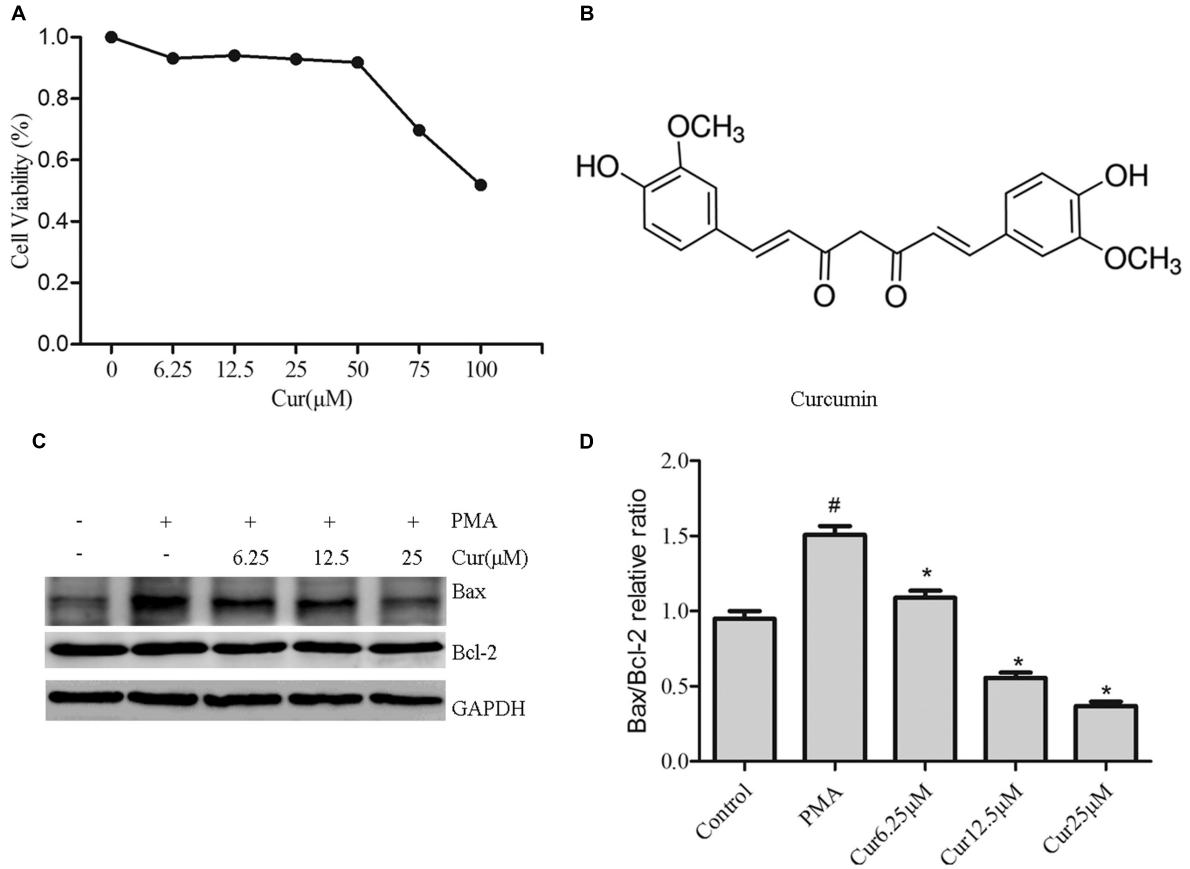

FIGURE 1 | Effects of curcumin on cell viability and apoptosis. THP-1 monocytes were incubated with various curcumin concentrations (0–100 μM) for 1 h and exposed to 100 nM of phorbol 12-myristate 13-acetate (PMA) for 48 h. **(A)** Cell proliferation was assessed using the CCK8 assay. Cells incubated in a medium without curcumin and PMA were defined as control and considered to have a 100% proliferation rate. **(B)** Chemical structure of curcumin. **(C)** Representative Western blot analysis of Bax and Bcl-2 in curcumin (6.25–25 μM)-treated THP-1 cells. **(D)** Densitometric analysis was used to quantify the ratio of Bax/Bcl-2. The results represent the mean ± SEM for three experiments. $^{*}P < 0.05$ vs. PMA group, $^{\#}P < 0.05$ vs. Control group.

Immunofluorescence Staining of NF-κB p65

Human monocytic THP-1 cells were cultured on 20-mm diameter glass coverslips in 12-well plates. Cells were pretreated with curcumin (6.25–25 μM) for 1 h and subsequently treated with PMA (100 nM) or vehicle control for 48 h. The cells were immunofluorescence-labeled using a cellular NF-κB translocation kit (Beyotime Biotech), according to the manufacturer's protocol. Briefly, after washing and fixing, the cells were incubated with a blocking solution at 4°C overnight and subsequently with the NF-κB p65 antibody for 2 h. After washing thrice, a rabbit IgG antibody conjugated with Cy3 was added and incubated for 1 h. To stain the nucleus, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The activation of NF-κB p65 was visualized with an inverted fluorescence microscope (Olympus DP70) at excitation wavelengths of 350 and 540 nm for DAPI and Cy3, respectively. The red and blue images were overlaid to create a two-color image, in which purple fluorescence indicated the areas of colocalization.

Statistical Analysis

All values were expressed as mean ± SEM. One-way ANOVA and subsequent *post hoc* Tukey's test were employed to analyze the differences between sets of data. Statistics was analyzed using the SPSS 20.0 software. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effects of Curcumin on Cell Viability and Apoptosis

We first examined the effect of curcumin on the viability of PMA-induced THP-1 cells. PMA-induced macrophages were treated with curcumin (6.25–100 μM) or the vehicle for 48 h. Cell viability was assessed using the CCK8 assay. As shown in **Figure 1A**, curcumin at 50 μM significantly reduced cell viability after 48 h of incubation compared with control ethanol. On this basis, the experiments in cultured THP-1 cells were conducted using 6.25, 12.5, and 25 μM of curcumin.

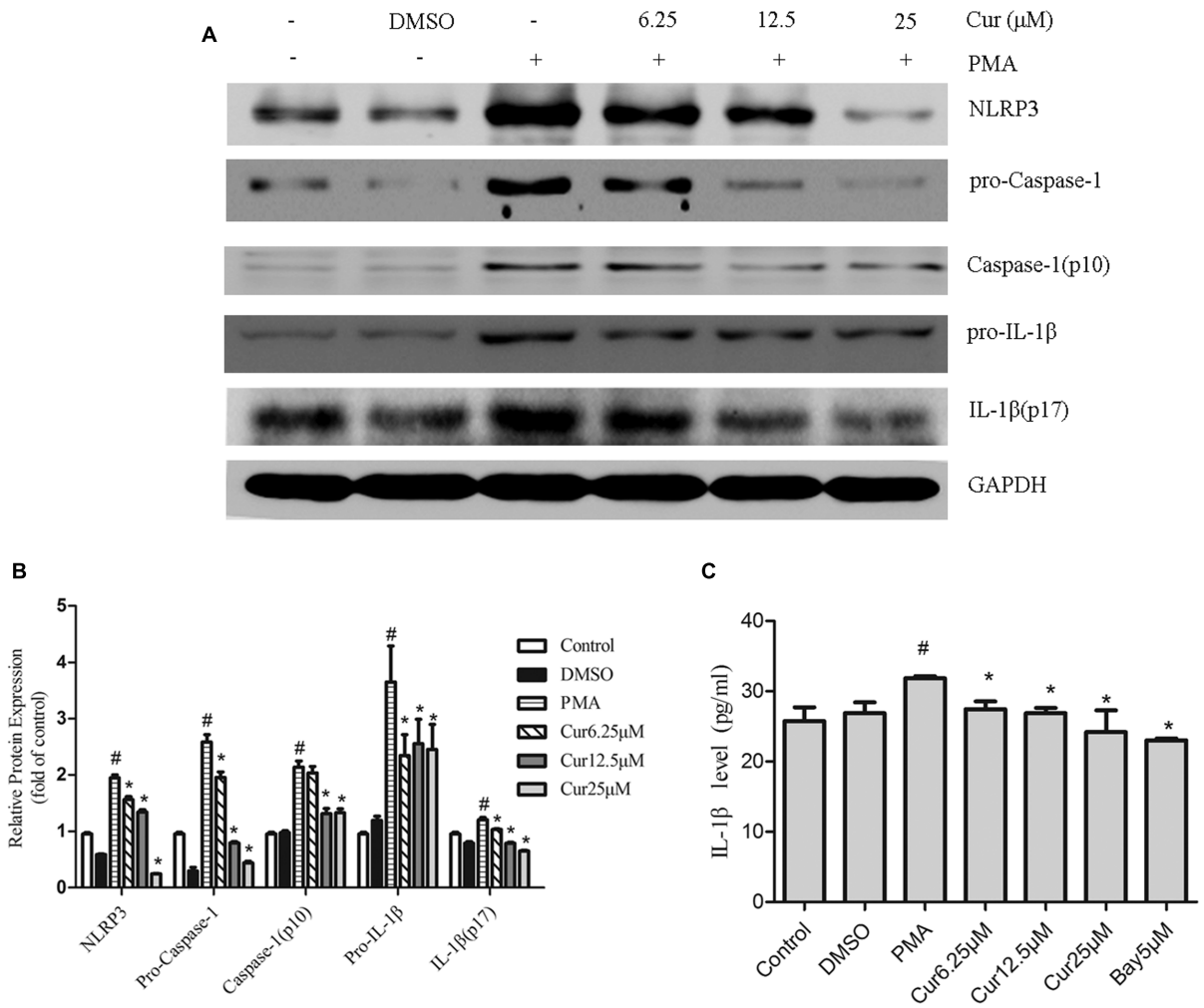

FIGURE 2 | Curcumin attenuates the activation of the NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome. THP-1 macrophages were stimulated by incubation with curcumin (Cur) at the indicated concentration (6.25–25 μM) for 1 h, followed by PMA for 48 h. The condition referred to as control refers to THP-1 treated with vehicle or dimethyl sulfoxide (DMSO) for 48 h. **(A)** Representative Western blot analysis of NLRP3 and the cleavage of caspase-1 and interleukin (IL)-1β protein expression after PMA-induced inflammasome activation. **(B)** Densitometric analysis was used to quantify the level of NLRP3 and cleavage of caspase-1 and IL-1β. **(C)** Concentrations of IL-1β in cell culture supernatants were detected by ELISA. The results represent the mean ± SEM for three experiments. $^{*}P < 0.05$ vs. PMA group, $^{\#}P < 0.05$ vs. Control group.

The structure of curcumin used in this study is shown in **Figure 1B**.

To confirm the effect of curcumin on the apoptosis of PMA-induced macrophages, we explored the effect of Bax and Bcl-2 expression by Western blot analysis (**Figures 1C,D**). Significantly, cucurmin-treated cells showed dose-dependent reduction of Bax/Bcl-2 ratio.

Curcumin Attenuates the Activation of the NLRP3 Inflammasome

To examine the effect of curcumin on NLRP3 inflammasome activation, we stimulated THP-1 cells with PMA in the presence or absence of curcumin. Results displayed that curcumin effectively reduced the cleavage and secretion of IL-1β level in a dose-dependent manner (**Figures 2A–C**). Upon activation, NLRP3, which contains a caspase recruitment domain, causes the cleavage of pro-caspase-1, an essential step to produce and release IL-1β (Schroder and Tschopp, 2010). Consistently, western blot analysis confirmed the reduction of cleaved caspase-1 (p10) and NLRP3 inflammasome level by curcumin (**Figures 2A,B**). These observations suggested that curcumin effectively attenuated the cleavage and secretion of IL-1β level, at least partially, via the inhibition of NLRP3 inflammasome activation.

NF-κB Pathway Is Involved in the Activation of the NLRP3 Inflammasome in PMA-Induced Macrophages

To investigate the associated mechanism of curcumin effect on NLRP3 inflammasome, cells were pre-incubated with 5 μM Bay

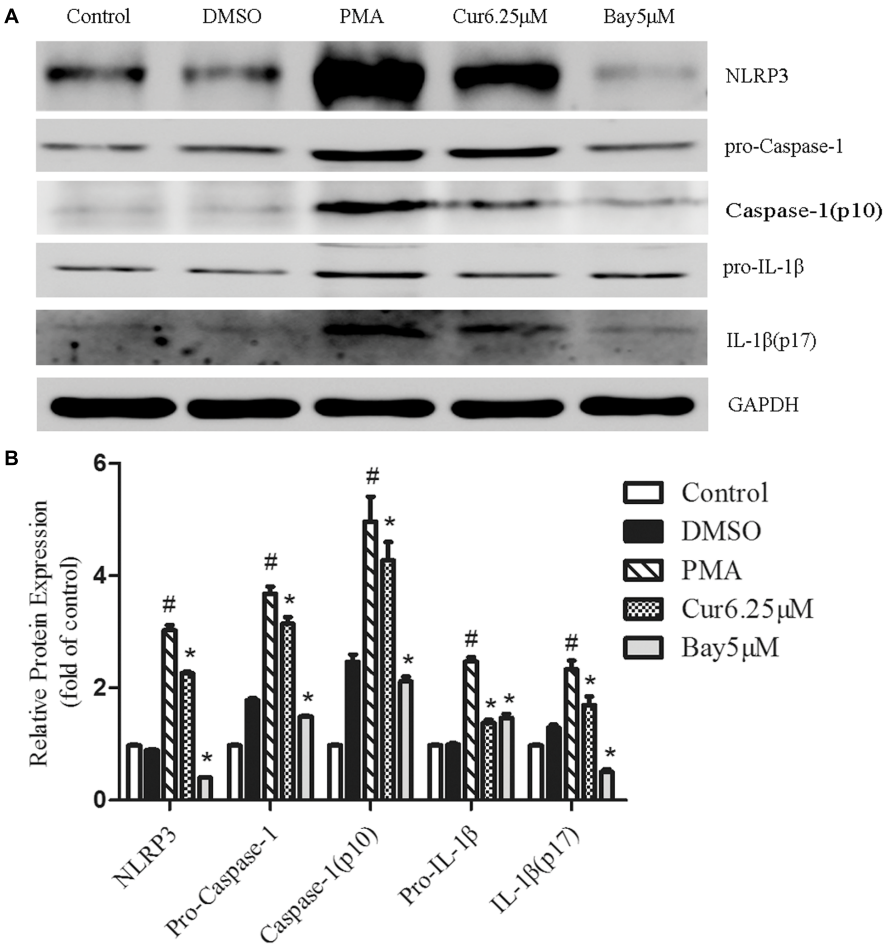

FIGURE 3 | Nuclear factor kappa B (NF-κB) pathway activation participates in the activation of the NLRP3 inflammasome in PMA-induced macrophages. Cells were incubated with 6.25 μM of curcumin (Cur) for 1 h or 5 μM of NF-κB specific inhibitor Bay 11-7082 (Bay) for 30 min and exposed to 100 nM of PMA for 48 h before collection. **(A)** Representative Western blot analysis of NLRP3 and the cleavage of caspase-1 and IL-1β protein expression after PMA-induced inflammasome activation. **(B)** Densitometric analysis was used to quantify the level of NLRP3 and cleavage of caspase-1 and IL-1β. The results represent the mean ± SEM for three experiments. $^{*}P < 0.05$ vs. PMA group, $^{\#}P < 0.05$ vs. Control group.

11-7082 (NF-κB pathway inhibitor) for 30 min before PMA addition. When cells were cultured in the presence of Bay 11-7082, complete inhibition of NLRP3 expression and cleavage of caspase-1 and IL-1β secretion were observed, which were congruent with the curcumin-pretreated groups (**Figures 3A,B** and **2C**). This result suggested that suppression of NF-κB-signaling pathway activation mitigated NLRP3 inflammasome in PMA-induced macrophages.

Curcumin Inhibits the Activation of the TLR4/MyD88/NF-κB-Signaling Pathways

Rapidly growing evidence presented that the TLR4/NF-κB signal transduction pathway is considered an early event essential for inflammasome activation and subsequent IL-1β secretion (Bauernfeind et al., 2009). NLRP3 inflammasome activation during pathological conditions also involves TLR signaling; thus, we monitored the changes in TLR4 expression in PMA-induced THP-1 cells and noticed that PMA stimulation increased the expression of TLR4, which was not observed in control group (**Figure 4A**). Quantification of Western blots showed that TLR4 expression level was reduced in a dose-dependent manner in curcumin treatment group (**Figures 4A,B**). TLR signaling involves the recruitment of MyD88 adapter protein and final activation of NF-κB (Barton and Medzhitov, 2003); hence, we examined the expression of these associated downstream-signaling molecules in cell cultures by Western blot analysis. PMA increased the expression of TLR4 and MyD88, as well as the phosphorylation level of IκB-α and nuclear p65 in the THP-1 cells (**Figure 4A**). Curcumin treatment effectively downregulated the PMA-induced upregulation of TLR4 and MyD88, and phosphorylation level of IκB-α and p65 subunit of NF-κB (**Figures 4A–E**). Additionally, curcumin reduced the nuclear localization of p65 (**Figure 4F**). Taken together, the results implied that curcumin-modulated TLR4/MyD88/NF-κB signalings during PMA stimulation in a dose-depended manner.

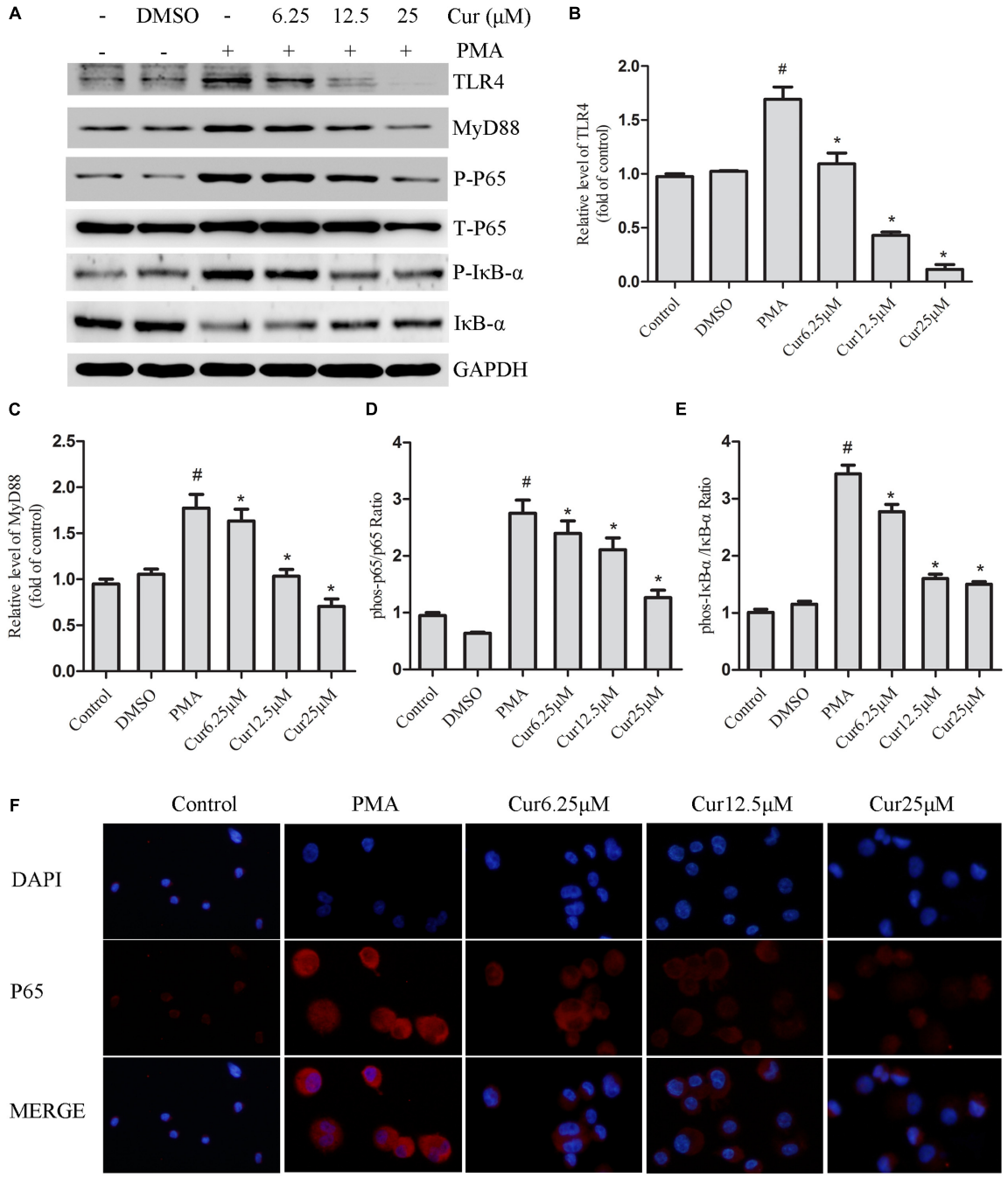

FIGURE 4 | Curcumin inhibits the activation of the TLR4/MyD88/NF-κB-signaling pathways. (A) Representative Western blot analysis of TLR4, MyD88, p-IκB-α, IκB-α, p-P65, and P65 was normalized based on the internal control GAPDH. **(B–E)** Densitometry measurements of protein analysis. The results represent the mean ± SEM for three experiments. $^{*}P < 0.05$ vs. PMA group, $^{\#}P < 0.05$ vs. Control group. **(F)** Differentiated THP-1 cells were treated with indicated agent's immunostained with DAPI (Blue) and anti-NF-κB p65 (Red) and observed using an inverted fluorescence microscope, 200×.

Curcumin Decreases the Expression of P2X7R in PMA-Induced Macrophages

Purinergic 2X7 receptor is essential for activating the NLRP3 inflammasome (Di Virgilio, 2007). Therefore, we hypothesized that curcumin inhibited the activation of the NLRP3 inflammasome via the mechanism of P2X7R. First, P2X7R siRNA was administered to knock-down the P2X7R expression of PMA-induced macrophages. We successfully and markedly decreased basal P2X7R expression (**Figures 5A,B**). Our data indicated that P2X7R-silenced cells significantly attenuated the

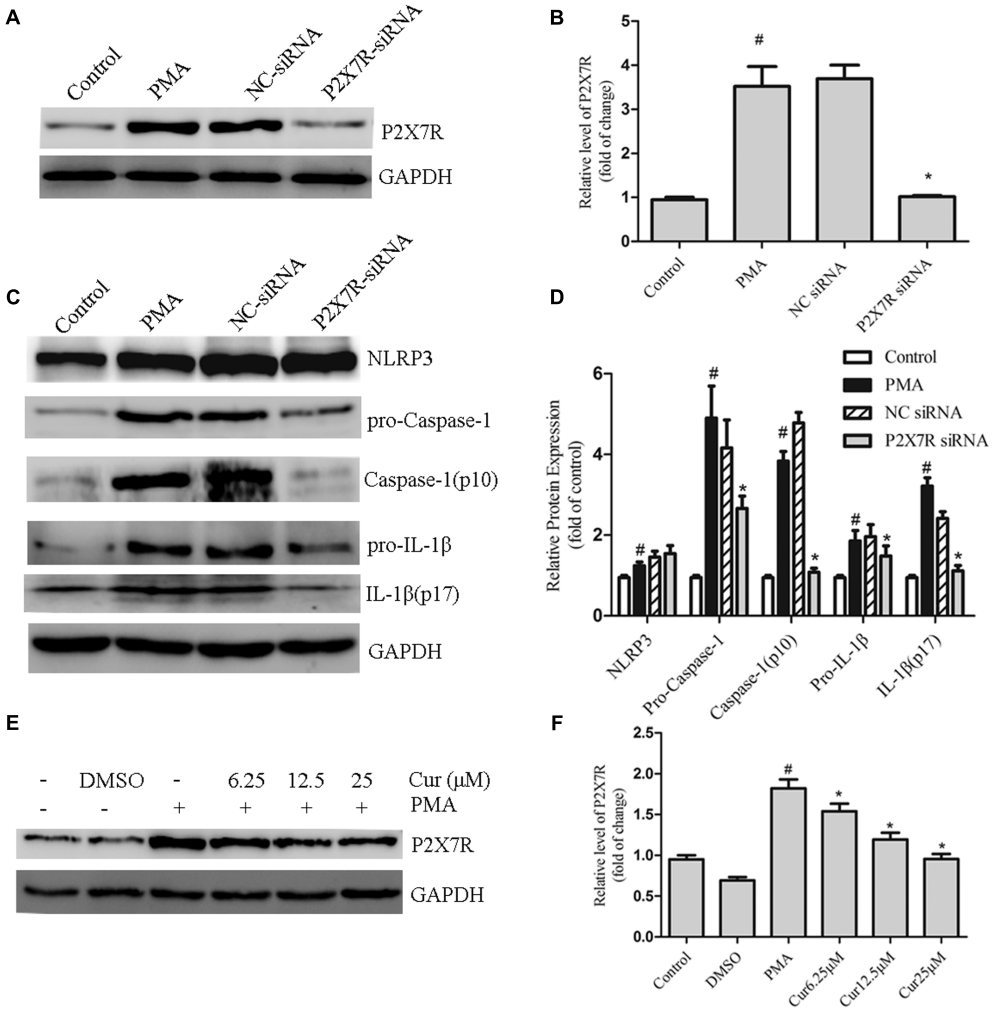

FIGURE 5 | Curcumin decreases the expression of P2X7R in PMA-induced macrophages. (A,B) THP-1 cells silenced for purinergic 2X7 receptor (P2X7R) were processed to obtain a whole-cell extract as described under the Section "Materials and Methods". The expression of P2X7R in cells were transfected with P2X7R or negative control (NC) siRNA. **(C,D)** The expression of NLRP3 and cleavage of caspase-1 and IL-1β in cells were transfected with P2X7R or negative control (NC) siRNA. **(E,F)** THP-1 cells were pretreated with curcumin in the presence of PMA for 48 h. The expression of P2X7R was determined by Western blot analysis. $^{*}P < 0.05$ vs. PMA group, $^{\#}P < 0.05$ vs. Control group.

activation of caspase-1, which was evidenced by decreased IL-1β production. However, P2X7R-silenced cells did not significantly decrease the expression of NLRP3 (**Figures 5C,D**). Second, we investigated the interaction between curcumin and P2X7R in PMA-induced macrophages. Curcumin inhibited the expression of P2X7R in a dose-dependent manner (**Figures 5E,F**). Our data indicated that curcumin can inhibit the NLRP3 inflammasomes via (at least patially) decreasing P2X7R.

P2X7R Activation Regulates Nuclear Translocation of NF-κB in PMA-Induced Macrophages

To clarify the relationship of P2X7R on the regulation of TLR4/MyD88/NF-κB signalings, we further used P2X7R-silenced cells by siRNA. Our result showed that P2X7R-silenced cells significantly decreased the phosphorylation level of p65 subunit of NF-κB. Nevertheless, the expression of TLR4 and MyD88 (**Figures 6A–C**) was not significantly changed. This finding indicated that P2X7R activation was only involved in regulating the nuclear translocation of NF-κB in PMA-induced macrophages.

DISCUSSION

In this research, curcumin effectively suppressed the expression of NLRP3 inflammasome in PMA-induced macrophages. Moreover, in PMA-induced macrophages, TLR4/MyD88/NF-κB and P2X7R pathways were necessary for the inhibition of NLRP3 inflammasome expression by curcumin. Therefore, these results supported a novel effect of curcumin on the expression

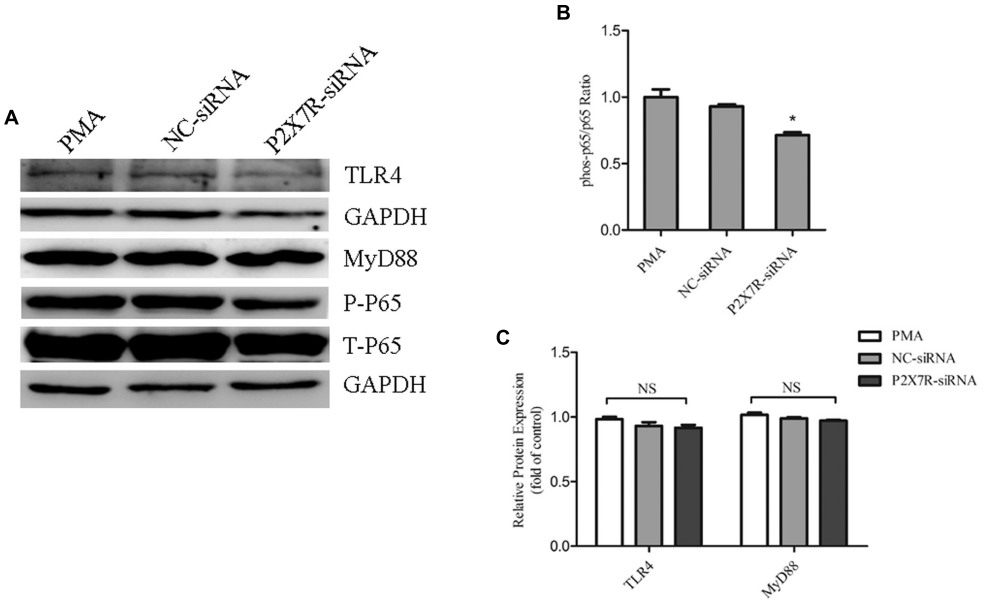

FIGURE 6 | Purinergic 2X7 receptor inhibition reduces the phosphorylation of p65 in PMA-induced macrophages. THP-1 cells silenced for P2X7R were used to obtain a whole-cell extract as described under Materials and Methods. **(A)** Representative Western blot analysis of TLR4, MyD88, p-P65, and P65 was normalized based on the internal control GAPDH. **(B,C)** Densitometry measurements of protein analysis. The results represent the mean ± SEM for three experiments. *$P < 0.05$ vs. PMA group.

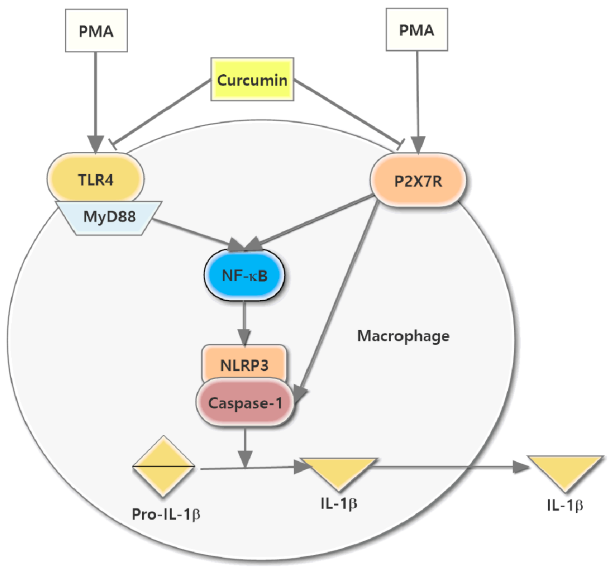

FIGURE 7 | Schematic model for the reduction of NLRP3 inflammasome expression in PMA-stimulated macrophages treated with curcumin. The curcumin-downregulated P2X7R expression and TLR4/MyD88/NF-κB signaling, which together inhibited NLRP3 expression, caspase-1 activation, and the secretion of IL-1β.

of NLRP3 inflammasome, which suggested curcumin as a promising therapeutic agent for ameliorating the development of atherosclerosis.

Monocytes play a key role in invading atherosclerotic lesions and differentiating them into macrophages (Boyle, 2005; Johnson and Newby, 2009). Plaque-residing macrophages produce chemokines/cytokines, such as IL-1β, which further regulate monocyte/T-cell infiltration into the lesion (Zernecke et al., 2008). PMA-induced IL-1β production is dependent on the NLRP3 inflammasome (Tulk et al., 2015), thereby suggesting that NLRP3 inflammasome can prevent mature IL-1β production. In addition, Zheng et al. (2014) showed that knocking down NLRP3 genes in apolipoprotein E-deficient mice results in reduced inflammatory cytokines and plaque content of lipid and macrophages, as well as increased plaque content of collagen; therefore, NLRP3 is associated with unstable qlaque of atherosclerosis. In our study, PMA treatment-induced NLRP3 inflammasome and the cleavage of caspase-1 and IL-1β expression; moreover, curcumin treatment could markedly mitigate the expression of NLRP3 inflammasome, as well as the cleavage of caspase-1 and IL-1β expression. These findings inferred that curcumin exhibits its anti-inflammatory activity through suppressing the expression of NLRP3 inflammasome in PMA-induced macrophages.

Toll-like receptor 4, which is broadly expressed on the plasma membranes of immune cells, plays a vital role in initiating the sterile inflammation related to atherosclerosis (Guo et al., 2015; Luo et al., 2015). Within the TLR4-signaling pathway, the MyD88-dependent signaling pathway is an important activator of NF-κB and the subsequent regulatory effects of NF-κB signaling (Barton and Medzhitov, 2003). In our study, the expression of TLR4 and MyD88 protein, phosphorylation level of IκB-α and

NF-κB p65, and nuclear localization of p65 were significantly increased at 48 h after PMA stimulation compared with the control group. Curcumin treatment obviously suppressed all these indicators. Moreover, Bay 11-7082 (a NF-κB-specific inhibitor) markedly reduced NLRP3 inflammasome activation, which was dramatically attenuated by curcumin treatment. Considering these findings, we suggested the protective effects of curcumin against NLRP3 inflammasome activation by regulating the TLR4/MyD88/NF-κB signaling in PMA-induced macrophages.

Purinergic 2X7 receptor, a member of the P2X subfamily, plays an important role in macrophage regulating cytokine production, which is activated by extracellular ATP to induce NLRP3 inflammasome assembly and caspase-1-dependent processing, and release of pro-inflammatory cytokines IL-1β and IL-18 (Di Virgilio, 2007; Franceschini et al., 2015; Gicquel et al., 2015). P2X7R activation can also activate the NLRP3 inflammasome to promote the progression of atherosclerosis (Piscopiello et al., 2013; Peng et al., 2015). To confirm whether curcumin inhibited NLRP3 inflammasome activation through P2X7R-signaling cascade, the receptor was silenced. As expected, siRNA interference of P2X7R significantly impaired NLRP3 inflammasome activation and IL-1β secretion in PMA-induced THP-1 cells; this result was consistent with the results of Peng et al. (2015). In addition, curcumin significantly inhibited PMA-induced P2X7R expression in macrophages. These results showed that curcumin reduced the activation of NLRP3 inflammasome via regulating P2X7R in PMA-induced THP-1 cells. P2X7R-silenced cells could also decrease the phosphorylation level of p65 subunit of NF-κB, but not TLR4 or MyD88 pathway in PMA-induced THP-1 cells; this observation indicated the activation of p65 is a common downstream of TLR4/MyD88 and P2X7R-signaling pathways. Together, curcumin-downregulated P2X7R expression and TLR4/MyD88/NF-κB signaling regulated the NLRP3 expression, caspase-1 activation, and IL-1β secretion in PMA-induced macrophages.

Indeed previous reports suggest that curcumin can inhibit the NLRP3 inflammasome activation and subsequent release of mature IL-1β both in J774A.1 cells, as well as murine peritoneal macrophages in *in vivo* experiments (Gong et al., 2015). One important difference between our study and that of Gong et al. (2015) is the focus on treatment of cell models. Gong et al. (2015) measured NLRP3 inflammasome production, which was subsequently stimulated with lipopolysaccharide combined with multiple NLRP3 inflammasome activators in mouse macrophages. Additionally, we detected NLRP3 inflammasome production in THP-1 cells stimulated with PMA, which imitated the process of monocyte differentiation to macrophages, which is important for atherosclerosis development. Another important difference is the signaling pathway; they showed that inhibition of NLRP3 inflammasome activation by curcumin involves the downregulation of ERK signaling. We observed that inhibition of NLRP3 inflammasome activation by curcumin involves the downregulation of TLR4/MyD88/NF-κB- and P2X7R-signaling pathways. Altogether, the results of Gong et al. and the present study strongly implicated curcumin as a potent antagonist of NLRP3 inflammasome activation in different pathophysiological processes.

Given the effect of curcumin on the expression of NLRP3 inflammasome and regulation of TLR4/MyD88/NF-κB and P2X7R, we concluded that curcumin reduced NLRP3 inflammasome, cleaved caspase-1 induction, and consequently reduced IL-1β secretion through TLR4/MyD88/NF-κB and P2X7R pathways in PMA-induced macrophages; the schematic model is shown in **Figure 7**. This study suggested that curcumin might act as an effective candidate for inflammation in atherosclerosis and have advantages for potential clinical applications.

AUTHOR CONTRIBUTIONS

FK designed and performed the experiments, analyzed the data, and drafted the manuscript. BY and JC assisted in the experiments. XC, LL, and SH assisted in data analysis. WH revised the paper. ZH designed the study, drafted and revised the manuscript. All authors read and approved the final manuscript.

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