REGULATION OF INFLAMMATION, ITS RESOLUTION AND THERAPEUTIC TARGETING

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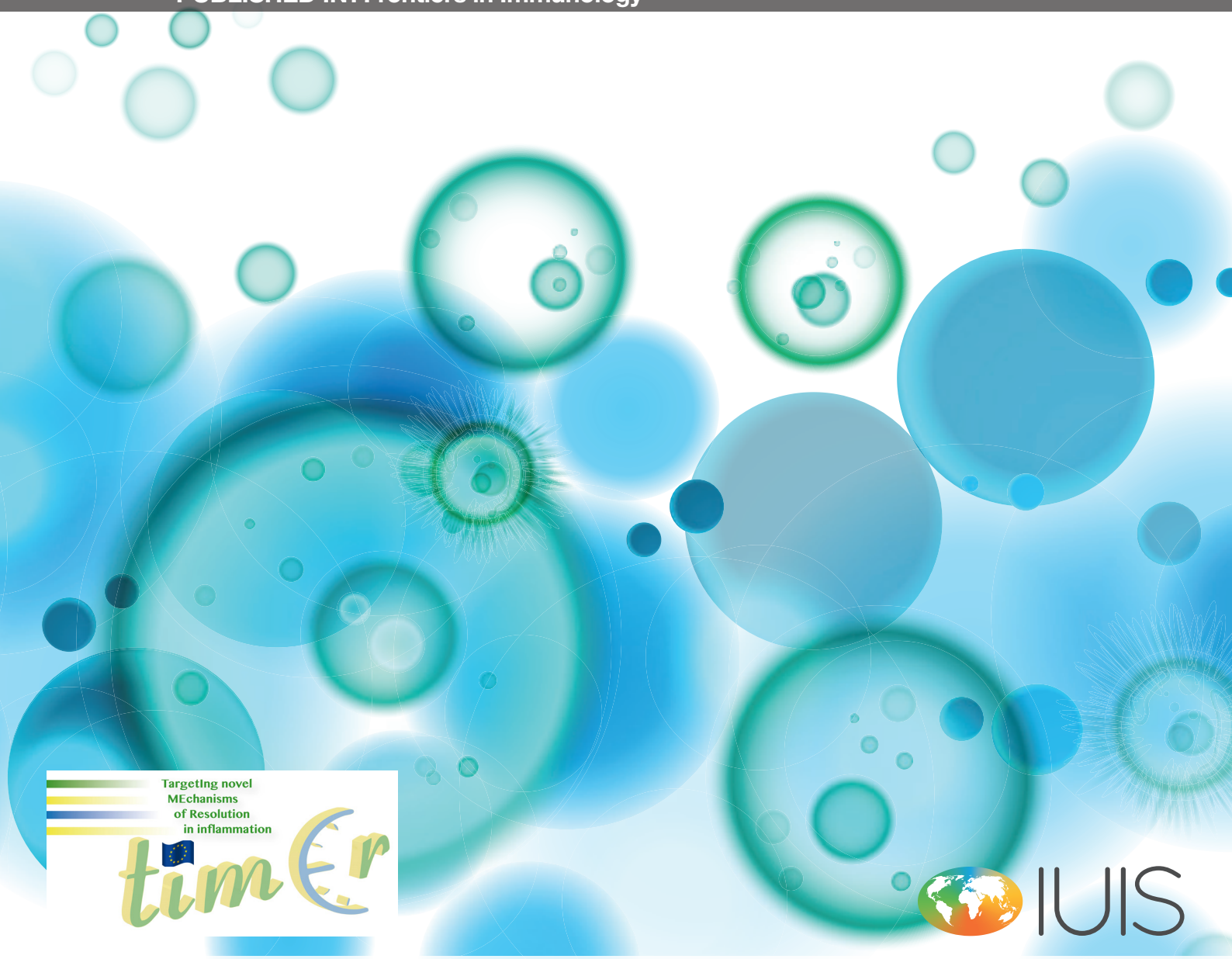

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REGULATION OF INFLAMMATION, ITS RESOLUTION AND THERAPEUTIC TARGETING

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Inflammation is a fundamental protective mechanism and at the same time the driving force of a variety of major diseases in humans. Indeed, acute self-resolving inflammation usually plays a positive role for the host, as exemplified by infectious diseases where its positive role is well established and testified by its perception as innate immunity. On the other hand, non-resolving inflammation and consequent chronicization is a key determinant of immunopathology and clinical manifestations of most major diseases in humans. As a consequence, it is increasing appreciated that the problem with inflammation is not how often it starts, but how often it fails to resolve. Appropriate resolution of inflammatory responses, which also drives activation of tissue damage repair mechanisms and return of local tissues to homeostasis, is a necessary process for ongoing health. Interestingly, cells sustaining these processes are also key to the proinflammatory responses, and the underlying "pro-resolving" molecular pathways are triggered as part of the pro-inflammatory response. This clearly indicates resolution of inflammation as an active process requiring functional repolarization of inflammatory cells that calls our attention on the underlying molecular mechanisms.

The increasing number of anti-inflammatory drugs best-sellers in the pharma market is a clear indication of the relevance of having inflammation under check; nonetheless, there is still a great need for better acting pharmacological tools for the control of inflammation. Indeed, the remarkable success of biological drugs targeting proinflammatory cytokines has indicates that tools able to block proinflammatory mediators have promising applications, but at the same time has made clear that there are intrinsic limitations to this approach which frequently vanish undermine the activity of single targeting drugs, including the well-known redundancy of inflammatory mediators. Under self-limiting conditions inflammation spontaneously resolves in an active process. Some cellular and molecular mechanisms involved in inflammation resolution have been uncovered in the recent past, and include generation of specific cytokines, apoptosis of inflammatory leukocytes, lipid mediators, macrophage repolarization and others are likely to be revealed in the next future, since loss-of-function mutations of an increasing

number of genes results in the development of spontaneous inflammation in experimental animals. We argue that "pushing for" inflammation resolution by exploiting active naturally-occurring pro-resolving processes may have significant advantages over the attempt to simply "push back" inflammation by passive blockade of proinflammatory mediators.

At present the research in the field of inflammation aims at identifying and validates new molecules involved in the resolution of inflammation as a basis for the development of innovative therapeutic strategies in chronic inflammatory and autoimmune diseases. This involves the discovery of new natural or synthetic "pro-resolving" molecules from plant and animals and the investigation of endogenous inflammation "pro-resolving" mechanisms, including atypical chemokine receptors, decoy receptors, and microRNA. An extensive effort is focused on the regulation by "pro-resolving" agents on two molecular systems of key relevance in inflammation: the chemokine system, which regulates recruitment, permanence and egress of leukocyte in tissues; and the Toll Like Receptor (TLR)/IL-1R system, which is central for the activation of infiltrating leukocytes.

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EDITORIAL
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Editorial: Regulation of Inflammation, Its Resolution and Therapeutic Targeting

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Editorial on the Research Topic

Regulation of Inflammation, Its Resolution and Therapeutic Targeting

Inflammation underlies the pathogenesis of diverse human diseases ranging from infection, immune-mediated disorders to cardiovascular pathology, neurodegeneration, and cancer. Progress in the field of immunity and inflammation has led to a change in paradigm concerning resolution. Resolution has emerged as an integrated actively orchestrated process with multiple players. These include metabolites of the arachidonic acid cascade, anti-inflammatory cytokines, decoy, and scavenger receptors. Inflammatory cells undergo genetic reprograming during resolution with for instance orientation of macrophages to a pro-resolving mode. Failure of resolution has emerged as a fundamental mechanism of disease. Smoldering non-resolving inflammation is, for instance, an essential constituent of the tumor microenvironment. Under self-limiting conditions, inflammation spontaneously resolves in an active process. Some cellular and molecular mechanisms involved in inflammation resolution have been uncovered in the recent past and include generation of specific cytokines, apoptosis of inflammatory leukocytes, lipid mediators, macrophage repolarization, and others are likely to be revealed in the next future, since loss-of-function mutations of an increasing number of genes results in the development of spontaneous inflammation in experimental animals. "Pushing for" resolution of inflammation by exploiting active naturally occurring pro-resolving processes may have significant advantages over the attempt to simply "push back" inflammation by passive blockade of pro-inflammatory mediators.

This Topic of Frontiers offers the reader views on key aspects of the regulation of resolution of inflammation. Its foundations lay in the European Union supported project TIMER (Targeting novel mechanisms of resolution in inflammation)[1] and the effort of the International Union of Immunological Societies (IUIS)[2] that have fostered the collaboration of several groups in Europe and Brazil, supporting the preclinical work on molecules and mechanisms involved in resolution of inflammation as a basis for the development of innovative therapeutic strategies in chronic inflammation and autoimmune disease. These studies have been laid the foundations in the clinical trials that have been initiated.

The topic is placed in the context set by a review from Sugimoto et al. that focuses on the events required for an effective transition from the pro-inflammatory phase to the onset and establishment of

[1] http://www.eumbrella.org/.
[2] http://www.iuisonline.org/.

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resolution, suggesting that the mediators promoting inflammation can simultaneously propel the program for an active resolution. A set of articles then focus on specific molecular players involved in this process. Molgora et al. summarize evidence indicating IL-1R8 as a key anti-inflammatory molecule, which needs further investigation in human pathology as its targeting holds promise of innovative therapies in several inflammatory conditions. Alves-Filho and Palsson-McDermott review expression and enzymatic activities of PKM2 that can be regulated at multiple levels, including transcription, posttranslational modifications, and allosteric regulation of conformational stability. PKM2 represents a novel potential target for the development of anti-inflammatory drugs, as recent studies have unraveled a notable involvement of PKM2 in controlling the transcriptional activity of HIF-1α and STAT3 pathways during inflammation (Alves-Filho and Palsson-McDermott). Still on STAT3, Muhl reviews the preclinical data suggesting that providing recombinant STAT3-activating cytokines directly targeting hepatocytes, especially IL-11 and IL-22, may evolve as additional novel pro-regenerative therapeutic option in hard-to-treat patients where standard therapy with *N*-acetylcysteine alone falls short. Notably, the benefit of focused short-term application of IL-11 or IL-22 in acute disorders, such as APAP-induced ALI, should likely outweigh the inherent danger of these cytokines to promote in the long run tumor growth, which has been detected for IL-22 and hepatocellular carcinoma patients (Muhl). Proudfoot and Uguccioni discuss how synergy between chemokines or DAMP molecules, together with the low-affinity interaction with GAGs can tune the response of leukocytes to chemokines, controlling leukocyte extravasation into damaged or inflamed tissues. Dampening inflammation targeting the chemokine system can be achieved either targeting chemokines or their receptors. Blood-sucking parasites inhibit the recruitment of immune cells by producing a class of chemokine-binding proteins known as Evasins, whose advantages and disadvantages for potential development for therapeutic use is here discussed by Bonvin et al. On the receptor side, allosteric antagonists of chemokine receptors, discussed by Allegretti et al., might provide both functional selectivity and probe/concentration dependence. Vertebrates have adopted a number of mechanisms for removing chemokines from inflamed sites to help precipitate resolution. Over the past 15 years, it has become apparent that essential players in this process are the members of the atypical chemokine receptor (ACKR) family. Broadly speaking, this family is expressed on stromal cell types and scavenges chemokines to either limit their spatial availability or to remove them from *in vivo* sites. Here, Bonecchi and Graham provide a brief review of these ACKRs and discuss their involvement in the resolution of inflammatory responses and the therapeutic implications of our current knowledge. Resolution of inflammation also requires a functional switch in inflammatory cells biology. Neutrophils are classically considered to be essential players in the host defense against invading pathogens. However, several investigations have shown that impairment of neutrophil migration to the site of infection, also referred to as neutrophil paralysis, occurs during severe sepsis, resulting in an inability of the host to contain and eliminate the infection. On the other hand, the neutrophil antibacterial arsenal contributes to tissue damage and the development of organ dysfunction during sepsis. Sônego et al. provide an overview of the main events in which neutrophils play a beneficial or deleterious role in the outcome of sepsis. Finally, Ferreira et al. provide a comprehensive comparison of the anti-inflammatory effectiveness of two PEGylated TLR7 partial agonists, concerning distinct lung pathological conditions and several routes of administration. The results suggest that the putative clinical application of TMX-302 in lung disorders should be examined with caution because of its direct pro-inflammatory effects. Moreover, in this context, TMX-306 seems to be comparatively more effective and safer than TMX-302, deserving further investigations in drug development particularly for silicosis (Ferreira et al.).

The increasing number of anti-inflammatory drugs best sellers in the pharma market is a clear indication of the relevance of having inflammation under check; nonetheless, there is still a great need for better acting pharmacological tools for the control of inflammation. Indeed, the remarkable success of biological drugs targeting pro-inflammatory cytokines has indicated that tools able to block pro-inflammatory mediators have promising applications. However, there are intrinsic limitations of single targeting drugs because effects frequently vanish, likely due to the well-known redundancy of inflammatory mediators. TIMER has investigated a number of players with potential of developing as innovative targets in this setting and "will not end with the end of dedicated European funding in December 2015, as the legacy of this EU-funded project endures in the preclinical work and in the collaboration that has been fostered," as his coordinator concluded in the TIMER final meeting.

AUTHOR CONTRIBUTIONS

All authors contributed to this editorial.

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REVIEW
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Resolution of Inflammation: What Controls Its Onset?

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An effective resolution program may be able to prevent the progression from non-resolving acute inflammation to persistent chronic inflammation. It has now become evident that coordinated resolution programs initiate shortly after inflammatory responses begin. In this context, several mechanisms provide the fine-tuning of inflammation and create a favorable environment for the resolution phase to take place and for homeostasis to return. In this review, we focus on the events required for an effective transition from the proinflammatory phase to the onset and establishment of resolution. We suggest that several mediators that promote the inflammatory phase of inflammation can simultaneously initiate a program for active resolution. Indeed, several events enact a decrease in the local chemokine concentration, a reduction which is essential to inhibit further infiltration of neutrophils into the tissue. Interestingly, although neutrophils are cells that characteristically participate in the active phase of inflammation, they also contribute to the onset of resolution. Further understanding of the molecular mechanisms that initiate resolution may be instrumental to develop pro-resolution strategies to treat complex chronic inflammatory diseases, in humans. The efforts to develop strategies based on resolution of inflammation have shaped a new area of pharmacology referred to as "resolution pharmacology."

Keywords: resolution, chemokine depletion, eicosanoids, pro-resolving mediators, tissue homeostasis

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INTRODUCTION

Inflammation is a reaction of the host to infectious or sterile tissue damage and has the physiological purpose of restoring tissue homeostasis (1). However, uncontrolled or unresolved inflammation can lead to tissue damage, giving rise to a plethora of chronic inflammatory diseases, including metabolic syndromes and autoimmunity pathologies with eventual loss of organ function (2). In fact, signs of persistent unresolved inflammation are not only typical of classical inflammatory diseases but also an underlying feature of a variety of human conditions not previously thought to have an inflammatory component (3), including Alzheimer's disease (4), atherosclerosis (5), cardiovascular disease (6), and cancer (7). This justifies the increasing interest in studying inflammatory processes. In this context, an important milestone has been reached with the awareness that engagement of resolution of acute inflammation is crucial to avoid persistent chronic inflammation and ensure proper return to homeostasis (8).

Historically, the first acknowledged report on resolution of inflammation was published in 1907 (9). This report shows that, in experimental irritant-induced pleurisy, a fluid containing fibrin and leukocytes was formed, disappearing after 5 days, with the clearance of "polynuclear leukocytes" and the persistence of mononuclear cells in the pleural cavity (9). For many years, resolution of inflammation was considered a passive phenomenon, merely associated with the removal of inflammatory stimuli, end of chemoattractant production, dilution of chemokine gradients over time, and prevention of further leukocyte recruitment. Some years later, the existence of endogenous inhibitors of leukocyte trafficking was reported, acting as a counteractive mechanism against promoters of cell recruitment, such as chemoattractants and adhesion molecules [reviewed in Ref. (10)]. Since then, several studies, especially those from Serhan's lab at Harvard, showed that the resolution of inflammation is an active process brought about by the biosynthesis of active mediators, which act on key events of inflammation to promote the return to homeostasis (11–14). In this context, homeostasis is recovered after the production of pro-resolving mediators that act on specific receptor targets to (i) shutdown polymorphonuclear leukocyte recruitment, (ii) counteract signaling pathways associated with leukocyte survival to promote apoptosis (or programmed cell death), and (iii) activate the clearance of apoptotic cells (especially by macrophages through a non-phlogistic process), yielding (iv) macrophage reprogramming from a proinflammatory to a pro-resolving phenotype (15, 16).

Inadequate or insufficient resolution can lead to chronic inflammation, excessive tissue damage, and dysregulation of tissue healing, leading to fibrosis. Additionally, it has been implicated in multiple disease states, including the development of autoimmunity (2, 8, 17). Thus, understanding the mechanisms required for the resolution of inflammation may not only unveil new mechanisms of pathogenesis but also support the development of drugs that are able to manage inflammatory processes in directed and controlled ways. Resolution of inflammation requires pro-resolving molecular pathways that are triggered as part of the host response, during the inflammatory phase. This concept challenges a linear model of induction and resolution of inflammation, suggesting a more complex balance between proinflammatory and anti-inflammatory events that are initiated, at least partly, in parallel (18). The inflammatory cells involved in the active phase of inflammation undergo a functional repolarization and contribute to the onset of resolution. Additionally, an accumulating body of evidence suggests that many proinflammatory mediators that promote the inflammatory phase can simultaneously initiate a program for active resolution. For this reason, it is important to understand that adequate resolution of inflammation follows on a coordinated and florid proinflammatory phase with marked leukocyte accumulation. In this context, Sehran, who uncovered the most important pro-resolving lipid mediators, and Savill elegantly stated that "the beginning programs the end" meaning that the events occurring early in acute inflammation engage an active and coordinated "resolution program" (18). In this review, we reason on the events required for an effective transition from the proinflammatory phase to the onset and establishment of resolution (**Figure 1**).

CELLULAR EVENTS IN THE RESOLUTION OF ACUTE INFLAMMATION

The molecular and cellular events of the inflammatory response are well known and typically characterized by increased blood flow, capillary dilatation, leukocyte infiltration, and production of chemical mediators. Acute inflammation is mainly characterized by the presence of neutrophils, which are highly motile leukocytes, able to rapidly migrate to the site of injury or infection. Although neutrophils are essential for proper elimination of the inflammogen, exaggerated influx of leukocytes can be more deleterious than the infection or injury itself and has been considered a bad marker of tissue homeostasis (19). Therefore, the key histological feature in the resolution of acute inflammation is the depletion of neutrophils from the local inflamed sites. This is achieved through programmed processes that occur in an overlapping fashion and are actively regulated at multiple levels (20, 21). The *cardinal signs of resolution* entail the limitation or cessation of blood-borne cell extravasation, the counter regulation of chemokines and cytokines, the switching off of signaling pathways associated with leukocyte survival, the induction of leukocyte apoptosis and their subsequent removal through efferocytosis by macrophages, the reprogramming of macrophages from classically activated to alternatively activated cells, the return of non-apoptotic cells to the vasculature or lymph, and finally the initiation of healing processes. Altogether, these events avoid excessive tissue damage and culminate in the return to tissue homeostasis, giving little opportunity for the development of chronic, non-resolving inflammation. On the other hand, failure of one or more steps in the resolution of inflammation may be involved in the pathogenesis of several human chronic inflammatory diseases (8).

PRO-RESOLVING MEDIATORS

Similar to the onset phase of inflammation, resolution of inflammation is coordinated and regulated by a large panel of mediators. The pioneer authors in the field of resolution and other investigators worldwide have focused on defining the endogenous mediators of resolution and the mechanisms through which the body regulates effector cells (PMNs, monocytes, and macrophages). It is worth noting that anti-inflammatory effects and pro-resolving effects are not totally overlapping: anti-inflammation mainly refers to an inhibitory/blocking action (e.g., stopping immune cell extravasation, which is a hallmark of acute inflammation), whereas pro-resolving actions indicate an inherent stimulation and activation of specific processes, such as apoptosis or efferocytosis. In both cases, the end point is the inhibition of inflammation, but pro-resolving mediators are those that genuinely enable resolution to take place (12, 22, 23). In the same vein, there is a mechanistic difference between an anti-inflammatory drug that blocks some specific pathways and a pro-resolving drug that is expected to activate a plethora of actions. Hence, the distinction is between blocking/inhibiting particular mediators, which can cause tissue damage, and agonism/activating cellular processes that participate in limiting or preventing damage, the latter enabling an amplifying effect. It is reasoned that pro-resolving-based

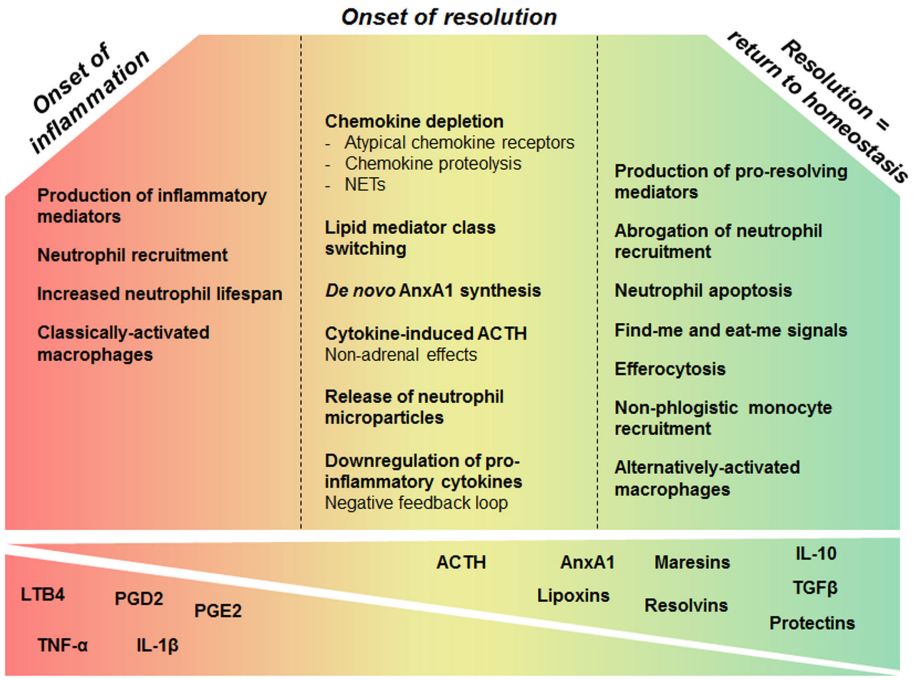

FIGURE 1 | Overview of cellular and molecular processes that govern inflammation and its resolution. During early phase of inflammation, production of inflammatory mediators promotes leukocyte accumulation and survival in the inflammatory site. While the inflammatory response evolves, several mechanisms enable the fine-tuning of these phenomena creating a favorable environment for the resolution phase leading to return to tissue homeostasis. Chemokine proteolysis, sequestration by atypical receptors, and degradation by neutrophil extracellular traps (NETs) are important mechanisms to shape chemokine gradients restricting the influx of neutrophils, once sufficient numbers of cells have been recruited. In addition, inflammatory mediators may induce a negative-feedback loop downregulating the production of inflammatory cytokines. Prostaglandins generated in the active phase of inflammation are involved in the switch from proinflammatory lipid production to the synthesis of lipoxins and other pro-resolving lipids, within inflammatory exudates. Mediators released early in inflammation, like ACTH, can also enable the induction of the pro-resolving phase. Upon activation, neutrophils release microparticles containing pro-resolution mediators that control further granulocyte ingress and turn on a resolution and tissue reparative programs. AnxA1 is a major component of the pro-resolving properties of neutrophil-derived microvesicles. Many resolution mediators downregulate survival pathways and activate apoptosis of granulocytes. Apoptotic neutrophils release pro-resolving mediators that contribute to inhibition of continued neutrophil infiltration and to recruitment of monocytes in a non-phlogistic manner. Upon apoptosis, neutrophils also promote their own clearance by expressing find me and eat me signals that attract scavengers and allow the identification of the dying cell, respectively. In response to local mediators and upon efferocytosis, proinflammatory macrophages switch to resolution-phase macrophages. These events will reestablish tissue homeostasis.

therapies will promote both anti-inflammatory and pro-resolution actions, differing from traditional anti-inflammatory agents that solely inhibit key proinflammatory mediators (20). In addition, we have recently pointed out that pro-resolving molecules are characterized by "mild-to-moderate actions," since they balance pro- and anti-inflammatory responses to reach an equilibrium (22).

According to the first consensus report from leading authorities on definitions and mechanisms in resolution (3) and subsequent reviews (16, 21), pro-resolving mediators should ideally fulfill some fundamental criteria that include:

- Stop: the limitation or cessation of neutrophil tissue infiltration;
- Sink: the counter regulation of chemokines and cytokines;
- Kill: the induction of apoptosis in spent neutrophils and their subsequent efferocytosis by macrophages;
- Skew: the reprogramming of macrophages from classically activated to alternatively activated cells;
- Leave: the return of non-apoptotic cells to the blood or lymphatic vasculature and egress of immune cells – following efferocytosis, the macrophages and dendritic cells leave the site of inflammation;
- Inform: the instruction of suppressive immune cells and adaptive immune response to help dealing with subsequent encounters;
- Heal: the induction of tissue repair – return to homeostasis without fibrosis or scar formation marks the final step of resolution.

Molecules that fulfill the criteria above, which qualify a pro-resolving mediator, are very diverse in nature (21) and include specialized lipid mediators [lipoxins (e.g., LXA_4), resolvins (e.g., RvD1), protectins, and maresins] (14), proteins and peptides [e.g., annexin A1 (AnxA1), adrenocorticotropic hormone, chemerin peptides, and galectin-1] (24), gaseous mediators (e.g., H_2S and CO) (25), a purine (adenosine) (26–28), as well as neuromodulators (acetylcholine and other neuropeptides) released under the control of the vagus nerve (29, 30).

Failure to produce adequate amounts of these anti-inflammatory and pro-resolving mediators or yet a failure to bind to their receptor could lead to the persistence of inflammation, playing a significant etiopathogenic role in chronic inflammatory and autoimmune diseases. This is highly plausible for inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC), chronic relapsing inflammatory conditions of the gastrointestinal tract that are characterized by intestinal inflammation and epithelial injury (31, 32). Resolution mediators (e.g., AnxA1, lipoxins, and resolvins) regulate intestinal mucosal injury, inflammation, and repair, supporting the resolution of inflammation in the gut. Therefore, defective expression of pro-resolution mediators may contribute to the chronic inflammatory response associated with IBD. Notably, colonic mucosa from UC patients demonstrates defective LXA_4 biosynthesis, which may contribute to the inability of these patients to resolve persistent colonic inflammation (33). Complete loss of AnxA1 protein was detected in colonic tissues from chronic CD patients, which correlated with the clinical status, response to therapy, TNF-α expression, and lymphocyte activation (34). Vong and coworkers (35) documented an increase in mucosal synthesis of AnxA1 and LXA_4, in individuals in medically induced remission from UC. Besides, during anti-TNF-α therapy, AnxA1 expression was upregulated in patients with a successful intervention, whereas non-responsive patients did not show the same expression profile (34). The contribution of AnxA1 to the remission of IBD was validated with a model of dextran sulfate sodium (DSS)-induced colitis in TNFR knockout (KO) mice, mimicking the anti-TNF-α therapy. Mucosal levels of AnxA1 increased in the absence of TNF-α signaling, allowing early recovery of colitis as compared to wild-type (WT) mice (36). According to these findings, changes in pro-resolving mediator levels may predict therapeutic efficacy. Moreover, inflammation-resolution agonists prevent immune-mediated tissue damage and restore tissue homeostasis. Interestingly, pharmacological treatment with LXA_4 or Resolvin E1 (RvE1) effectively promoted the resolution of trinitrobenzenesulphonate (TNBS)-induced colitis (37, 38). The beneficial effect of lipid mediators in colitis was accompanied by decreased leukocyte infiltration and proinflammatory cytokines. In addition, TNBS-specific IgG serum levels decreased after treatment with RvE1, suggesting diminished antigen presentation and antibody production (38). Moreover, AnxA1 peptides encapsulated in nanoparticles accelerated the recovery of experimentally induced colitis and the healing of colonic biopsy-induced wounds (39).

Persistent airway inflammation in lung diseases, including asthma, may also be due to a defect in counter regulatory signaling (40, 41). Clinical findings suggest that severe asthma is associated with diminished expression of LXA_4, its receptor FPR2, and 15-lipoxygenase, the major enzyme involved in LXs generation (42–46). Thus, LXA_4-deficient production and/or signaling might have a role in the progression of the disease. In a recent study, AnxA1 and LXA_4 plasma levels were lower in wheezy infants than in control group (47). Once persistent wheezing in children may progress to asthma, this reduced level of pro-resolving molecules could be an early event in asthma progression (48).

In some cases, failure in the activity of specific mediators may contribute to the inflammatory process even when the expression is normal or higher, when compared to healthy controls. For example, CD-related inflammation is characterized by reduced activity of the immunosuppressive cytokine transforming growth factor (TGF)-β1. TGF-β is a crucial cytokine in inflammation resolution due to its immunoregulatory activities, essential to tolerance and homeostasis, and its role in epithelial restitution and fibrosis (49). Indeed, *in vitro* and *in vivo* studies have demonstrated that TGF-β1 acts as a potent negative regulator of mucosal inflammation (50). Although TGF-β is found in high levels in human IBD tissue, it has reduced activity due to the overexpression of an inhibitor of TGF-β1 signaling, SMAD7 (51). As a result, TGF-β is unable to reduce the chronic production of proinflammatory cytokines that drives the inflammatory process in IBD and, consequently, inflammation is maintained (51). Notably, therapeutic strategies that restore TGF-β signaling pathway may downregulate the inflammatory response and induce remission in patients with CD (51, 52).

POSITIVE NETWORKS IN RESOLUTION

Evidence is accumulating that a *pro-resolving cascade* becomes operative during resolution, whereby one pro-resolving mediator would induce another one. We reported one of the first evidence that fundamental pro-resolving mediators, such as AnxA1 and LXA_4, induce the production of further anti-inflammatory molecules *in vivo*, such as IL-10 (53). Later, Brancaleone and colleagues (54) provided strong evidence that the engagement of FPR2/ALX by LXA_4 induces AnxA1 phosphorylation and mobilization in human PMN. Similarly, the pro-resolving mediator RvE1 stimulates endogenous LXA_4 production (55).

Other examples and *modus operandi* of this cross talk in resolution are emerging, as the cross talk between AnxA1 and glucocorticoid (GC)-induced leucine zipper (GILZ) during certain inflammatory events (56). GILZ mediate and mimic several anti-inflammatory actions of GCs (57). Besides demonstrating that GILZ expression depends on AnxA1, we identified that the lack of endogenous GILZ during the resolution of inflammation is compensated by AnxA1 overexpression. In the model of lipopolysaccharide (LPS)-induced pleurisy, GILZ deficiency was associated with an early increase of AnxA1 and equal neutrophil influx and resolution as compared to WT mice. Likewise, we demonstrated that dexamethasone-induced resolution was not altered in GILZ KO mice due to compensatory expression and action of AnxA1 (56). These studies indicate that pro-resolution mediators not only communicate in positive loops but also enact compensatory actions to guarantee the effective engagement of resolution pathways.

We predict that a further definition of the positive loops of resolution is crucial for the discovery of new pharmacological targets that could resolve inflammation, especially in the context of chronic inflammatory diseases. A better understanding of the key controlling points of resolution networks may allow us to design specific strategies to promote resolution.

HOW DOES RESOLUTION START?

Briefly, the acute inflammatory response can be divided in two stages: initiation (productive and transition phases) and resolution (**Figure 1**) (58). Interestingly, molecular and cellular mechanisms involved in the first phase of inflammation contribute to the initiation of the pro-resolving response. It has now become evident that coordinated programs of resolution initiate shortly after the beginning of the inflammatory response (18). In this context, several anti-inflammatory and pro-resolving mediators are endogenously produced to temper the inflammatory events. However, here we intend to highlight the existence of events and pathways that do not fulfill all criteria to be classified as pro-resolving, but do contribute to the initiation of resolution. These mechanisms provide the fine-tuning of inflammation, creating a favorable environment for the resolution phase to take place, and for homeostasis to return. As "contributors of resolution" these events, pathways, and mediators deserve special attention since they may be key targets for the pharmacological input or enacting of resolution, especially when it has not turned on, such as in chronic inflammatory settings.

Aside its well-known proinflammatory functions, nuclear factor kappa B (NF-κB) also has a crucial role in the initiation of resolution of inflammation. NF-κB proteins are a family of transcription factors of central importance in inflammation and immunity (59, 60). NF-κB and its activating IκB kinase (IKK)β play important roles in driving the inflammatory response by activating the expression of proinflammatory and anti-apoptotic genes (61). However, several reports have shown that NF-κB and IKKβ also influence anti-inflammatory response, pointing to their involvement in both onset and resolution of acute inflammation (62–64). The functional transcription factors consist in homo- or hetero-dimers comprising five subunits (p50, p52, p65, cRel, and RelB), which utilize Rel homology domain (RHD) for DNA binding and dimerization (65). Dimers containing at least one subunit with transactivating domains (TAD) in their C-terminus (p65, RelB, or cRel) are required to induce gene transcription. In contrast, dimers that contain only subunits without TAD (p50 and p52) are transcriptionally inactive and may prevent transcriptionally active NF-κB dimers from binding to κB sites (66). In resting cells, NF-κB dimers are sequestered to the cytoplasm and maintained inactivated by reversible association with its inhibitor IκB or unprocessed forms of cytoplasmic p50/p105 (NF-κB1) and p52/p100 (NF-κB2) (60, 65, 67). NF-κB activation in response to proinflammatory stimuli is regulated by IKK, which phosphorylates IκB and promotes its proteasome degradation and the release of NF-κB for nuclear translocation and gene transcription activation (61).

Nuclear factor kappa B activates many promoters containing highly divergent κB-site sequences. The fact that the regulation of gene expression is dimer-specific explains, in part, how NF-κB pathways can modulate both inflammation and resolution (65, 68). Differential expression of NF-κB subunits and the differential effects of NF-κB dimers may be intimately associated with the temporal regulation of inflammatory responses (69). p65/p50 heterodimer is the predominant form of functionally active NF-κB with proinflammatory activity, since this dimer enhances the transcription of genes related to the proinflammatory phase. On the other hand, p50/cRel, p65/cRel, or p50/p50 seems to be involved in the transcription of genes related to the recovery phase (70). Accordingly, the genes regulated by p50/cRel and p65/cRel are activated in later points after inflammatory stimulation, providing the necessary period between the burst of the proinflammatory response and the recovery phase (69, 71). p50/p50 homodimer exerts important anti-inflammatory and pro-resolving effects and competes with p65/50 heterodimer for DNA binding (72, 73). Unlike p65/p50, p50/p50 lacks the transactivation domain and may repress proinflammatory genes (74–76). Bohuslav and colleagues demonstrated that increased expression of p50 subunit of NF-κB directly results in the downregulation of LPS-induced TNF production (72). Recently, the enhancement of efferocytosis mediated by RvD1 was associated with p50/p50-mediated suppression of TNF-α expression (77). In this context, RvD1 modulates at least two different NF-κB pathways leading to enhanced localization of p50 in the nucleus, while it suppresses dissociation from IκBα and concurrent nuclear translocation of p65 (77). Moreover, upon LPS stimulation, macrophages express p65/p50 heterodimer in predominance over p50/50 homodimer, thereby provoking the proinflammatory state. However, in later time points, these macrophages show p105 degradation, nuclear translocation of p50, and formation of p50/p50 homodimer, presumably as an adaptive cellular response to proinflammatory insult.

During the proinflammatory phase, besides inducing proinflammatory genes, p65/p50 also induces the transcription of genes that will provide the control of the recovery phase, such as Rel, the gene that codifies cRel (71). For example, Muxel and colleagues showed that the expression of p65/cRel, crucial for inflammation resolution, is induced by p65/p50, which is earlier expressed in LPS-stimulated macrophages (69). The authors identified that temporal regulation of cRel promoted the synthesis of melatonin (*via* p65/cRel) by macrophages, a modulator of phagocyte function preventing over-activation of this cell type (78, 79). In addition, NF-κB negatively regulates NLRP3-inflammasome activation and IL-1β production (63). In macrophages, NF-κB prevents premature and excessive NLRP3-inflammasome activation, acting as a negative regulator of IL-1β secretion (63). Although the precise molecular mechanism underlying NF-κB-mediated inhibition of NLRP3-inflammasome activation remains unclear, NF-κB has been suggested to promote autophagy (80), a cellular process that negatively regulates NLRP3 inflammasome activity (81–83). Reinforcing this observation, a recent study revealed that NF-κB restricts inflammasome activation in macrophages *via* elimination of damaged mitochondria (84). This allows NF-κB to restrain its own inflammation-promoting activity in macrophages (84).

Clearly, NF-κB may have dual function in inflammation, which is likely the result of the central role of this molecule in the convergence of several inflammatory signals (62). This results in divergent effects of NF-κB pharmacological inhibition in inflammatory models. On the one hand, NF-κB inhibitors may attenuate inflammation and promote resolution in different experimental models of inflammation (62). For example, NF-κB inhibitors possess anti-inflammatory effects in models of LPS-induced

lung injury (85), traumatic brain injury (86), colitis (87), and pulmonary arterial hypertension (88). Our research group showed that inhibition of NF-κB promotes resolution in established murine models of neutrophilic and eosinophilic inflammation associated with enhanced apoptosis of inflammatory cells (89, 90). On the other hand, inhibition of NF-κB during the resolution of inflammation prolonged the inflammatory response and prevented apoptosis (62). In addition, IKKβ has also been shown to have an anti-inflammatory role, such as the suppression of M1 macrophage activation during infection through the inhibition of signal transducer and activator of transcription (STAT)1 pathway (91). In accordance with this observation, IKKβ ablation results in severe neutrophilia and inflammation mediated by IL-1β (92). Notably, mice lacking IKKβ had hyperproliferative granulocyte–macrophage progenitors and pregranulocytes and a prolonged lifespan of mature neutrophils that correlated with the induction of genes encoding pro-survival molecules (92). Of clinical relevance, enhanced inflammation and neutrophilia were observed in human subjects that were treated with IKKβ inhibitors.

Notably, proinflammatory and resolution phases of inflammation are under the control of both transcriptional and post-transcriptional mechanisms, which regulate the expression of proteins that initiate and resolve inflammation. Reviewing this topic in 2010, Anderson (93) pointed out that post-transcriptional controlling mechanisms link the initiation/productive phase to the resolution phase of inflammation. mRNA translation is a highly regulated process governed by post-transcriptional mechanisms. Transcription is the first step in the regulation of gene expression, but since mRNA can be long-lived, turning off its synthesis does not rapidly redirect or stop the progress of inflammation. On the other hand, the second step, i.e., post-transcriptional regulation, can rapidly suppress protein expression by promoting mRNA degradation or by inhibiting its translation (93). Post-transcriptional control mechanisms may rapidly limit the expression of potentially toxic inflammatory mediators and help protecting the host against the pathological overexpression of potentially injurious proteins. For instance, a number of cytokine mRNAs can be regulated at the level of mRNA stability (94). mRNA decay and translational repression of target transcripts are promoted by RNA-induced silencing complex (RISC) that is composed by argonaute proteins bound to small non-coding RNAs, microRNAs (miRNAs). Importantly, the mechanisms used to ensure limited production of the proteins involved in the inflammatory response are highly variable, and in some cases, interact with each other to define protein expression levels. It remains not fully understood whether post-transcriptional controlling mechanisms play a role in the resolution of inflammation, but exciting possibilities for pharmacological intervention against the overproduction of many inflammatory proteins are likely to emerge from this elucidation (95).

Importantly, miRNAs triggered by immune mediators have a central role in modulating NF-κB signaling pathways and might be involved in controlling the switch from a strong early-inflammatory response to the resolution phase of the inflammatory process, in a timely and orchestrated manner (96, 97). The endotoxin-responsive gene miR-146a was the first one to be discovered to suppress the activation of the NF-κB pathway (98). miR-146a has been described as a negative regulator of the canonical NF-κB inflammatory cascade by targeting IL-1 receptor-associated kinase (IRAK) 1 and TNF receptor-associated factor (TRAF) 6 (98, 99). Moreover, miR-146a targets RelB, which is mostly implicated in the non-canonical NF-κB pathway, and controls monocyte responses during inflammatory challenge (100, 101). Some studies indicate that miR-146a can regulate proinflammatory gene expression by controlling RelB-dependent reversible chromatin remodeling (102, 103). Notably, deletion of miR-146a gene results in the production of higher levels of inflammatory cytokines by macrophages (104). Remarkably, the expression of many miRNAs is induced in an NF-κB-dependent manner after inflammatory stimulus or pathogen infection, promoting the control of the strength and longevity of an inflammatory response (97, 98, 104–109). miR-146a was the first reported miRNA whose expression can be induced through the NF-κB-dependent pathway in response to various immune mediators, such as LPS, IL-1β, and TNF-α (98, 105, 110–113). Since then, many studies have further identified subsets of miRNAs related to the TLR-induced NF-κB-dependent pathway. Another example, miR-9 expression is directly induced by LPS *via* the TLR4-MyD88-NF-κB-dependent pathway in human monocytes and neutrophils. In turn, miR-9 operates a feedback control of the NF-κB-dependent responses by fine-tuning NF-κB1 expression. Bazzoni and colleagues suggest that miR-9 induction probably acts as a tuning mechanism to prevent negative regulation by p50 homodimers, as occurs in monocytes in systemic anti-inflammatory response syndrome (SIRS) (109).

Because miRNA-mediated post-transcriptional control is important to fine-tune the expression of genes involved in inflammation, dysregulation of expression levels of miRNAs can lead to chronic infections, autoimmunity, allergic inflammation, or immune deficiency. Recent studies have identified dysregulated miRNAs in tissue samples of IBD patients and have demonstrated similar differences in circulating miRNAs in the serum of these patients [reviewed in Ref. (114)]. In fact, dysregulated expression of tissue and blood miRNAs in IBD already numbers >100 (114) and may be involved in the reduced apoptosis of T-cells, which is an important mechanism in T-cell homeostasis, and cell activation (115).

Also important for resolution initiation, the pituitary hormone adrenocorticotrophin (ACTH) is released quite early during inflammation, in response to proinflammatory cytokines, including IL-1β (116). For a long time, ACTH has only been thought to modulate host response through the rapid generation of adrenal-derived GCs, which are *de novo* synthesized from cholesterol. However, recent works have revealed important immune-modulatory properties of ACTH, through the activation of specific receptors in the periphery (117), expressed on macrophages and other stromal cells such as chondrocytes [reviewed by Montero-Melendez (118)]. Molecules that activate these receptors on macrophages are able to promote resolution of inflammation with a downstream impact on experimental arthritis (119, 120).

Chemokine Depletion Decreases Infiltration of Neutrophils into Tissue

As discussed above, successful inflammation depends on the regulation of neutrophil recruitment, allowing the proper elimination of the inflammogen but avoiding the tissue damage induced by excessive neutrophil influx and toxic content release. According to Headland and Norling – who recently reviewed this subject (21) – restricting the influx of neutrophils, once sufficient number of cells has been recruited, is a process through which chemokine and cytokine gradients are reduced, proinflammatory lipid mediators are switched to pro-resolving mediators, and circulating neutrophils are no longer activated and recruited to the inflammatory site. Chemokines are low molecular weight cytokines that orchestrate the migration of target cells to the site of inflammation. Chemokine depletion through mechanisms, such as chemokine cleavage by proteolysis and chemokine sequestration, is necessary to achieve a resolving environment and to abrogate neutrophil influx (16). Chemokines directly induce cell migration through a set of conventional chemokine G protein-coupled receptors. However, chemokines are also recognized by a small subfamily of atypical chemokine receptors (ACKR), previously called decoys, interceptors, scavengers, or chemokine-binding proteins (121). The binding of chemokines to their respective atypical receptors does not promote leukocyte migration due to the inability of ACKR to initiate classic G protein-dependent signaling pathways. Instead, ACKR sequestrate chemokines from the environment, an important mechanism to shape chemokine gradients. Therefore, ACKR are now emerging as crucial regulatory components of chemokine networks in a wide range of physiologic and pathologic contexts (122).

Chemokine proteolysis is another important mechanism for chemokine depletion and consequently the decrease of neutrophil recruitment and activation. Matrix metalloproteinases (MMPs) are traditionally associated with extracellular matrix protein degradation in several physiological and pathological processes. However, it is now clear that MMPs mediate homeostasis of the extracellular environment by modulating the biological activity of many bioactive molecules involved in cell function (123, 124) and innate immunity (125), including chemokines (123, 126–130), TNF-α (124, 131), α-defensin (132), and mannose-binding lectin (133). In this context, Dean and colleagues (134) proposed that macrophages aid the regulation of acute inflammatory responses by precise proteolysis of chemokines through MMP-12. Macrophage-specific MMP-12 cleaves CXC chemokines in the ELR motif, which is fundamental for receptor binding, thus rendering the mediators unable to recruit neutrophils (134). In some cases, cleaved chemokines continue to bind to their corresponding receptors, but fail to induce downstream signaling and chemotaxis, thus acting as antagonists dampening inflammation (126, 127).

Pro- and Anti-Inflammatory Networks Help to Turn on the Resolution Program

A great number of evidence indicates that proinflammatory molecules can be involved in the initiation of the resolution program. In order to limit the undesirable consequences of an excessive inflammatory process, many mediators involved in the onset of the inflammatory response simultaneously trigger a program that actively resolves inflammation. In this context, our group has observed, in two complementary studies, the intricate balance and cross talk between pro- and anti-inflammatory cytokines during a systemic inflammatory response. In 2003, we described a network of TNF-α, IL-1β, and IL-10 during severe intestinal ischemia and reperfusion injury (135). Both, IL-1β and TNF-α triggered an anti-inflammatory cascade resulting in the production of IL-10. We identified that IL-1β plays a major role in driving endogenous IL-10 production and protecting against TNF-α-dependent systemic and local acute inflammatory response. IL-1β has been implicated in inflammatory events, such as the expression of adhesion molecules and neutrophil influx following reperfusion of ischemic tissues. However, some studies have failed to show a protective effect of IL-1β inhibition during ischemia/reperfusion (I/R) injury (136–138). In our investigations, we associated neutralizing strategies or selective receptor antagonism to prevent the actions of IL-1β with an overall enhancement of tissue injury, proinflammatory cytokine expression (TNF-α), and lethality (135). Members of the IL-1 family of cytokines (e.g., IL-1β, IL-18, and IL-36γ) display a dual role in regulating IBD, reinforcing the concept that proinflammatory cytokines may contribute to both proinflammatory responses and resolution of inflammation. These cytokines are upregulated in the inflamed mucosa during experimental colitis as well as in human IBD. Remarkably, they not only contribute to intestinal inflammation (139) but also to resolution of inflammation, as demonstrated by the increased susceptibility to DSS-induced colitis by mice lacking IL-1β, IL-18, and IL-36 receptors or components of their processing (140–144). In humans, polymorphisms leading to decreased Nlrp3 expression, and consequent hypoproduction of IL-1β, are associated with increased risk of developing CD (145).

Moreover, we and others have observed that TNF-α is central to the pathogenesis of reperfusion-associated injury and lethality (135, 146, 147). However, this proinflammatory cytokine also contributes to the production of IL-10 during intestinal ischemia and reperfusion (147). Furthermore, we reported that TNF-α modulates IL-1β production: first, inhibition of TNF-α was accompanied by enhanced reperfusion-induced production of IL-1β (147); second, administration of exogenous IL-10 was linked to decreased TNF-α concentration and enhanced IL-1β. Based on these results, we hypothesized that TNF-α could be inducing an intermediate molecule that controls IL-1β production (147). It is interesting to note that recent investigations have identified a central role for TNF-α in upregulating a pro-resolving master receptor that transduces the actions of AnxA1, LXA_4, and RvD1 (148).

Several studies have identified a mechanism feedback for IL-10 as a potent repressor of proinflammatory cytokine production by macrophages, acting therefore as a key anti-inflammatory mediator (149, 150). In murine bone marrow-derived macrophages (BMDM) activated by LPS, IL-10 attenuated proinflammatory cytokine production *via* reduction of mRNA stability. IL-10 initiates a STAT3-dependent increase of the expression of the RNA

destabilizing factor tristetraprolin (TTP) accompanied by the release from p38 MAPK-mediated inhibition. As a result, IL-10 diminishes mRNA and protein levels of TNF-α and IL-1β (151).

Resolution of Inflammation Is Accompanied by an Active Switch in the Mediators That Predominate in Exudates

In a classical acute inflammatory response, proinflammatory lipid mediators, such as the classical eicosanoids [prostaglandins (PGs) and leukotrienes (LTs)], are generated during the initial phase of the inflammatory response through enzymatic modification of arachidonic acid (AA) by cyclooxygenases (COX) and lipoxygenases (LO) (152). These proinflammatory molecules have important roles in initiating leukocyte trafficking and stimulating blood flow changes, increasing vasopermeability to yield edema formation, all leading to neutrophil influx to the site of inflammation (14). In addition, PGs and LTB_4 are involved in the initiating steps that permit leukocytes to leave postcapillary venules *via* diapedesis (153). Thereby, a switch in lipid mediators from proinflammatory PGs to lipoxins, which are anti-inflammatory/pro-resolving mediators, is crucial for the transition from inflammation to resolution (154). As Serhan pointed out in a scholar review (20), during inflammation, neutrophils undergo a phenotype switch to produce different profiles of lipid mediators depending on the cells and substrates present in the local environment. Neutrophils in the peripheral blood generate and release LTB_4 on activation, as one of their main bioactive products. During spontaneous resolution of acute inflammation, there is a switch in PMN-LO pathway products expression, from LTs to lipoxins and resolvins. Evidence indicates that first-phase proinflammatory eicosanoids "reprogram" the exudate PMN to produce pro-resolving lipid mediators and hence promote resolution. For instance, Levy and colleagues suggested that when circulating PMNs begin diapedesis, they are exposed to autacoid gradients (e.g., PGE_2) that initiate phenotypic changes *via* gene expression regulation (12). In this context, local PGE_2 and PGD_2 stimulate the processing of 15-LO mRNA in leukocytes to produce functional enzymes for the synthesis of lipoxin. AA is then converted to anti-inflammatory lipid mediators, such as LXs (e.g., lipoxin A_4 and lipoxin B_2), which harness dual anti-inflammatory and pro-resolving actions, *in vitro* and *in vivo* (20). Lipoxins are generated by transcellular biosynthesis, involving two or more cell types, since the required enzymes are differentially expressed in the cells. Thus, at the sites of injury or inflammation, LXs are generated *via* biosynthetic routes engaged during cell–cell interactions. Mobilization of LX biosynthetic circuit occurs, for example, when infiltrating PMNs (which express 5-LO) interact with tissue resident cells (which express 15-LO) in inflamed target organs. In an autocrine, paracrine, or juxtacrine manner, newly formed LXs can interact with specific receptors on leukocytes to regulate their function (12).

Cyclooxygenase-2 apparently has a dual role in the inflammatory process, initially contributing to the onset of inflammation and later helping to resolve the process. Gilroy and colleagues reported that COX-2 expression and PGE_2 levels transiently increased in the early stage of carrageenan-induced pleurisy in rats (155). Later in the response, COX-2 was induced again to even greater levels and generated anti-inflammatory PGs, such as PGD_2 and 15-deoxy-Delta(12,14)-PGJ_2 (15d-PGJ_2), but only low levels of proinflammatory PGE_2. Anti-inflammatory actions mediated by 15d-PGJ_2, a terminal product of COX-2 pathway, represent another negative feedback that explains how once-initiated immunologic and inflammatory responses are switched off and terminated. 15d-PGJ_2, a terminal product of COX-2 pathway, is abundantly produced in inflamed sites, suggesting its potential role in facilitating the resolution of inflammation (156). 15d-PGJ_2 exerts potent anti-inflammatory actions, in part by antagonizing the activities of NF-κB, STAT3, and activator protein 1 (AP1), while stimulating the anti-inflammatory nuclear factor E2-related factor 2 (Nrf2). Besides targeting the transcriptional machinery, 15d-PGJ_2 is a potent inhibitor of protein translation. Interestingly, 15d-PGJ_2-mediated translational repression triggers a stress response program that results in the assembly of stress granules containing untranslated mRNAs. Stress granules have an important role in reprogramming gene expression to allow stressed cells to survive to noxious stimuli (157, 158). Altogether, these mechanisms might combine to effectively dampen inflammation (93). Thus, 15d-PGJ_2, especially formed during the late phase of inflammation, might inhibit cytokine secretion and other events by antigen-presenting cells such as dendritic cells or macrophages. Production of the 15d-PGJ_2 is a consequence of a series of dehydration (oxidation) of PGD_2 (159). The latter is a major COX-2 product formed in various cells (e.g., mast cells) and tissues during inflammatory processes by the action of PGD_2 synthase, which catalyzes the isomeric conversion of PGH_2 to PGD_2. The pathogenic relevance of PGJ_2 is suggested by clinical findings of reduced levels of PGD_2 in some human diseases, such as the cerebrospinal fluid of patients suffering from multiple sclerosis and schizophrenia (160). Other evidence of clinical relevance comes from atherosclerosis, where PGE_2 is over-expressed in symptomatic plaques of patients who underwent carotid endoarterectomy, while in asymptomatic ones, the PGD_2 pathway prevails, known to be associated with NF-κB inactivation and MMP-9 suppression. These clinical findings suggest that PGE_2-dominated eicosanoid profile is associated with cerebral ischemic syndromes, possibly through MMP-induced plaque rupture (161).

Although therapeutic inhibition of COX-2 by non-steroidal anti-inflammatory drugs (NSAIDs) may have beneficial effects in the early phase of inflammation by preventing prostanoid production, it may also be "resolution-toxic," by disrupting the production of anti-inflammatory PGs and LXs (3, 155, 162, 163). Disturbance of physiologic lipid mediator class switching by COX-2 inhibitors has deleterious consequences in humans (164) as well as in murine peritonitis (163), arthritis (165), and lung acute injury (ALI) models (166). In the study from Fukunaga and colleagues, COX-2 inhibition resulted in an exacerbation of ALI with longer recovery times. Reinforcing the dual role of COX-2 during inflammation, inhibition of COX-2 activity by pharmacologic treatment or gene targeting decreased early PMN trafficking to the lung but paradoxically led to dramatic increases in inflammation at later time points, mainly due to the disruption of LXA_4 production (166). Furthermore,

COX-2 inhibition decreased macrophage phagocytosis of apoptotic PMNs *in vitro* and reduced prostaglandin E2 and LXA_4 expression (163). During peritonitis, treatment with specialized pro-resolving lipid mediators [aspirin (ASA)-triggered lipoxins, RvE1, and protectin D1] rescued the resolution deficit promoted by COX-2 inhibition (163).

Aspirin is unique among other NSAIDs because it irreversibly inhibits COX-2 by acetylation of an amino acid serine residue preventing prostanoid generation (167) yet enabling the biosynthesis of endogenous anti-inflammatory mediators. Therefore, the generation of ASA-triggered specialized lipid mediators (AT-SLM) (11, 168–170) may enhance resolution and counteract the loss in prostaglandin production by ASA (18). Low-dose ASA triggers the resolution phase by activating endogenous epimers of specialized pro-resolving lipid mediators in humans and several animal models (3). Low-dose ASA triggers 15-epi-LXA_4 in skin blisters in humans to reduce PMN infiltration by inducing anti-adhesive nitric oxide, thereby dampening leukocyte/endothelial cell interaction and subsequent extravascular leukocyte migration (171). In addition, low-dose ASA administration to mice triggered the formation of 15-epi-LXA_4, which in turn attenuated I/R-mediated vascular inflammation (172). In a randomized controlled study, low-dose ASA administration to volunteers augmented plasma ATL levels while inhibiting thromboxane (173). These observations support the idea that low-dose ASA may be considered "resolution friendly" (18), since it mimics endogenous biosynthetic mechanisms to trigger new mediators, leading to a favorable net change (173) for pro-resolution (174, 175).

Neutrophils: Important Cells to Turn on Resolution

Aborted neutrophil recruitment is one of the steps required to reconstitute tissue homeostasis, followed by apoptosis and clearance by macrophages. Interestingly, neutrophils have pivotal roles in attenuating inflammatory diseases and seem to orchestrate both elimination of microorganisms and resolution of inflammation (21). In view of that, wound healing is delayed in neutrophil depletion models, indicating a critical role of these cells in the resolution of inflammation (176). Moreover, depletion of neutrophils aggravates different types of experimental UC (177, 178) and extends joint inflammation in a murine model of gout (179). Among the anti-inflammatory functions of neutrophils, it is worth mentioning its capacity to disrupt chemokine gradients *via* several mechanisms. For instance, neutrophils release proteases that not only degrade extracellular matrices and cells surrounding the inflammatory milieu but also deactivate inflammatory cytokines (180). Additionally, neutrophils modulate the cytokine production stimulated by bacterial peptidoglycans and LPS (181). *In vitro* studies have shown that PMN lysates and neutrophil elastase can degrade recombinant human IL-1β and TNF-α but not IL-10, and alpha1-antitrypsin can inhibit this process (180). Neutrophil-derived proteases are also involved in the downregulation of IL-1β and TNF-α produced by mononuclear cells, an effect that is independent on ROS production or phagocytosis (180). Serine proteases released by activated neutrophils may also be associated with NETs, which are web-like structures composed of nuclear material in complex with neutrophil proteins that display exquisite antibacterial properties (182). A recent article by Schauer and colleagues revealed that at the very high neutrophil densities that occur at the site of inflammation, NETs build aggregates that trap and degrade proinflammatory mediators *via* the proteolytic action of inherent neutrophil serine proteases (179). However, it remains to be investigated if this anti-inflammatory effect can be reproduced in physiological conditions where concentrations of NET may be lower. Conversely, NETs are also related to proinflammatory effects that in part induce further neutrophil recruitment (183). Recent observations suggest that NETs are effective activators of the inflammasome machinery in both human and murine macrophages, resulting in the release of active IL-1β and IL-18 (184). Indeed, pharmacological and genetic strategies that prevent NETosis have been shown to be protective in murine models of lupus (185), cardiac infarction (186), deep vein thrombosis (187), atherosclerosis (183), and diabetes (188). In addition, a recent work suggests that damage-associated molecular patterns (DAMPs) released during liver I/R result in formation of NETs which subsequently exacerbate organ damage and initiate inflammatory responses (189). Moreover, the presence of DNAse-sensitive NETs in skin wounds impairs wound healing in diabetes (188). Timely degradation/removal of NETs is critical since its components may serve as autoantigen or DAMPs leading to inflammatory and chronic autoimmune diseases, including systemic lupus erythematosus (SLE) [reviewed in Ref. (189–194)]. Furthermore, mitochondrial ROS-dependent NETosis may promotes externalization of proinflammatory oxidized mtDNA and subsequent activation of type I interferon (IFN) synthesis, what may contribute to lupus-like disease (195). Finally, serum of SLE patients show an increase in various NET proteins [e.g., defensins, high-mobility group box protein 1 (HMGB1), and bactericidal proteins] compared to healthy-donor blood, indicating that NETosis may be implicated in the genesis and/or amplification of the disease (196, 197). Therefore, like uncleaned apoptotic and necrotic cell remnants, uncleaned NETs may contribute to inflammation and autoimmunity.

Another neutrophil-related mechanism that is worth mentioning here is the release of S100A8 and S100A9 proteins and their calprotectin heterocomplex, upon stimulation. These proteins have been shown to have dual biological functions on inflammation (198–201). Abundant in neutrophils, calprotectin is released at sites of infection where it exerts antimicrobial activity, which is attributed to its ability to chelate manganese and zinc (200–205). In addition, calprotectin activates the innate immune system through activation of the receptor of advanced glycation end products (RAGE) and TLR4, resulting in downstream NF-κB activation and secretion of proinflammatory cytokines, such as TNF-α and IL-17 (206–208). Diverging properties of calprotectin related to PMN recruitment and functions have been described. Calprotectin was shown to activate the recruitment of PMNs and stimulate their adhesion by activating MAC-1 β2 integrin (209). Moreover, the functional blockage of calprotectin reduced PMN recruitment stimulated by LPS *in vivo* (210). Conversely, studies have pointed to the ability of S100A8 and S100A9 to repel PMNs (fugetaxis) and inhibit their

chemotaxis toward chemokines *in vitro*. Additionally, calprotectin inhibited LPS-induced recruitment of PMNs in the rat air-pouch model of inflammation *in vivo* (211, 212). S100A9 differentially modified the responsiveness of neutrophils and dendritic cells to LPS, suggesting that the effects of calprotectin may be cell specific. While S100A9-deficient neutrophils exhibited a reduced secretion of cytokines (e.g., TNF-α and MCP-1) in response to LPS stimulation, inflammatory cytokine production in dendritic cells was exacerbated by S100A9 deficiency (213). Circulating concentrations of calprotectin increase with acute inflammation and during sepsis (214, 215), which has led some authors to suggest a proinflammatory role for this protein (216). Supporting this notion, Pepper and coworkers (217) showed that calprotectin plays a critical role during glomerulonephritis, amplifying autocrine and paracrine proinflammatory effects on BMDMs, renal endothelial cells, and mesangial cells. Indeed, calprotectin have an established clinical role as a biomarker in IBD (218).

In contradiction to these findings, anti-inflammatory, antinociceptive, and protective properties of calprotectin have also been described. In addition, regulation of S100A8 by GCs reinforces the idea of an anti-inflammatory role for this protein (219). For instance, calprotectin was suggested to be involved in the regulation of inflammatory processes in joints, since it produced marked anti-inflammatory and protective effects in models of adjuvant-induced arthritis in rats (220). Indeed, calprotectin deficiency was found in wound fluid from patients with non-healing venous leg ulcers, when compared with that from patients with healing open-granulating acute wounds (221). Sun and colleagues proposed protective and anti-inflammatory functions for calprotectin in sepsis. The authors showed that mice treated with S100A8 increased their survival rates and reduced tissue damage, inflammation, and oxidative injuries to major organ systems in a model of LPS-induced endotoxemia (222). Calprotectin was also shown to inhibit the oxidative metabolism of LPS-activated PMNs *in vitro*, which could contribute to reduce the oxidative organ injury seen in sepsis (223–225). Calprotectin suppressed NF-κB expression, proinflammatory cytokines, and inflammation in experimental autoimmune myocarditis (226), while the loss of calprotectin exacerbated T-cell activation and cardiac allograft rejection (227). In opposition, calprotectin aggravated post-ischemic heart failure through activation of RAGE-dependent NF-κB signaling (228). The diverging biological functions reported for calprotectin and its subunits suggest that their effects might be concentration dependent and influenced by the cellular and biochemical composition of the local milieu (229).

A novel and intriguing pro-resolving mechanism centered on neutrophils involves the generation of membrane borne microvesicles, also called microparticles or ectosomes (21). In 2004, Gasser and Schifferli (230) found that these microvesicles blocked the inflammatory response of macrophages exposed to zymosan and LPS. Further studies on neutrophil microparticles revealed that these microstructures could carry a variety of anti-inflammatory and pro-revolving mediators, enabling important modulatory functions in inflammation. Dalli and colleagues (231) defined the proteomic content of neutrophil microparticles. These authors observed that neutrophils have the ability to respond to a specific stimulus by producing microparticles loaded with a distinct proteomic profile, supporting the notion that microparticles production is a regulated process and might be endowed with very discrete functions (231). Some proteins, such as alpha-2-microglobulins, were identified to be selectively confined in vesicles generated from neutrophils adhered to an endothelial monolayer, whereas AnxA1 was more enriched in vesicles from exudate neutrophils. AnxA1 +ve vesicles possess anti-inflammatory properties (232) and allow the proper externalization of this pro-resolving mediator to gain access to extracellular surface receptors (i.e., FPR2) and to exert anti-inflammatory effects (39). AnxA1 acts as an exquisite brake for neutrophil adhesion to the microvascular wall, preventing over-exuberant cell transmigration to the inflammatory site (21, 233–235). We recently identified new properties for AnxA1 +ve vesicles, specifically those abundant in human synovial fluids collected from patients suffering from rheumatoid arthritis: these vesicles ensure the delivery of AnxA1 (and presumably other factors) to the chondrocyte in deep cartilage, enabling the activation of reparative circuits (236). In a recent review, we discussed the newly discovered modulatory roles of AnxA1 on neutrophil recruitment and other features of the resolution of inflammation (237).

Distinct Macrophage Populations Mediate Acute Inflammation and Resolution Phases of Inflammation

Macrophages are one of the first cells to sense injury, infection, and other types of noxious conditions, triggering the immune response through the production of proinflammatory mediators (1). During resolution, macrophages play an anti-inflammatory role and are required for the clearance of apoptotic cells. Following efferocytosis, macrophages undergo a functional repolarization, switching from a pro- to an anti-inflammatory phenotype (238). Accordingly, efferocytosis is coupled with increased release of TGF-β and IL-10 and lower levels of proinflammatory cytokines, such as IL-6 (238–240). This change in the phenotype of macrophages also activates pro-resolving mechanisms, because they generate LXA_4, which stimulates phagocytic activities without releasing proinflammatory mediators. This is an important non-phlogistic process typical of resolution, and shared, for instance, by GCs (241).

In addition to participating in the lipid mediator class switching discussed above, PGE_2 is also important in macrophage reprogramming, mediating the transition from the acute to the resolution phase of inflammation. Early data from Kunkel's group showed a suppressive effect of PGE_2 on macrophage TNF-α and IL-1β production (242, 243), and this has been confirmed by other investigators (238). This inhibitory feature allows proinflammatory cytokines to regulate their own production using PGE_2 as a self-induced modulator (242). Recently, MacKenzie and colleagues (244) reported that the addition of PGE_2 to LPS-stimulated macrophages represses proinflammatory cytokine production but induces IL-10. In particular, PGE_2 displayed a biphasic effect on IL-6 transcription: at early time points, this eicosanoid promoted IL-6 transcription but at later time points, it repressed the induction of IL-6 mRNA (244). Another study showed that PGE_2 from activated bone marrow stromal

cells promotes IL-10 in LPS-stimulated macrophages, an effect mediated by prostaglandin EP2 and EP4 receptors (245). PGE_2 in combination with LPS was able to induce the mRNA for Arginase 1, LIGHT (TNFSF14), and SPHK1, potential markers of alternatively activated and regulatory macrophages (245), again suggesting long-lasting roles for this prostaglandin in macrophage reprogramming.

PGE_2 has also been implicated in tissue maintenance and regeneration. This is supported by reports that indicate that increased levels of PGE_2 were associated with increased regenerative capacity. In this regard, Zhang and colleagues showed that the inhibition of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), a prostaglandin-degrading enzyme, potentiates tissue regeneration in multiple organs in mice. In a model of DSS-induced colitis, PGE_2 elevation diminished colon ulcers, suppressed mucosal inflammation, and reduced colitis symptoms, in conjunction with increased cell proliferation in the DSS-damaged mucosa. Interestingly, the pharmacological induction of higher levels of PGE_2 was associated with markedly increased rate and extent of liver regeneration in mice after partial hepatectomy as compared to control groups (246). In the lung, PGE_2 is the major eicosanoid produced by fibroblasts, alveolar macrophages, and other lung cells, playing important roles in tissue repair processes and in immune-inflammatory response limitation (247). PGE_2 directly inhibits several major pathobiologic functions of lung fibroblasts and myofibroblasts, including proliferation, migration, collagen secretion, and myofibroblast differentiation [reviewed in Ref. (248)]. Of note, diminished PGE_2 production and/or signaling can be observed in human and animal lung fibrosis, reinforcing its relevance for proper resolution (249, 250).

What the Future Reserves for Resolution

Undoubtedly, the inflammatory system is greatly complex. The history of the discovery of proinflammatory mediators reminds us that several decades of research were required to define the biology and pharmacology of the currently known mediators of inflammation. Since Sir Henry Dale and Patrick Laidlaw described some physiological effects of histamine *in vivo*, in 1910, immunological research has tremendously advanced (251). Pharmacological research has accompanied this progress, as historically represented by the discovery of antihistamines by Daniel Bovet and the identification of anti-H2R antagonists by Sir James Black, both awarded with the Nobel Prize in Physiology and Medicine (251). Subsequently, we made progress in the immunological and pharmacological fields of research, appreciating and shaping the concept of resolution of inflammation, and the mechanisms underpinning it. Fundamental concept here is the acceptance that resolution of inflammation is an active process evoked by specific classes of pro-resolving mediators, which differ from classical "anti-inflammatories" due to their ability to stimulate selective molecular and cellular programs of resolution. In the last decade, it has become evident that the enormous complexity of the proinflammatory system is mirrored at the level of pro-resolution pathways. Despite these remarkable advancements in the field, it seems that we have just started to scratch the surface of resolution mediators and other new cellular players are likely to be identified and defined in the near future. Likewise, we need to identify the major triggering pathway of these pro-resolving events, a phenomenon likely to be tissue- and/or disease-specific, as well as appreciate the complex networks among pro-resolving mediators. Such knowledge would be instrumental in developing pro-resolution based strategies to treat complex chronic inflammatory diseases in man, thus establishing a new area of pharmacology to be referred to as "resolution pharmacology" (22).

AUTHOR CONTRIBUTIONS

MS, LS, VP, MP, and MT conceived and wrote the manuscript, and realized the figure.

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REVIEW
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Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response

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Chemokines and their receptors are key mediators of the inflammatory process regulating leukocyte extravasation and directional migration into inflamed and infected tissues. The control of chemokine availability within inflamed tissues is necessary to attain a resolving environment and when this fails chronic inflammation ensues. Accordingly, vertebrates have adopted a number of mechanisms for removing chemokines from inflamed sites to help precipitate resolution. Over the past 15 years, it has become apparent that essential players in this process are the members of the atypical chemokine receptor (ACKR) family. Broadly speaking, this family is expressed on stromal cell types and scavenges chemokines to either limit their spatial availability or to remove them from *in vivo* sites. Here, we provide a brief review of these ACKRs and discuss their involvement in the resolution of inflammatory responses and the therapeutic implications of our current knowledge.

Keywords: chemokines, immunity, inflammation, scavenging, atypical receptors

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INTRODUCTION

An effective inflammatory response requires carefully regulated initiation, maintenance, and resolution phases (1). Inflammation is characterized by a stepwise recruitment of leukocytes, with neutrophils typically being the first recruited cellular population, followed by macrophages and lymphocytes. The precise molecular control of inflammation has not yet been fully worked out, although it is clear that the primary regulators of *in vivo* leukocyte migration to inflamed tissue sites are the chemokines, or chemotactic cytokines (2). Chemokines are members of a large family of proteins defined by the presence of a conserved cysteine motif in their mature protein sequences. Chemokines are divided into CC, CXC, XC, and CX3C subfamilies according to the specific nature of the cysteine motif (3). Chemokines are exclusive to vertebrates (4), and the primordial chemokine is almost certainly CXCL12, which was evolved to regulate stem cell migration during embryogenesis. From this one ancestral gene, the family has expanded to the point at which mammals have around 45 different chemokines, which are involved, in sometimes extremely complex and subtle ways, in regulating immune and inflammatory cell migration *in vivo*. Chemokines can be broadly defined as being either inflammatory or homeostatic according to the contexts in which they function (3, 5). Inflammatory chemokines are not normally expressed at significant levels but are induced extremely rapidly following tissue insult, or infection, and serve to recruit inflammatory leukocytes to any compromised body site. In all likelihood, all cells are capable of producing inflammatory chemokines

and thus initiating inflammation. In contrast, homeostatic chemokines are involved in the basal recruitment of cells involved in immune responses, and these control much more specific cellular navigation processes.

Chemokines interact with their target cells by binding to receptors belonging to the 7-transmembrane-spanning family of G protein-coupled receptors (6). Thus far, 10 receptors for CC chemokines, 7 for CXC chemokines, and single receptors for the XC and CX3C chemokines have been identified. Again, these receptors can be defined as being either inflammatory or homeostatic according to the class of chemokines they bind. One complex feature of chemokine receptors, particularly those involved in regulating inflammatory leukocyte migration, is that they display promiscuous ligand binding. In addition, individual chemokines can bind to more than one receptor and individual receptors are expressed on numerous different leukocyte cell types (6). Moreover, the formation of receptor dimers and oligomers at the cell surface can modify their chemokine binding and signaling activity, further complicating biology (7). This biological complexity, and the likely existence of biased-signaling in terms of receptor/ligand interactions (8, 9), suggests that chemokine receptor involvement in inflammatory responses is complex and potentially redundant.

In the context of an inflammatory response, it is clear from a number of studies that numerous inflammatory chemokines are simultaneous expressed at damaged sites. These then attract leukocytes by interacting with inflammatory chemokine receptors and initiate inflammatory responses. While inflammation typically is transient, and resolves efficiently, occasionally, it can be associated with chronic inflammatory disease. The fact that chemokines and their receptors are the primary drivers of inflammatory leukocyte recruitment therefore highlights them as important therapeutic targets (10). Despite this exciting opportunity, progress toward development of clinically useful receptor antagonists has been extremely disappointing (11). Indeed, 25 years since the cloning of the first inflammatory chemokine receptor (12), there are still no chemokine receptor antagonists licensed for use in treating inflammatory diseases. While there are many pharmacological reasons for this, one over-riding reason is that we currently have a relatively poorly developed understanding of precisely how chemokines and their receptors orchestrate inflammatory responses and of the layers of complexity introduced as different inflammatory leukocytes enter, and exit, inflamed sites. Thus, a much more comprehensive understanding of this process is required for it to be effectively therapeutically targeted.

The resolution of the inflammatory response is a key step at which inflammation can transition, from an acute and transient response, to one that is chronic and pathological. Accordingly, there have been numerous studies into the molecular regulation of the resolution of the inflammatory response, which has highlighted lipid mediators, such as resolvins (13), as important regulators. In terms of removal of chemokines during resolution, this is achieved in two separate ways. First, most chemokines (and indeed other inflammatory cytokines) are removed from inflamed tissue by drainage through the lymphatic system (14). This almost certainly accounts for the high levels of inflammatory mediators and chemokines in the plasma of patients with chronic inflammatory pathologies. However, recent data have highlighted active roles for chemokine-scavenging atypical chemokine receptors (ACKRs) in the resolution of inflammatory responses (15). In this review, we discuss the roles for ACKRs in the resolution of the inflammatory response and highlight their potential therapeutic value.

ATYPICAL CHEMOKINE RECEPTORS

Atypical chemokine receptors (6, 16), (**Table 1),** in contrast to canonical chemokine receptors, are mainly expressed by non-leukocyte cell types, such as erythrocytes, lymphatic or vascular endothelial cells, although some expression of ACKRs (especially ACKR2 and ACKR3) is detected on leukocytes (6, 17, 18). ACKRs bind chemokines with high affinity and do not induce cell migration as a result of their structural inability to couple to G proteins. In fact, ACKR activation of β-arrestin-dependent pathways modulates chemokine bioavailability by transporting chemokines to intracellular degradative compartments or, in the case of polarized cells, to the opposite side of the cell monolayer (19). ACKRs can also modulate the chemokine system by regulating the expression, or signaling, of other canonical chemokine receptors (18).

Four molecules have been officially named and included in the ACKR subfamily: ACKR1, previously called duffy antigen receptor for chemokines (DARC); ACKR2, also known as D6 or CCBP2; ACKR3, also called CXC-chemokine receptor 7 (CXCR7) or RDC1; and ACKR4, previously called CC chemokine receptor-like 1 (CCRL1) and also known as CCX-CKR. Two other molecules, CCRL2 and PITPNM3, tentatively included in the ACKR family as "ACKR5" and "ACKR6," respectively, will not be covered by this review as they are awaiting functional confirmation (16). It may be that additional ACKRs exist and these will be incorporated into the systematic nomenclature as they are identified (16). One of the problems in routinely identifying such receptors, for example, by de-orphanizing known orphan GPCRs is their lack of canonical signaling. Thus, each of the known atypical receptors has been identified through serendipity rather than through directed signaling-based screening approaches.

Here, we will review the involvement of the four characterized ACKRs in inflammation and its resolution.

TABLE 1 | Ligands and expression patterns for the ACKRs.

Gene	Ligands	Expression
ACKR1	CCL2, 5, 7, 11, 13, 14, 17; CXCL5, 6, 8, 11	Erythrocytes, vascular endothelial cells, and Purkinje cells
ACKR2	CCL2, 3, 3L1, 4, 5, 7, 8, 11, 12, 13, 17, 22	Lymphatic endothelial cells, leukocytes (especially B1 B cells), keratinocytes, and trophoblasts
ACKR3	CXCL11, 12	Hematopoietic cells, lymphatic endothelial cells, mesenchymal cells, and neuronal cells
ACKR4	CCL19, 21, 25; CXCL13	Lymphatic endothelial cells and epithelial cells

ACKR1 (DARC)

ACKR1 binds over 20 inflammatory chemokines belonging to the CC and CXC families (20). It is expressed by erythrocytes and endothelial cells lining small veins and venules (21). From a structural perspective, it is completely lacking the DRYLAIV motif in the second intracellular loop and has a low percentage of sequence homology with the other chemokine receptors (22). Thus, in contrast to the other ACKRs, the genes for which sit within chromosomal loci incorporating other canonical chemokine receptors, ACKR1 appears to share limited evolutionary relationship to the other receptors.

ACKR1 expressed by erythrocytes regulates the bioavailability of circulating chemokines by binding them with high affinity (23, 24). African people, referred to as "Duffy null" or negative because they lack ACKR1 expression on erythrocytes (but not endothelial cells), have higher concentrations of circulating chemokines (25), and genome-wide association studies have linked the ACKR1 variant Asp42Gly with serum CCL2 and CXCL8 levels (23). During inflammatory conditions, ACKR1 can function as a "sink" but also as a buffer for chemokines, increasing their systemic bioavailability and avoiding excessive changes in the concentration of circulating chemokines (26). In addition, it was found that ACKR1 expressed by endothelial cells is able to induce chemokine internalization and trancytosis (19, 27), thereby facilitating presentation of inflammatory chemokines on the luminal surface of vascular endothelial cells.

In the context of resolution of inflammation, the role of ACKR1 was studied in models of acute or chronic inflammation in WT and ACKR1 KO mice. Lack of the receptor results in reduced neutrophil recruitment to the lung after intratracheal administration of CXCL8 or LPS (28, 29) and in a model of acid-induced injury (30). Reduced neutrophil recruitment was also found in ACKR1 KO mice in a model of acute kidney damage induced by ischemia or LPS and was associated with renal protection (31). In a model of bone fracture, ACKR1 KO mice have decreased levels of pro-inflammatory cytokines (IL-1β, IL-6, and CCL2) and fewer macrophages around fractures (32). ACKR1 plays also a role in chronic inflammation, as demonstrated by the use of the ApoE KO mouse model of atherogenesis. ACKR1 KO mice are partially protected from atheroma development, and this is associated with decreased levels of inflammatory chemokines in the aorta and modest changes in T lymphocytes and inflammatory monocyte numbers in plaques (33). A role for ACKR1 was also found in infectious diseases: it is the receptor for the human malarial parasites *Plasmodium vivax* and *Plasmodium knowlesi* and individuals lacking ACKR1 (Duffy negative), or carrying polymorphic variants, are less susceptible to *P. vivax* infection (34).

The emerging picture is that ACKR1 expressed by erythrocytes acts as a chemokine buffer and can limit excessive leukocyte extravasation. In contrast, endothelial ACKR1 promotes acute and chronic inflammation by reducing chemokine concentrations in the inflamed tissues and creating a gradient that increases neutrophil and monocyte extravasation.

ACKR2 (D6 OR CCBP2)

ACKR2 is able to bind a broad panel of inflammatory CC chemokines. It is expressed by lymphatic endothelial cells, trophoblasts in the placenta, and some leukocytes such as alveolar macrophages and innate-like B cells (35). ACKR2 is a chemokine scavenger receptor which functions, in a catalytic manner, by transporting chemokines to degradative intracellular compartments (36, 37). It is able to dynamically adapt its scavenger function to the extracellular chemokine concentration activating a β-arrestin-dependent pathway that increases its plasma membrane localization without affecting the internalization rate (38, 39). ACKR2 promotes the resolution of inflammation and regulates lymphatic vessel function (40) and density (14), and ACKR2 KO mice in different pathological contexts exhibit dysregulated inflammatory reactions due to the lack of chemokine clearance and associated accumulation of inflammatory cells (41).

In response to phorbol ester, ACKR2 KO mice develop a severe skin inflammatory response resembling psoriasis (42), and after injection of complete Freund's adjuvant, KO mice develop larger granulomas compared to WT mice (43). ACKR2 also controls inflammatory responses in the gut (44, 45) and in the lung (46). ACKR2 expressed by trophoblasts inhibits inflammation in the placenta, where it protects from inflammation-associated miscarriage and allogeneic embryo rejection (47, 48). After myocardial infarction, ACKR2 prevents excessive infiltration of classical monocytes and neutrophils by scavenging CCL2, promoting cardiac remodeling (49). ACKR2 is also important for the control of inflammation in infectious diseases such as *Mycobacterium tuberculosis* (50). The role of ACKR2 in the context of autoimmune diseases is still controversial. It was reported that ACKR2 KO are resistant to the induction of experimental autoimmune encephalomyelitis (EAE) (51) and have reduced renal inflammation in a model of diabetic nephropathy (52). More recently, it appears that ACKR2 deficiency does not suppress autoreactive T-cell priming and autoimmune pathology, but can enhance T-cell polarization toward Th17 cells (53).

In addition to these data indicating that ACKR2 promotes resolution of the inflammatory response by chemokine clearance and inhibition of excessive leukocyte recruitment, it was reported that ACKR2, expressed by leukocytes, inhibits their pro-inflammatory phenotype. ACKR2 restricts neutrophil migration (54) and regulates macrophage efferocytosis and cytokine secretion (55). Finally, a key role for ACKR2 in regulating the promotion of inflammation-dependent cancers has been shown using mouse models of both cutaneous (56) and colorectal cancer (45). In these contexts, ACKR2 functions essentially as a tumor suppressor gene by limiting tumor-promoting tissue inflammatory responses.

ACKR3 (CXCR7 OR RDC-1)

ACKR3 binds two chemokines, CXCL12, the ligand of CXCR4, and CXCL11, one of the ligands of CXCR3 (57). It is expressed by endothelial cells, some hematopoietic cells, mesenchymal cells, and neurons. ACKR3 mainly signals through β-arrestin pathways

activating extracellular signal-regulated kinases (ERKs) or protein kinase B (PKB or Akt) (58). ACKR3 modulates CXCL12 activity in several ways. It downregulates CXCL12 concentrations by scavenging and modulates CXCR4 expression and signaling activity by forming heterodimers with CXCR4 (59). Elegant studies using zebrafish embryos have demonstrated important and evolutionary conserved roles for ACKR3 in the regulation of key cellular populations during embryogenesis (60). These studies have shed important light on the importance of ACKR3 for the generation of tissue gradients during cellular migration within the embryo. ACKR3 KO mice have defects in brain, heart, and kidney development (61–63).

During inflammatory conditions, both leukocytes and endothelial cells increase ACKR3 expression. Peripheral blood lymphocytes from patients with inflammatory bowel disease have enhanced ACKR3 expression, which was also upregulated upon stimulation (CD3) or costimulation (CD3/CD28) (64). ACKR3 was found expressed by macrophages in the atherosclerotic plaque and was associated with a pro-inflammatory phenotype that includes production of inflammatory chemokines and phagocytic activity (65). ACKR3 is also prominently expressed in a wide range of tumors both within the tumor cells and by cells of the tumor vasculature (66). It has therefore been highlighted as a potential therapeutic target in oncology.

In relation to endothelial cells, ACKR3 is expressed in rheumatoid arthritis synovium, in which it promotes the inflammatory process increasing angiogenesis (67). In addition, ACKR3 is induced in brain microvascular endothelial cells during experimental inflammatory conditions, such as permanent middle cerebral artery occlusion and EAE, and favors leukocyte extravasation by enhancing leukocyte adhesion to the endothelial surface (68). It should be noted that CXCR7 is also expressed by neurons and astrocytes in various brain regions and, during EAE, it is upregulated by oligodendrocyte progenitors, important cells for the remyelination process (69).

In summary, ACKR3 expression promotes inflammation inducing a leukocyte pro-inflammatory phenotype, enhancing angiogenesis and leukocyte extravasation.

ACKR4 (CCRL1 OR CCX-CKR)

ACKR4 binds the homeostatic chemokines CCL19, CCL21, CCL25, and CXCL13. It is expressed by thymic epithelial cells, bronchial cells, and keratinocytes. ACKR4 is a constitutively internalizing receptor with chemokine-scavenging function (70). After chemokine binding, it recruits β-arrestin 2, but it is not known if it activates signal transduction pathways.

Few data are available on the *in vivo* role of ACKR4 in the context of inflammation. It appears to be important in the correct trafficking of dendritic cells for the induction of adaptive immune responses. Indeed, ACKR4 expression in lymph nodes is necessary for creating a gradient of the CCR7 ligands, CCL19 and CCL21, in the subcapsular sinus (71). In addition, using ACKR4 in KO mice, it was demonstrated that homeostatic chemokine clearance is necessary to control excessive Th17 responses that can lead to immunopathologies (72).

CONCLUDING REMARKS

The identification and characterization of the ACKRs has represented a major advance in our understanding of the overall orchestration of chemokine-driven immune and inflammatory responses. These receptors have been shown to play important roles in regulating cell migration in developmental, inflammatory, immune, and pathological contexts (**Table 2**). In this context, these receptors control the chemokine system by scavenging, transporting, or storing chemokines, but also by regulating the activity of canonical chemokine receptors with which they share the ligands by forming heterodimers or modulating their expression levels or signaling activity.

The essential roles that they play, particularly in the context of resolving inflammatory responses, highlights them as potential therapeutic targets. While the normal pharmacological approach is to develop chemokine receptor antagonists, in the case of the atypical receptors what would be more useful would be small molecule inducers of either expression or activity. Such inducers could work through known cytokine pathways that induce ACKRs (73) or by capitalizing on our developing understanding of the kinetics of cell surface mobilization of these receptors (37, 38).

If developed, these could be used to increase ACKR function and thus neutralize chemokine activity in a number of inflammatory pathologies. Topical application of such regulators could be envisaged as having therapeutic potential in, for example, psoriasis and intranasal administration in the context of lung inflammatory responses. Furthermore, it may be possible to adapt these molecules for use in cancer therapy to restrict cancer access to pro-tumorigenic inflammatory leukocytes.

TABLE 2 | Phenotype of ACKRs knockout mice in inflammation and infection models.

Gene deletion	Phenotype	Reference
ACKR1	Reduced neutrophil recruitment in acute inflammation models	(28–30)
	Renal protection in ischemia or LPS induced acute kidney damage	(31)
	Reduced macrophages infiltration in bone fracture model	(32)
	Reduced atheroma development in the Apo E KO	(33)
ACKR2	Severe skin inflammatory reaction similar to psoriasis	(42)
	Increased granulomatous inflammatory response	(43)
	Increased gut and lung inflammation	(44–46)
	Increased tissue damage after myocardial infarction	(49)
	Increased inflammation-associated miscarriage and allogeneic embryo rejection	(47, 48)
	Uncontrolled *Mycobacterium tuberculosis* infection	(50)
ACKR3[a]	Exacerbates neointimal hyperplasia	(65)
ACKR4	Excessive Th17 responses	(72)

[a]*Tamoxifen-inducible knockout.*

Atypical chemokine receptors therefore represent novel therapeutic targets likely to benefit in a number of pathologies with unmet clinical need.

AUTHOR CONTRIBUTIONS

GG and RB wrote the text and approved the final submission.

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REVIEW
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Regulatory Role of IL-1R8 in Immunity and Disease

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Interleukin-1 receptor family members (ILRs) and toll-like receptors (TLRs) are characterized by the presence of a conserved intracellular domain and the toll-IL-1resistance (TIR) domain and are key players in immunity and inflammation. ILR and TLR signaling is tightly regulated at different levels. All cell types of the innate immune system express ILRs and TLRs. In addition, IL-1 family members are emerging as key players in the differentiation and function of innate and adaptive lymphoid cells. IL-1R8, also known as TIR8 or SIGIRR, is a fringe member of the ILR family and acts as a negative regulator of ILR and TLR signaling, which dampens ILR- and TLR-mediated cell activation. IL-1R8 is a component of the receptor recognizing human IL-37. Here, we summarize our current understanding of the structure and function of IL-1R8, focusing on its role in different pathological conditions, ranging from infectious and sterile inflammation to autoimmunity and cancer-related inflammation.

Keywords: cytokine, interleukin-1, toll-like receptors, inflammation, infection, inflammation-associated cancer

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INTRODUCTION

Interleukin-1 receptor family members (ILRs) and toll-like receptors (TLRs) are members of a superfamily characterized by the presence of a common intracellular signaling domain, called toll-IL-1 resistance (TIR) domain, and Ig-like domains or leucine-rich repeats in their extracellular part (1) (**Figure 1**). ILRs and TLRs are phylogenetically conserved receptors responsible for the initiation and amplification of events leading to inflammation and innate and adaptive immune responses. TLRs work as sensors for exogenous infectious agents and host tissue injury, recognizing specific pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Ten TLRs have been identified to date in humans and 12 in the mouse. The ILR subfamily is a growing family of receptors and accessory proteins (AcP) for the cytokines of the IL-1 family. The nomenclature of ILRs has recently been revised (2) and will be used here: IL-1R1 (IL-1RI), IL-1R2 (IL-1RII), IL-1R3 (IL-1RAcP), IL-1R4 (ST2), IL-1R5 (IL-18Rα), IL-1R6 (IL-1Rrp2, IL-36R), IL-1R7 (IL-18Rβ), IL-1R8 (also known as TIR8 or SIGIRR), IL-1R9 (TIGIRR-2), and IL-1R10 (TIGIRR-1). The IL-1 system includes molecules with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), three receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), and an anti-inflammatory cytokine (IL-37) (**Figure 1**).

Upon ligand binding, TLRs and ILRs dimerize through their TIR domains, inducing the recruitment of TIR domain-containing adapter proteins, in particular MyD88, MAL, TRIF, TRAM, and SARM, which couple to downstream protein kinases [e.g., IL-1R-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6)]. The signal leads to the activation of key transcription factors associated with inflammatory and immune responses, such as nuclear factor-κB

FIGURE 1 | The IL-1 receptor (ILRs) and toll-like receptor (TLRs) superfamily. Ligands, receptors, accessory proteins, and regulators are shown. Ligands of the ILR family include IL-1α, IL-1β, IL-38, IL-33, IL-36α, IL-36β, IL-36γ, and IL-18. Microbial compounds (LPS, CpG, poly IC, flagellin, and others), β-amyloid, and danger signals are ligands for TLRs. IL-1R, IL-33R, IL-36R, and IL-18R complexes transduce positive signals. IL-R2, sIL-1R1, IL-1Ra, IL-36Ra IL-18BP, and IL-1R8 are negative regulators acting at different levels. IL-37 is an anti-inflammatory cytokine, which signal is dependent on the formation of a tripartite complex (IL-37/ IL-1R5/IL-1R8). IL-1R3 is an accessory protein, which activity is necessary for IL-R1, IL-1R2, IL-1R4, and IL-1R6 function. IL-1R8, IL-1R9, and IL-1R10 are still orphan receptors.

(NFκB), activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK), p38 mitogen-associated protein kinase, extracellular signal-regulated kinases (ERKs), mitogen-activated protein kinases (MAPKs), and members of the interferon (IFN)-regulatory factor (IRF) (3–5).

IL-1 family and TLR signaling are a crucial network that covers a wide spectrum of functions in several tissues and cell types, ranging from tissue homeostasis regulation to protective responses against infections and modulation of inflammation. Given the huge capacity of ILR and TLR triggering to drive inflammatory responses, the strict regulation of this system plays a significant role in both physiological and pathological conditions.

Both ILR and TLR functional activations are modulated by several and diverse mechanisms. Among these, IL-1R2 exerts regulatory functions acting as decoy receptor for IL-1, dominant-negative molecule, and scavenger (6, 7). In addition, it is also present in the cytoplasm where it binds pro-IL-1α and pro-IL-1β, preventing their cleavage and activation (8). IL-1R8, also known as TIR8 or SIGIRR, whose function will be detailed below, is a fringe member of the family that lacks conventional signaling capacities and behaves as a negative regulator of the family, acting intracellularly. Available information suggests that IL-1R8 interferes with the association of TIR-containing adaptor molecules to the receptor complex, thus dampening the signaling pathway leading to signal transduction (9, 10). In addition, IL-1R8 is a component of the receptor recognizing the anti-inflammatory cytokine IL-37 (11). IL-37 is an anti-inflammatory cytokine that acts as a natural brake of inflammation, signaling through IL-1R5/IL-18Rα and IL-1R8 was recently described as a coreceptor, required for the formation of the tripartite complex IL-37–IL-1R5/IL-18Rα–IL-1R8 (11). IL-18BP is an extracellular protein that binds IL-18, preventing its interaction with the receptor IL-1R5/IL-18R, and thus neutralizing its activity (12–14). IL-1Ra and IL-36Ra are receptor antagonists that bind IL-1R1 and IL-1R6, respectively (5, 15–18).

IRAK-M and MyD88s are intracellular signaling molecules that negatively regulate ILR and TLR pathways (19, 20). Finally,

specific miRNAs (miR-155, miR-21, miR-146a, miR-132, miR-9, and miR-147) target ILR and TLR signaling proteins (21–23). The abundance of these regulatory mechanisms underlines the relevance of the negative regulation of both ILRs and TLRs, which if uncontrolled, may activate detrimental inflammation and cause tissue damage. For instance, local and systemic inflammation induced by IL-1 underlay a broad list of diseases, ranging from rheumatic diseases and autoinflammatory syndromes to cardiovascular diseases, type 2 diabetes, and infections and sepsis, and targeting of IL-1 has relevant therapeutic implications (24–27).

Here, we will review our current understanding of the structure and function of the negative regulator IL-1R8 (TIR8/SIGIRR), focusing on its relevance in different inflammatory or immune-mediated pathological disorders and emphasizing recent discoveries.

IL-1R8 (TIR8/SIGIRR) GENE AND PROTEIN

IL-1R8 was identified by our group and reported as TIR8 in 1998 (Accession number: AF113795), and in parallel by John Sims' group in 1999 and reported as SIGIRR (28). IL-1R8 is localized on human chromosome 11, band p15.5, and is composed of 10 exons spanning about 11,700 bp (28). It is therefore not part of the IL-1R family cluster, which is located in humans on chromosome 2. The murine gene is localized on chromosome 7, band F4, and encompasses nine exons spanning about 4000 bp. The human protein is 410 amino acids long and displays unique features compared with other ILRs. It is composed of a single extracellular Ig domain, in contrast with the other family members which have three, a transmembrane domain, a cytoplasmic TIR domain, and an unusually long tail (95 residues), which is missing in other TIR domain-containing receptors. The IL-1R8 TIR domain lacks two conserved residues (Ser447 and Tyr536), which are replaced by Cys222 and Leu305 suggesting unconventional signaling. IL-1R6 and IL-1R3 display a similar amino acid substitution in their TIR domain, but the functional relevance of this replacement has not been addressed yet (28, 29).

IL-1R8 sequence is highly conserved among vertebrates, from chicken to human in terms of sequence and pattern of expression (30). Human and murine protein sequences are 82% identical and share 23% overall identity with IL-1R1. In teleost fish, the receptor DIGIRR, which has two Ig-like domains in its extracellular region and an Arg–Tyr-mutated TIR domain, exerts regulatory activities *in vitro*, negatively modulating LPS, and IL-1β-dependent NFκB activation, therefore resembling a "transitional" form between the signaling molecule IL-1R1 and the negative regulator IL-1R8 (31).

IL-1R8 has five potential N-glycosylation sites in the extracellular domain in humans and four in the mouse and is extensively *N*- and *O*-glycosylated. Zhao et al. recently showed that loss of N-linked glycosylation was associated with an inactive isoform of IL-1R8, generated by alternative splicing in colon cancer cells. Moreover, loss of complex glycan modifications was sufficient to suppress IL-1R8 activity *in vivo*, highlighting that posttranscriptional modifications are required for the functional activity of IL-1R8 (32).

IL-1R8 is widely expressed in several epithelial tissues, in particular by epithelial cells of the kidney, digestive tract, liver, lung, and in lymphoid organs. Among leukocytes, it is expressed by monocytes, B and T lymphocytes, dendritic cells, and NK cells (28, 33). Little is known about the regulation of IL-1R8 expression and the stimuli and pathways involved. In general, both IL-1R8 mRNA and protein expression are reduced in inflammatory conditions. IL-1R8 was downmodulated in ulcerative colitis in humans and colitis in the mouse, intestinal bacterial infections, and exposure to flagellin (34, 35). The expression of IL-1R8 (and other anti-inflammatory molecules) was reduced in leukocytes of psoriatic arthritis patients and, together with TLR4, it was reduced in asymptomatic bacteriuria patients (36, 37). Nanthakumar et al. showed that IL-1R8 level was decreased in intestinal cells of necrotizing enterocolitis patients compared with fetal human enterocytes, whereas pro-inflammatory proteins were expressed at high levels, in line with the exacerbated inflammatory response of the immature intestine (38). In the mouse, acute lung infection by *Pseudomonas aeruginosa* caused *IL-1R8* mRNA downregulation in the lung and in neutrophils (39). IL-1R8 transcript was also reduced upon intestinal infection by *Toxoplasma gondii* (40). In a model of pyelonephritis induced by *Escherichia coli*, it was shown that renal IL-1R8 mRNA was downregulated in the early phase of infection and it started to return to basal level 24 h postinfection (41). Moreover, *in vitro* experiments in human bladder epithelial cells (BECs) demonstrated that IL-1R8 mRNA and protein expression were downregulated upon stimulation with LPS (42). Finally, colon tumorigenesis was shown to be associated with a lower expression level of IL-1R8 on the intestinal cell surface compared with the healthy counterpart. This was due to a mechanism of alternative splicing that caused the generation of an inactive mutant form of IL-1R8 that will be further discussed below (32).

Concerning the mechanism of IL-1R8 downregulation, Kadota et al. showed that LPS treatment reduced the binding between SP1, a zinc finger protein, and the proximal promoter of IL-1R8 (34). SP1 would normally directly interact with IL-1R8 promoter and favor gene transcription, but in presence of LPS the binding was inhibited and IL-1R8 expression transiently decreased in epithelial cells. Recently, the role of SP1 in the regulation of IL-1R8 mRNA expression was also confirmed in human primary monocytes and neutrophils. The inhibition of SP1 binding to IL-1R8 promoter induced by LPS was due to the activation of p38, which is downstream of TLR4. Indeed, treatment of both monocytes and neutrophils with a p38 inhibitor (SB203580) abolished the LPS-induced downregulation of IL-1R8 mRNA (43). Conversely, sepsis and sterile systemic inflammation have been associated with higher level of IL-1R8 expression by monocytes compared with homeostatic conditions, which correlated with reduced TNFα and enhanced IL-10 production in response to LPS and Pam_3CysSK_4 (44).

IL-1R8 is differentially expressed in polarized T lymphocytes. Murine Th2 cells displayed higher levels of IL-1R8 compared with Th1 or naive T cells (45). In *P. aeruginosa*-infected mice, IL-1R8 was upregulated by the neuropeptide vasoactive intestinal peptide (VIP) in a cAMP-independent manner in the cornea, in macrophages, and in Langerhans cells (46). The probiotic

microorganism *Lactobacillus jensenii* was found to upregulate IL-1R8, *via* TLR2 in porcine Payer's patch antigen-presenting cells, and to favor the expression of IL-10 and TGF-β, thus inducing tolerogenic properties (47). Finally, in murine Payer's patch DCs, but not splenic DCs, LPS was shown to induce the upregulation of IL-1R8, Tollip, and IL-1R4. This could be a potential mechanism used by Payer's patch DCs to modulate the inflammation driven by TLR signaling (48).

Recently, Costello et al. proposed a mechanism involved in IL-1R8 regulation in the context of neuroinflammation. Amyloid β treatment increased the expression of TLR2 and decreased the expression of IL-1R8 in microglia. Interestingly, TLR2 neutralization led to an increase of IL-1R8 mRNA in microglia and hippocampal tissue (49). The transcription factor peroxisome proliferator-activated receptor (PPAR)γ is a key anti-inflammatory mediator that regulates Aβ responses in the brain. Binding sites for the transcription factor PPARγ were identified in the IL-1R8 gene and treatment with the PPARγ inhibitor (GW9662) reduced the anti-TLR2-mediated IL-1R8 upregulation, supporting the involvement of PPARγ in the modulation of IL-1R8 expression. The PI3K/Akt pathway was also involved in the regulation of IL-1R8, since PI3K inhibitor (LY294002) abolished the effect of TLR2 neutralization. The expression of IL-1R8 and TLR2 is therefore inversely correlated and IL-1R8 upregulation mediated by TLR2 neutralization may be a compensation mechanism adopted to limit the deleterious effect of Aβ (49).

IL-1R8-MEDIATED ANTI-INFLAMMATORY ACTIVITY OF IL-37

IL-1R8 was considered an orphan receptor, lacking a specific ligand. IL-37 has been recently demonstrated to bind IL-1R8 and to generate the tripartite complex IL-37–IL-1R5/IL-18Rα–IL-1R8 (11) (**Figure 2**). IL-37 is an anti-inflammatory cytokine that dampens the inflammatory response triggered by TLRs and cytokines in peripheral blood mononuclear cells (PBMCs), in macrophages and epithelial cells, and IL-37-transgenic mice (IL-37tg mice) are protected in different inflammatory pathological conditions (50). Recently, advanced imaging analysis revealed a rapid interaction of IL-37 with both IL-1R5/IL-18Rα and IL-1R8 in human PBMCs and bone marrow-derived macrophages (BMDMs) of IL-37tg mice upon stimulation with LPS (11). In particular, following a pro-inflammatory stimulus, the tripartite complex IL-37–IL-1R5/IL-18Rα–IL-1R8 was formed on the cell membrane of PBMCs and cell lines (RAW, HEK293, and A549). IL-1R8 and IL-1R5/IL-18Rα were both required to support the anti-inflammatory activity of IL-37 in PBMCs, THP-1 macrophages, and A549 epithelial cells. Indeed, silencing of IL-1R8 or IL-1R5/IL-18Rα or both in these cell types impaired the IL-37-mediated reduction of inflammatory cytokines (e.g., IL-β and TNF), upon stimulation with LPS. Finally, proteomic and transcriptomic analysis demonstrated that the IL-37–IL-1R5/IL-18Rα–IL-1R8 complex triggered multiple signaling events leading to anti-inflammatory responses, such as inhibition of MAPK, NFκB, mTOR, TAK1, and Fyn and activation of STAT3, Mer, PTEN, and p62(dok). Thus, IL-1R8 acts as a coreceptor for IL-1R5/IL-18Rα upon IL-37 binding, and it is required for the anti-inflammatory activity of IL-37 (11, 51) (**Figure 2**). This mechanism is relevant *in vivo*, since IL-1R8 deficiency abolished the protection of IL-37tg mice against endotoxin challenge or the protective role of IL-37 in a model of non-resolving *Aspergillus fumigatus* infection and pulmonary damage (11, 52). Moreover, in a model of OVA-induced asthma, IL-37-driven anti-inflammatory effects were abolished in mice lacking either IL-1R5/IL-18Rα or IL-1R8 (53).

IL-37-overexpressing mice showed improved response to insulin and increased glucose tolerance and were protected from obesity. In addition, in adipocytes and macrophages IL-37 activated AMPK (54). Since IL-37–IL-1R8 signaling inhibited mTOR signaling, whereas AMPK, STAT6, and transcription factors of the Foxo family were activated (11), these results indicate that the IL-37–IL-1R8 axis is also involved in regulating inflammation-dependent modification of cell metabolism, favoring a pseudo-starvational state in macrophages and DCs.

Thus, these results demonstrate that IL-1R8 is part of the receptor complex of the anti-inflammatory cytokine IL-37 and mediates an anti-inflammatory signaling activation.

REGULATION OF ILR AND TLR SIGNALING BY IL-1R8

IL-1R8 exerts its regulatory activity by inhibiting NFκB and JNK activation induced by TIR-containing ILRs or TLRs upon ligand binding, but not by other receptors such as TNF receptors. In particular, IL-1R8 was shown to tune the activation of IL-1R1, IL-1R5/IL-18Rα, IL-1R4/ST2, TLR4, TLR7, TLR9, TLR3, and TLR1/2 (29, 45, 55–57) (**Figure 3**).

The knowledge about the mechanism of inhibition exerted by IL-1R8 is still fragmentary. Upon stimulation with IL-1, the IL-1R8 extracellular domain was shown to block the dimerization between IL-1R1 and IL-1R3/IL-1RAcP, and the intracellular TIR domain was shown to bind the TIR-containing adaptor protein MyD88 and downstream signaling molecules (IRAK and TRAF6), thus modulating IL-1 signaling (55, 57). Similarly, the targeting of IL-1R4/ST2 was shown to be dependent on both the extracellular immunoglobulin and TIR domains (45). In contrast, only the TIR domain was necessary for the inhibition of TLR4 signaling, as demonstrated by mutagenesis studies (55, 57). Indeed, a nonsense mutation (Q111*) and a frameshift mutation (P2fs) cause the generation of a truncated form of IL-1R8, which lose inhibitory activity (55, 57, 58). A computational approach revealed that IL-1R8 targeting of TLR4 and TLR7 signaling was dependent on IL-1R8 intracellular TIR domain and in particular on the BB-loop, which is shared by all TIR domain-containing proteins. The model proposed showed that IL-1R8 binds through its BB-loop region to TLR4 and TLR7 interfering with the dimerization site and replaces a MyD88 monomer, thus disturbing MyD88 homodimerization (59). Recent computational studies that predict the three-dimensional structures of the TLR family proteins suggested that IL-1R8 does not block the formation of the Myd88-dependent signalosome, but it inhibits NFκB activation by preventing the translocation of the signalosome from the receptor (58). This strategy would be similar to that used by

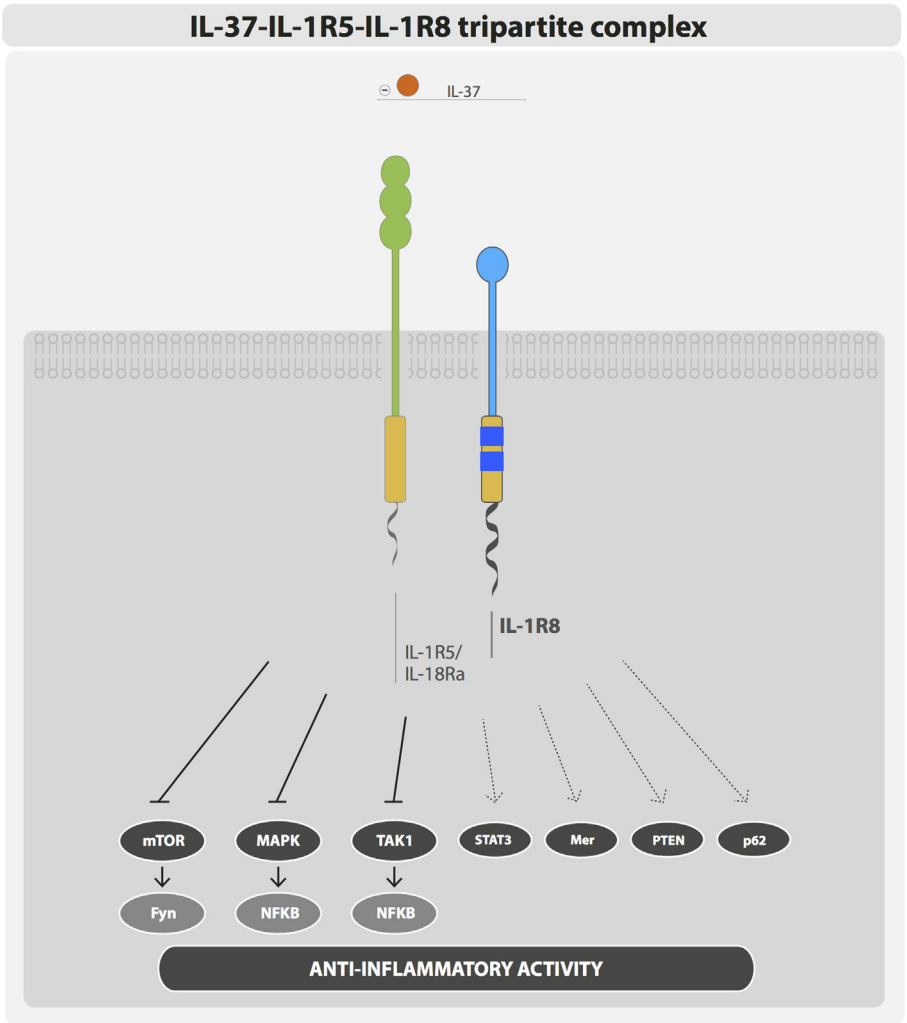

FIGURE 2 | IL-37–IL-1R5–IL-1R8 tripartite complex. IL-37 anti-inflammatory activity is exerted through the formation of a membrane bound tripartite complex composed of IL-37, IL-1R5, and IL-1R8.

IRAK-M, which blocks the dissociation of the myddosome from the receptor (19).

Moreover, it was demonstrated that IL-1R8 could potentially interact with all TIR domain-containing proteins in the TLR pathway, preventing the dimerization of Mal, TRAM, and TRIF, and inhibiting signalosome formation. However, the BB-loop of IL-1R8 is not involved in the interaction with Mal and TRAM. Thus, in addition to MyD88-dependent pathway, IL-1R8 can inhibit TRIF-dependent signaling. Indeed, IL-1R8 was shown to target TRIF-dependent TLR3 signaling, probably by blocking TRAM homodimerization and TLR4–TRAM and TRIF–TRAM interactions (10, 58, 60). One of the clinically observed oncogenic mutations of IL-1R8 (L282M) is located on the IL-1R8–Mal and IL-1R8–TRIF interaction sites and abolishes the interaction with TRIF. These results indicate that the BB-loop of IL-1R8 is relevant in the interaction with TLRs, but not necessarily with other TIR-containing molecules (58).

c-Jun N-terminal kinase and mTOR phosphorylation were enhanced in IL-1R8-deficient Th17 cells. IL-1R8 is therefore crucial in the modulation of metabolism, differentiation, expansion, and effector functions of Th17 cells (61). IL-1R8 was also demonstrated to target mTOR phosphorylation driven by IL-1 or TLR agonists derived from commensal flora in intestinal epithelial cells (IECs) (62). IL-1R8 therefore emerges as a regulator of the cell cycle, playing a crucial role in homeostatic conditions (**Figure 3**).

The interaction between IL-1R8 and other receptors of the family is still poorly defined and sometimes contradictory. This could be due to posttranscriptional modifications in different cell types that can affect its functions.

Thus, these results indicate that IL-1R8 interferes with the formation of TIR domain signalosome, prevents the dimerization of receptors, AcP, and adaptor molecules, and blocks signal transduction.

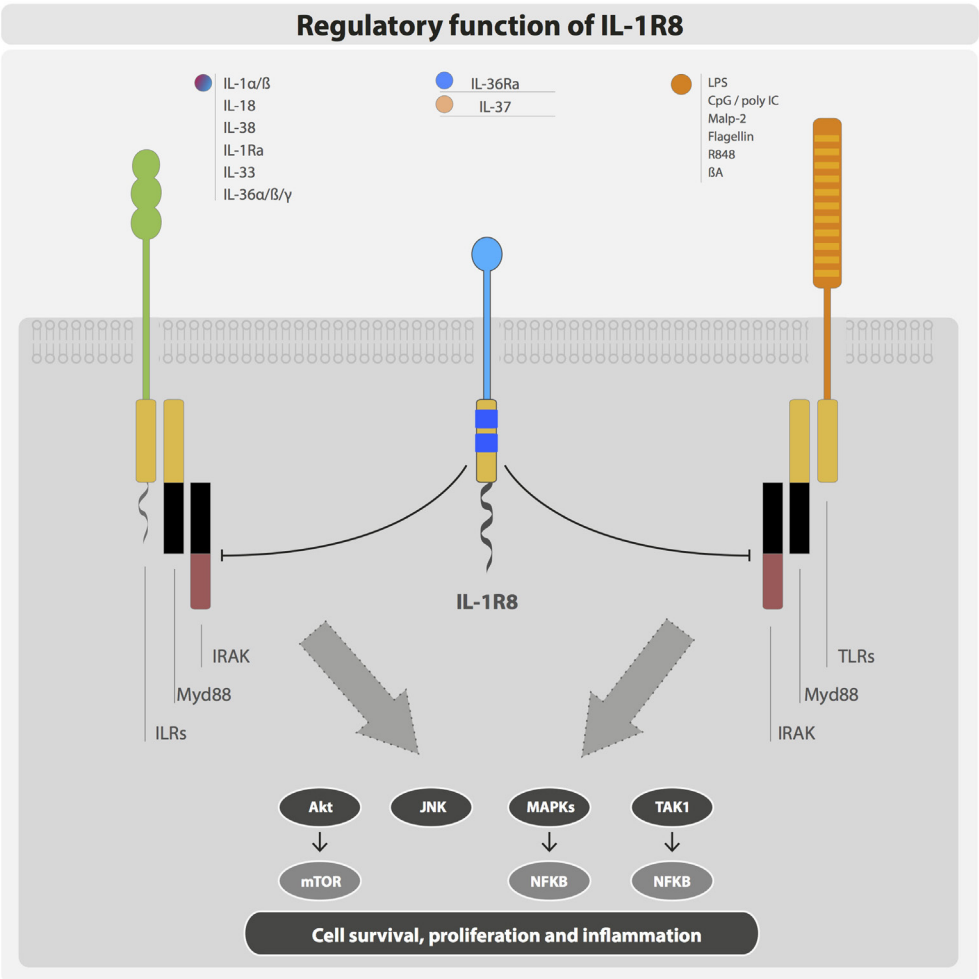

FIGURE 3 | **Negative regulation exerted by IL-1R8**. IL-1R8 is composed of a single extracellular domain, a transmembrane domain, a cytoplasmic TIR domain, and an unusually long tail (95 residues). The IL-1R8 TIR domain lacks two conserved residues (Ser447 and Tyr536), which are replaced by Cys222 and Leu305 suggesting unconventional signaling. IL-1R8 acts as a negative regulator of ILR and TLR signaling, inhibiting TIR-containing receptors and adaptor proteins, and thus blocking Akt, JNK, MAPKs, TAK1, and consequently mTOR and NFκB activation.

ROLE OF IL-1R8 IN INFECTION-DRIVEN INFLAMMATION

IL-1R8 emerged as a non-redundant molecule in infectious conditions, playing a key role in the regulation of TLR and ILR responses to pathogens by dampening inflammation and tissue damage (**Figure 4**).

In *Mycobacterium tuberculosis* infection, IL-1R8-deficient mice displayed higher mortality compared with the control group, even if no difference in tissue bacterial load in the lung, liver, or spleen was observed. The increased susceptibility was dependent on the exacerbated systemic inflammatory response. Indeed, IL-R8-deficient mice presented an overwhelming inflammatory response, which is characterized by enhanced macrophage and neutrophil lung infiltration and increased systemic levels of inflammatory cytokines. The *in vivo* depletion of crucial inflammatory mediators (IL-1 and TNFα) in *M. tuberculosis* infection significantly prolonged the survival of IL-1R8-deficient mice (63). In a model of keratitis induced by *P. aeruginosa*, IL-1R8 was involved in the regulation of IL-1R1 and TLR4 signaling in T cells and dampening Th1 response, thus preventing tissue damage and promoting resistance to infection (64). Similarly, in acute lung infections caused by *P. aeruginosa*, IL-1R8-deficient mice showed increased susceptibility to the pathogen, in terms of mortality and bacterial load, and increased production of pro-inflammatory cytokines, both locally and systemically. The enhanced susceptibility was dependent on the deregulation of IL-1 signaling, since IL-1R1 deficiency abolished the phenotype observed in IL-1R8-deficient mice. IL-1R8 therefore emerged as a non-redundant regulator of IL-1 mediated control of *P. aeruginosa* infection (39).

In *C. albicans* and *A. fumigatus* infections, the absence of IL-1R8 led to increased susceptibility to mucosal and disseminated or lung infections (65). IL-1R8-deficient mice showed increased mortality and fungal burden, enhanced activation

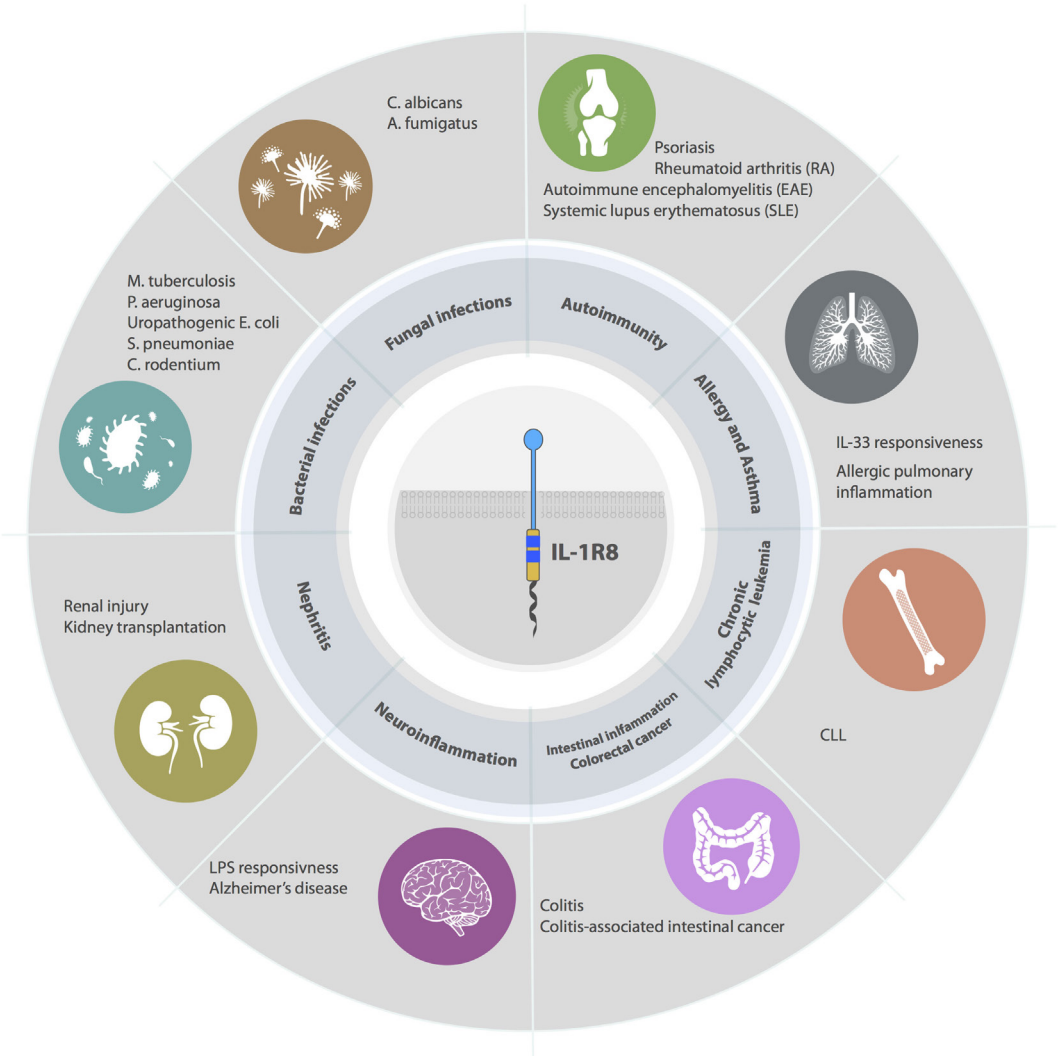

FIGURE 4 | **Roles of IL-1R8 in pathology**. IL-1R8-deficient mice have demonstrated that IL-1R8 acts a key modulator of acute and chronic inflammation in several pathological contexts. For instance, IL-1R8 plays a non-redundant role in models of bacterial infections, fungal infections, autoimmune diseases, allergy, asthma, renal inflammation, brain inflammation, intestinal inflammation, and cancer (colorectal cancer and CLL).

of IL-1 signaling and Th17 cell response, and reduced Treg activation. This correlated with higher levels of IL-12, IL-23, IL-6, IFN-γ, and IL-17 and reduced levels of IL-10. IL-1R8 was demonstrated to be a regulator of Th17 cells and IL-17A production by γδ T cells and a modulator of T cell polarization. The phenotype observed in IL-1R8-deficient mice could be due to a deregulated Th17 response, which is dependent on the uncontrolled IL-1 signaling. Indeed, *in vitro* experiments demonstrated that IL-17 production by $CD4^+$ T cells, primed with *Candida*-pulsed DCs, was reduced by the treatment with IL-1β- and IL-23-blocking antibodies (65).

The role of IL-1R8 is therefore attributable to the negative regulation of IL-1 signaling in *P. aeruginosa*, *C. albicans*, and *M. tuberculosis* infections, since IL-1 neutralization was sufficient to abolish the phenotype in IL-1R8-deficient mice.

Moreover, IL-1R8-deficient mice on a BALB/c background showed enhanced mortality, upon endotoxin challenge (55). In line with this, IL-1R8 overexpression in lung epithelial cells reduced the inflammatory response and improved the survival of BALB/c mice in a model of LPS-dependent acute lung injury (66). Since IL-1R8-deficient mice on a C57BL/6 × 129/Sv background revealed no difference in terms of LPS reaction, compared with controls, it has been hypothesized that IL-1R8-mediated regulation of LPS response may depend on the background of mice (56).

The relevance of these data in the mouse was supported by a case-population study design in Vietnam, showing that 3 SNPs (rs10902158, rs7105848, rs7111432) in IL-1R8 gene correlated with the development of both pulmonary tuberculosis and tuberculous meningitis. Moreover, coinheritance of these SNPs

with previously identified polymorphisms in TLR2 and TIRAP was associated with enhanced risk of susceptibility (67).

The protective role of IL-1R8 in the infections mentioned above is due to the regulation of ILR and TLR signaling that potentially cause detrimental inflammation and tissue damage. However, in a model of experimental urinary tract infection (UTI) induced by uropathogenic *E. coli*, IL-1R8-deficient mice displayed reduced renal bacteria outgrowth and diminished renal dysfunction UTI. IL-1R8 also modulated the recruitment of immune cells in the kidney, since a more sustained renal neutrophil influx was observed in the early phase of infection in IL-1R8-deficient mice. This is possibly due to the activity of IL-1R8 in dampening *E. coli* induced activation of tubular epithelial cells. Indeed, *in vitro* stimulation of IL-1R8-deficient tubular epithelial cells with LPS or heat-killed *E. coli* resulted in increased production of TNFα and chemokines (CXCL1, CCL2, and CCL3) and a mild increased expression of the adhesion molecule ICAM-1. Finally, IL-1R8 mRNA transcript was reduced in kidneys during the early phase of *E. coli* induced pyelonephritis (41). A recent *in vitro* study demonstrated that IL-1R8 regulates the responsiveness to LPS in a human BEC line, and, in line with previous studies, IL-1R8 mRNA and protein expression were downregulated upon stimulation with LPS (42). IL-1R8 silencing in BECs caused increased LPS-induced IL-6 and IL-8 production and this correlated with enhanced phosphorylation rate of JNK, p38, and ERK1/2. Finally, IL-1R8 siRNA transfected cells developed an impaired LPS tolerance, suggesting that IL-1R8 is involved in this process. Overall, these findings highlighted the importance of IL-1R8 in the negative regulation of urinary tract and renal response to bacterial infections (42).

Similar to what was observed in UTI, during pneumonia and sepsis induced by *Streptococcus pneumoniae* in the mouse, IL-1R8 deficiency was associated with delayed mortality, reduced bacterial load in the lungs, and reduced dissemination of the infection (68). Increased interstitial and perivascular inflammation was observed in IL-1R8-deficient lungs mice in the early phase of infection. Thus, IL-1R8 suppressed the protective antibacterial immune response in *S. pneumoniae* induced pneumonia (68).

Murine models of *Citrobacter rodentium* infection resemble human intestinal infections driven by enteric bacterial pathogens, such as enterohemorrhagic *E. coli* (EHEC) and *Salmonella typhimurium*. Upon *C. rodentium* infection, IL-1R8 deficiency correlated with accelerated IEC proliferation and enhanced pro-inflammatory and antimicrobial response. However, IL-1R8 was shown to be protective in this model, in terms of weight loss, intestinal damage, colitis score, and intestinal bacterial burden. IL-1R8 protective function was dependent on IL-1R1–MyD88 signaling, but not on TLR2 or TLR4 pathways, indicating that IL-1R1 may be the key receptor targeted by IL-1R8 in this context. Infected IL-1R8-deficient mice underwent a more rapid and dramatic loss of commensal flora, compared with controls. In infected mice, the microbiota depletion was directly dependent on the exacerbated antimicrobial response occurring in IL-1R8-deficient mice that favored pathogen colonization. Thus, IL-1R8-mediated regulation of IECs is responsible for the inhibition of a strong antimicrobial response that would otherwise lead to a rapid depletion of the commensal microbiota, during intestinal infection. In turn, the absence of competing microflora would favor the colonization by bacterial pathogens (69).

These studies indicate that IL-1R8 plays a crucial role in favoring the maintenance of a delicate equilibrium between the protective immune response against infections and the development of detrimental inflammation and host injury. The activity of IL-1R8 is therefore strictly dependent on the context and several lines of evidence suggest that during homeostasis the constitutive expression of IL-1R8 protects against inappropriate responses, whereas its downregulation during acute inflammatory stimulation enhances the effectiveness (and pathogenic potential) of antibacterial host defense.

ROLE OF IL-1R8 IN AUTOIMMUNITY AND ALLERGY

The interest in studying IL-1R8 involvement in autoimmunity arises from the fact that ILRs and TLRs are key players in the pathogenic mechanisms of autoimmune disorders (**Figure 4**). In particular, IL-1 regulates the differentiation and function of Th17 cells, which are involved in inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis, psoriasis, and inflammatory bowel disease (IBD) (70). IL-33 is a driver of type 2 inflammatory responses and is implicated in allergy and asthma (71).

Gulen et al. recently showed that IL-1R8 was induced during Th17 cell polarization and that it controlled Th17 cell differentiation, expansion, and effector functions through the direct inhibition of IL-1 signaling in T cells. An increased phosphorylation rate of JNK, mTOR, and 4EBP1 was observed in IL-1R8-deficient T cells, upon stimulation with IL-1. In particular, IL-1-induced mTOR pathway was critical for the IL-1R8-mediated modulation of Th17 response. Thus, IL-1R8 emerged as a key regulator of IL-1 activity in Th17 cells, and it was also observed to be involved in the Th17-mediated development of central nervous system (CNS) autoimmune disorders. Indeed, IL-1R8-deficient mice revealed higher susceptibility to experimental autoimmune encephalomyelitis (EAE), due to an increased Th17 infiltrate in the CNS and enhanced Th17 polarization and pathogenic functions (61).

Toll-like receptors and ILRs are involved in the pathogenesis of RA (72–74). IL-1R8 was shown to suppress the spontaneous release of cytokines in human RA synovial cells *in vitro*, suggesting its involvement in the modulation of chronic inflammation in RA. *In vivo* experiments supported this evidence, since IL-1R8-deficient mice developed a more severe disease in both zymosan-induced arthritis and collagen antibody-induced arthritis models, which was associated with increased cellular infiltration into the affected joints. IL-1Ra treatment reduced the susceptibility of IL-1R8-deficient mice in zymosan-induced arthritis, suggesting that IL-1 played a central role in this model. However, the phenotype of IL-1R8-deficient mice was not completely rescued by the treatment, possibly because other TLR (e.g., TLR2) or ILR ligands are implicated in zymosan-induced arthritis pathogenesis (74). In agreement with this study, IL-1R8 expression was reduced in peripheral blood of patients with psoriatic arthritis, compared with healthy donors (36). Moreover, IL-1R8 deficiency caused

enhanced susceptibility to psoriasis, associated with increased infiltration and activation of γδ T cells, in both Aldara- and rIL-23-induced psoriasis models. Interestingly, IL-R8 directly modulated IL-1-driven IL-17A expression by γδ T cells *in vitro* and *in vivo*, and IL-17A depletion abolished the phenotype observed in IL-1R8-deficient mice (75).

Increasing evidences implicated IL-1R8 in the pathogenesis of systemic lupus erythematosus. Indeed, altered TLR signaling in DCs and B cells is one of the driving mechanisms of this autoimmune disorder. In particular, immune complexes containing the lupus autoantigen U1snRNP or nucleosomes activate DCs and autoreactive B cells *via* TLR7 and TLR9, respectively (76, 77). In the mouse, IL-1R8 deficiency alone did not induce autoimmunity against DNA. However, IL-1R8 deficiency in C57BL/$6^{lpr/lpr}$ mice, which develop delayed autoimmunity due to impaired Fas-induced apoptosis of autoreactive B and T cells, caused increased activation of DCs and B cells and production of pro-inflammatory cytokines (CCL2, IL-6, and IL-12p40) and B cell antiapoptotic mediators (Baff/BlyS and Bcl-2). Moreover, IL-1R8 regulated B cell proliferation, upon exposure to RNA and DNA immune complexes or other TLR agonists. IL-1R8-deficient C57BL/$6^{lpr/lpr}$ mice also displayed and increased production of autoantibodies (anti-dsDNAIgG, anti-nucleosome, anti-Sm antigen, anti-snRNP, and rheumatoid factor) and presented a massive lymphoproliferative disorder, associated with enhanced autoimmune lung disease, lupus nephritis, and hypergammaglobulinemia, compared with IL-1R8-competent C57BL/$6^{lpr/lpr}$ controls (78). In line with this, IL-1R8 was also protective in a model of hydrocarbon oil-induced lupus, in which it modulated TLR7-mediated activation of DCs and expansion of autoreactive lymphocyte clones. IL-1R8 is therefore involved in the regulation of DC and B cell activation, by preventing exacerbated autoimmune reactions, lymphoproliferation, and tissue damage in SLE (79). The data in the mouse were supported by recent analysis of IL-1R8 involvement in SLE in human. A case study of a cohort of SLE patients revealed a reduced frequency of IL-$1R8^+$ $CD4^+$ T cells in the peripheral blood of SLE patients compared with healthy individuals. Moreover, the frequency of IL-$1R8^+$ $CD4^+$ T cells was further reduced in SLE patients with nephritis, compared with those without nephritis (80). Zhu et al. showed that B cells from SLE patients displayed an upregulation of TLR7 and TLR9 compared with healthy controls, but the response to corresponding ligands was normal or even reduced. The authors suggested that this could be explained by the enhanced IL-1R8 expression in SLE B cells, even though the pathological significance of IL-1R8 increase in this context is still unclear (81). A genetic analysis of allelic variants of the IL-1R8 gene in a large European-descent population showed no correlation between IL-1R8 polymorphisms and SLE, but the analysis was restricted to a single missense SNP (rs3210908) (82). More recently, another genetic variant of IL-1R8 (rs7396562) was identified, and it was demonstrated to correlate with the susceptibility to SLE, in a Chinese population (83).

IL-33 signaling is a key driver of type 2 immunity, which favors protective immune responses in parasite infections and tissue repair but is also involved in pathological conditions, such as asthma, allergy, and eosinophilia (84). IL-33 receptor (IL-1R4/ST2) affects innate and adaptive lymphoid cells (ILCs and Th2), inducing the production of type 2 cytokines (IL-4, IL-5, IL-13), and can be targeted by IL-1R8. Indeed, IL-1R8 inhibits IL-33-mediated signaling in Th2 cells, controlling the production of type 2 cytokines *in vitro* and *in vivo* (45). IL-1R8-deficient mice were shown to be hyper responsive to IL-33, in terms of lung inflammation, splenomegaly, and increased serum levels of IL-5 and IL-13 (45). Moreover, in a model of allergic pulmonary inflammation induced by OVA, IL-1R8 deficiency was associated with increased leukocyte lung infiltration, IL-5 and IL-4 levels, and OVA-specific IgE induction, due to an exacerbated Th2 response (45). These results indicate that IL-1R8 serves as a negative feedback control in Th2 polarization and restimulation, thus controlling allergic inflammatory responses. However, a genetic study performed on a cohort of Japanese asthma patients revealed that none of the alleles or haplotypes of IL-1R8 identified were associated with asthma susceptibility or asthma-related conditions (85).

These data demonstrate the relevance of the control mediated by IL-1R8 on T and B lymphocytes and on antigen-presenting cells in the development of autoimmune and allergic diseases.

ROLE OF IL-1R8 IN KIDNEY STERILE INFLAMMATION

IL-1R8 is expressed at high levels in the kidney, in particular, by tubular epithelial cells and immune cells such as DCs and macrophages. Immunohistochemical analysis revealed extensive IL-1R8 positivity in the majority of tubular epithelial cells of the renal cortex, showing a predominant expression at the apical side of renal proximal tubules (29). IL-1R8 was shown to be a key player in sterile kidney diseases, by regulating TLR activation by nucleosomes and DAMPs, released during ischemic cell necrosis and associated with pathological conditions, such as lupus nephritis, postischemic acute renal failure, or kidney transplantation (78, 79, 86, 87) (**Figure 4**).

In a postischemic renal failure model, IL-1R8 deficiency was associated with increased renal injury, due to a massive activation of myeloid cells, increased intrarenal cytokine and chemokine production and increased leukocyte recruitment. In this model of sterile inflammation, DAMPs activate immune cells, in particular, neutrophils and macrophages, mainly *via* TLR4 and TLR2. In a model of renal ischemia/reperfusion, bone marrow chimeric mice demonstrated a major role of IL-1R8 in the hematopoietic compartment, since $Il1r8^{+/+}$ animals transplanted with $Il1r8^{-/-}$ hematopoietic cells reproduced the phenotype of IL-1R8-deficient mice (86). In line with this, in a mouse model of fully mismatched kidney allotransplantation, IL-1R8-deficient grafts were less tolerated compared with control grafts, leading to acute rejection. Moreover, IL-1R8 deficiency was associated with an enhanced ILR- and TLR-driven posttransplant kidney inflammatory response, in particular, due to increased neutrophil and macrophage infiltrate and higher expression of TNFα and chemokines. An amplified adaptive response was also observed in IL-1R8-deficient mice, in which expansion and maturation of DCs was enhanced and the immune response against donor antigens was exacerbated. The higher allostimulatory activity of DCs may possibly explain the increased frequency of reactive T cells and reduced Treg development in absence of IL-1R8. Thus,

IL-1R8 plays a key role in the regulation of the allogeneic immune response *in situ* and is involved in graft survival (87).

In case of renal fibrosis induced by unilateral ureteral obstruction (UUO), IL-1R8 deficiency did not modulate the renal pathology. Indeed, IL-1R8-deficient mice did not show any difference compared with controls in this model, in terms of mRNA transcript of pro-inflammatory and profibrotic mediators, leukocyte recruitment, and renal injury (88). These data are in line with the evidence that TLR2, TLR9, and MyD88 signaling are not involved in the pathogenesis of postobstructive renal fibrosis (89).

ROLE OF IL-1R8 IN BRAIN INFLAMMATION

IL-1R8 is expressed in the brain by neurons, microglia, and astrocytes, and it was shown to be involved in the regulation of LPS responsiveness in the brain (16, 33, 90) (**Figure 4**). Indeed, IL-1R8 deficiency was associated with a massive LPS-induced inflammation in the brain. In response to LPS, IL-1R8 negatively regulated CD40, ICAM, and cytokine (IL-6 and TNFα) mRNA expression in microglial cells and cytokine production in hippocampal tissue. This is in line with an increased hippocampal expression of CD14 and TLR4, and NFκB activation in IL-1R8-deficient mice (91).

In addition, it has been observed that cognitive and synaptic functions, such as novel object recognition, spatial reference memory, and long-term potentiation (LTP), were impaired in IL-1R8-deficient mice, in absence of any external stimulus. This was associated with a higher expression of IL-1R1 and TLR4 and an enhanced activation of IL-1R1 and TLR4 downstream signaling molecules (IRAK1, c-Jun, JNK, and NFκB) (92). Indeed, treatment with IL-1Ra and anti-TLR4 antibody and the inhibition of JNK and NFκB rescued the deficit in LTP in IL-1R8-deficient animals, suggesting a central role of IL-1R1 and TLR4 signaling in this model. IL-1α and high mobility group box 1 (HMGB1), which activate IL-1R1 and TLR4, respectively, were proposed to play a central role in the phenotype observed and the expression levels of both molecules were increased in IL-1R8-deficient mice. These findings revealed a key role of IL-1R8 in modulating the inflammatory response associated with synaptic and cognitive decline and identified IL-1α and HMGB-1 as central mediators in this process (92). Moreover, IL-1R6 antagonist (IL-36Ra) inhibits the IL-1- and LPS-induced inflammatory response in glial cells, and this effect was absent in mixed glia prepared from IL-1R8-deficient mice, suggesting the involvement of IL-1R8 in the anti-inflammatory activity of IL-36Ra, possibly mediated through the production of IL-4 (16). Finally, a recent study showed that IL-1R8 acted as a negative regulator of β-amyloid (Aβ) peptide-induced TLR2 signaling in the brain. Aβ is the main component of neuritic plaques in Alzheimer's disease (AD) and the primary mediator of the AD-associated neuroinflammation (49). The response to the TLR2 agonist (Pam_3Cys_4) was increased in glial cells from IL-1R8-deficient mice and Aβ-induced inflammation in the brain was enhanced in IL-1R8-deficient mice, in terms of cytokine production (IL-6 and TNF-α). *In vitro* experiments demonstrated that Aβ treatment increased the expression of TLR2 and decreased the expression of IL-1R8 in microglia and this was mimicked by the treatment with the TLR2 agonist Pam_3Cys_4. Anti-TLR2 treatment of microglia attenuated the inflammatory response and the impairment in LTP, both induced by Aβ, confirming the central role of TLR2 in the Aβ-induced neuroinflammation. Interestingly, TLR2 neutralization also led to an increase of IL-1R8 mRNA (49). These findings highlighted the key role of IL-1R8 in the modulation of TLR2-induced inflammation in the brain and its relevance in a potential therapeutic approach targeting TLR2 in AD-related pathology.

ROLE OF IL-1R8 IN INTESTINAL INFLAMMATION AND INTESTINAL CANCER

IL-1R8 was demonstrated to be a key regulator of intestinal homeostasis (**Figure 4**). IECs are intrinsically hyporesponsive to bacterial products, thus not only preventing exaggerate inflammatory responses against the commensal flora but also limiting the enteric host defense (56). On the other hand, gut microflora-mediated activation of ILRs and TLRs provides the survival signals for IECs and this pathway is targeted by IL-1R8, which is therefore involved in controlling proliferation and survival in colon crypts (93). IL-1R8-deficient mice displayed constitutive NFκB and JNK activation and increased expression of Cyclin D1 and Bcl-xL. The effect was further enhanced upon treatment with IL-1 or LPS, and it was dependent on the commensal flora, since microbiota depletion rescued the phenotype (93). This phenotype in healthy mice was not confirmed by other studies (56, 94), probably because of the animal house-dependent variation of the microflora. The relevance of IL-1R8 expression in IEC in terms of response to intestinal infections and control of commensal microbiota has been described above.

In dextran sodium sulfate (DSS)-induced colitis, IL-1R8 deficiency is associated with an exacerbated intestinal inflammation, in terms of weight loss, intestinal bleeding, local tissue damage, and a reduced survival. This correlates with an increased leukocyte infiltration in the intestine and higher level of pro-inflammatory cytokines (TNFα, IL-6, IL-1β, IL-12p40, IL-17), chemokines (CXCL1, CCL2), and prostaglandins. Experiments with bone marrow chimeric mice demonstrated that the regulatory function exerted by IL-1R8 occurs in epithelial cells, in both DSS- and enteric pathogen-induced colitis (56, 93).

Epidemiological studies have shown that chronic inflammation, both dependent on infectious agents or not, can increase the risk of cancer. The hallmarks of cancer-related inflammation are comparable to those observed in chronic inflammatory conditions: inflammatory cells and mediators are present in the tumor tissue, and they are implicated in tissue repair, remodeling, and angiogenesis. This "smoldering inflammation" occurs even in tumors that are not directly caused by an inflammatory trigger. Cancer-related inflammation depends on two possible pathways: an intrinsic pathway, driven by oncogenic mutations that cause both neoplasia and inflammation, or an extrinsic pathway, driven by inflammatory conditions that favor tumor development (e.g., colitis-associated intestinal cancer) (95–98). Several studies have

revealed a crucial role of ILR and TLR signaling in this context, in which NFκB is one of the key orchestrators, and that IL-1R8 plays a protective role in the pathogenesis of cancer-related inflammation in different murine models of colon cancer (98). IL-1R8 was studied in a model of CAC, induced by the treatment with the procarcinogen azoxymethane (AOM), followed by DSS, which favors chronic inflammation (93, 94). This model mimics intestinal cancer that develops in chronic IBD patients, in particular, ulcerative colitis patients. In the AOM–DSS CAC model, IL-1R8 deficiency was associated with exacerbated inflammation in the intestine, leading to increased susceptibility to cancer development, in terms of number, size, and severity of lesions. IL-1R8 negatively regulated intestinal permeability, *in situ* production of pro-inflammatory cytokines and chemokines and prostaglandin E_2, and the expression of NFκB-induced genes involved in cell survival and proliferation (Bcl-xL and Cyclin D1) (93, 94). In this context, chemokines favored cancer progression, influencing the extent and type of leukocyte infiltrate (e.g., recruiting Th2 and Treg cells) and driving tumor cell and endothelial cell growth and migration (99, 100). Moreover, increased levels of IL-10 and TGF-β were observed in tumors of IL-1R8-deficient mice, reflecting an immunosuppressive microenvironment that inhibited T cell-dependent antitumoral immunity (100). The expression of IL-6, which promotes cancer growth, was also increased in the intestine of IL-1R8-deficient mice (101, 102). IL-1R8 overexpression in gut epithelial cells abolished the susceptibility of IL-1R8-deficient mice to CAC development, suggesting that the regulatory activity of IL-1R8 in IECs plays a central role in this model (93). Since commensal microflora-derived stimuli are necessary for the homeostasis of colon epithelium and are involved in colitis-associated carcinogenesis, IL-1R8 regulation may be possibly dependent on its direct modulation of microbiota-activated TLRs (103). However, IL-1R8-mediated targeting of other TLR- and ILR-related pathways cannot be excluded in this model.

IL-1R8 involvement in colon cancer was also investigated in the genetic $Apc^{min/+}$ model, in which tumor initiation is caused by loss of heterozygosity (LOH) of the tumor suppressor Apc and which mimics the familial adenomatous polyposis syndrome (104). In $Apc^{min/+}$ mice, IL-1R8 deficiency led to an increased susceptibility to cancer development, due to a more sustained activation of the Akt/mTOR pathway, which plays a crucial role in tumor initiation (105). In agreement with the CAC model, commensal bacterial played a pivotal role in colonic tumorigenesis, suggesting that IL-1R8 regulation might occur through the inhibition of TLR signaling, even if mTOR enhanced activation was also observed upon stimulation of epithelial cells with IL-1. Thus, IL-1R8 exerts an antitumoral activity by suppressing IL-1- and TLR-induced mTOR-mediated cell cycle progression and consequent genetic instability (62).

A recent study has investigated the role of IL-1R8 in human colorectal cancer, demonstrating that colon tumors express lower level of IL-1R8 compared with healthy tissues and that IL-1R8 is frequently inactivated in human colorectal cancer (32). Indeed, Zhao et al. identified a dominant-negative isoform of IL-1R8 ($IL\text{-}1R8^{\Delta E8}$) and RNA sequencing data demonstrated that the expression level of this isoform increased in human colon cancer, compared with healthy tissue. The $IL\text{-}1R8^{\Delta E8}$ isoform originated from a transcript that lacks the exon 8 of the gene and exhibited compromised integrity of the TIR domain, increased retention in the cytoplasm, and reduced N-linked glycosylation. The cytoplasmic retention caused a decrease in the cell surface expression of IL-1R8 and a consequent loss of its inhibitory activity. Moreover, $IL\text{-}1R8^{\Delta E8}$ isoform was shown to be able to interact with full-length IL-1R8, acting as an antagonist of IL-1R8 and thus suppressing its function. To investigate the mechanism responsible for $IL\text{-}1R8^{\Delta E8}$ isoform synthesis in tumor cells, sequence analysis were performed and predicted that exon 8 would be intrinsically a "weak" exon, with high probability of exclusion. Exon 8 also displayed a binding site for CTCF, a factor that favors the inclusion of weak exons, and since the binding can be reduced by methylation, hypermethylation was proposed to be the strategy followed by cancer cells that leads to $IL\text{-}1R8^{\Delta E8}$ isoform expression. Indeed, treatment with decitabine, a methyltransferase inhibitor, reduced $IL\text{-}1R8^{\Delta E8}$ isoform expression. To model the impact of $IL\text{-}1R8^{\Delta E8}$ isoform in colon carcinogenesis in the mouse, gut epithelium-specific IL-1R8 transgenic mice were generated, expressing a mutant form of IL-1R8 ($IL\text{-}1R8^{N85/101S}$) that mimics $IL\text{-}1R8^{\Delta E8}$ isoform or wild-type IL-1R8 as a control. In both AOM and AOM–DSS models, the presence of wild-type IL-1R8 in IECs protected the mice from the development of colon cancer. On the contrary, mice expressing $IL\text{-}1R8^{N85/101S}$ isoform had the same phenotype as IL-1R8-deficient mice, suggesting that complex glycan modifications and cell surface expression are necessary for IL-1R8 functional activity *in vivo* (32). Thus, IL-1R8 alternative splicing is an escape mechanism adopted by tumor cells to inactivate IL-1R8 through the generation of a dominant-negative isoform.

ROLE OF IL-1R8 IN CHRONIC LYMPHOCYTIC LEUKEMIA

Both genetic defects and microenvironment stimuli contribute to chronic lymphocytic leukemia (CLL) development and progression. Moreover, factors originating from the microenvironment are involved in the selection and expansion of the malignant clone (106, 107). Human malignant B cells expressed lower levels of IL-1R8 mRNA than normal B cells, and accordingly, in the well-established transgenic mouse model of CLL (TCL1), $CD19^+$ B cells expressed lower levels of IL-1R8 mRNA transcript, compared with controls (107–110). IL-1R8 deficiency did not affect B cell compartment in healthy mice, whereas it correlated with an earlier and more severe appearance of monoclonal B cell expansion and a reduced mouse life span in TCL1 transgenic mice, mimicking the aggressive variant of human CLL (110). These findings revealed IL-1R8 inhibitory role in CLL initiation and progression, even though the molecular mechanism is still unclear (**Figure 4**). Endogenous TLR or ILR ligands are known to be involved in CLL and may be candidate targets of IL-1R8.

CONCLUDING REMARKS

It is well established that IL-1R8 acts as negative regulator of ILR and TLR signaling, which are key pathways involved in inflammation and immunity. Early studies are consistent with the

fact that IL-1R8 is a conserved and widely expressed molecule that plays a key role in the modulation of inflammation, tissue damage, and host defense against infections, autoimmunity, and cancer. Its mechanism of action is probably dependent on the interaction with TIR domain containing-signaling molecules, preventing the signalosome formation and activation. Although IL-1R8 was considered an orphan receptor, IL-36Ra has been proposed as brain-specific IL-1R8 ligand. Recently, IL-1R8 has been shown to act as a coreceptor for IL-37–IL-1R5/IL-18Ra and to be required for the anti-inflammatory activity of IL-37 (11, 51, 52). Indeed, IL-37 needs IL-1R8 to trigger a rapid anti-inflammatory program, revealing a novel role for IL-1R8. In addition to its regulatory activity, IL-1R8 therefore emerges as a coreceptor molecule, which able to boost IL-37-mediated signaling. Since IL-37 ameliorates insulin resistance and obesity-induced inflammation (54), it will be important to address whether IL-1R8 is involved in these contexts, preserving glucose tolerance and insulin sensitivity and reducing inflammation in the adipose tissue.

IL-1R8 regulates the metabolism, activation and polarization of several innate and adaptive immune cell types, as well as of non-hematopoietic cells. Thus, it plays a non-redundant role in the regulation of both pathogen-induced and sterile inflammation, managing the delicate equilibrium between host defense and detrimental inflammation.

The IL-1 system and TLR ligands affect all cells of the immune system, as well as epithelial, endothelial, and stromal cells. Since IL-1R8 is expressed by most cell types, but its functional characterization is still incomplete, future studies will be important to identify the peculiar role of IL-1R8 in the regulation of ILR- and TLR-dependent activation in specific cell types.

The involvement of IL-1R8 in human pathologies needs to be further investigated, since it could emerge as a potential target in several inflammatory contexts. Results showing that IL-1R8 inactivation is an escape mechanism adopted by cancer cells in human colon cancer are of particular importance, since they represent the first strong genetic evidence of the relevance of IL-1R8 in human disease. Further analysis of IL-1R8 polymorphisms and epigenetic regulations of IL-1R8 gene represent important future directions to gain a more precise view of IL-1R8 involvement in human diseases.

Given the well-established role of IL-1R8 as a key anti-inflammatory molecule in a broad spectrum of contexts, its targeting holds promise of innovative therapies in several pathological conditions.

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REVIEW
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Pyruvate Kinase M2: A Potential Target for Regulating Inflammation

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Pyruvate kinase (PK) is the enzyme responsible for catalyzing the last step of glycolysis. Of the four PK isoforms expressed in mammalian cells, PKM2 has generated the most interest due to its impact on changes in cellular metabolism observed in cancer as well as in activated immune cells. As our understanding of dysregulated metabolism in cancer develops, and in light of the growing field of immunometabolism, intense efforts are in place to define the mechanism by which PKM2 regulates the metabolic profile of cancer as well as of immune cells. The enzymatic activity of PKM2 is heavily regulated by endogenous allosteric effectors as well as by intracellular signaling pathways, affecting both the enzymatic activity of PKM2 as a PK and the regulation of the recently described non-canonical nuclear functions of PKM2. We here review the current literature on PKM2 and its regulation, and discuss the potential for this protein as a therapeutic target in inflammatory disorders.

Keywords: PKM2, inflammation, HIF-1α, glycolysis and oxidative phosphorylation, immunometabolism, cancer

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INTRODUCTION

Cancer cells and most activated immune cells display a radical shift in metabolism becoming highly dependent on glucose, which is metabolized through an increased rate of aerobic glycolysis, a metabolic state termed the Warburg effect (1, 2). Normal cell metabolism involves generating energy through a relatively low rate of glycolysis giving rise to pyruvate, which enters the mitochondrial tricarboxylic acid (TCA) cycle. Pyruvate undergoes a series of oxidizing reactions, thereby generating ATP. In contrast, cells displaying Warburg metabolism will instead rely on an increased rate of glycolysis to generate energy. Pyruvate is now diverted away from the oxidative phosphorylation of the TCA cycle and is converted to lactate by lactate dehydrogenase (LDH) in the cytosol. Since this process allows for ATP generation during low oxygen, it may provide an explanation for the tolerance of cancer cells to extreme local hypoxia providing the cells with obvious growth advantages compared to surrounding tissue and immune cells. The high rate of glycolysis ensures that the increased demand for biosynthetic precursors, including proteins, lipids, and nucleic acids, is met. As glucose is broken down to pyruvate, intermediates of glycolysis are used for nucleotide and amino acid synthesis as well as for nicotinamide adenine dinucleotide phosphate (NADPH) production through the pentose phosphate pathway (PPP). Furthermore, fatty acids, required for membrane lipid synthesis, are synthesized from citrate in the cytosol generating acetyl-CoA. This metabolic reprograming renders the cells highly dependent on glucose, which can lead to nutrient competition within the tumor microenvironment, a scenario that has been shown to directly contribute to cancer progression (3). Interest in the metabolic state of immune cells during inflammation and infection has recently surged as it is becoming clear that resting immune cells display distinct

metabolic configurations compared to activated immune cells. Hence, the field of immunometabolism has evolved incorporating the concept that alterations in metabolism may influence the phenotype of immune cells and regulate transcriptional, as well as posttranscriptional events, upon activation.

Pyruvate kinase (PK) is the enzyme responsible for the final rate-limiting step of glycolysis, catalyzing phosphoenolpyruvic acid (PEP) and ADP to pyruvate and ATP. Due to the vast literature supporting the role of the PK isoform PKM2 as a key regulator of the metabolic changes observed in cancers [reviewed recently in Ref. (4, 5)], an interest in defining the potential role of this protein in inflammation has emerged. Here, we will review our understanding of PKM2's regulation and functions in cancer and immune cells, and examine the current literature on its role in inflammatory disorders while discussing the potential in targeting PKM2 function therapeutically.

PKM2 GENE EXPRESSION

Pyruvate kinase isozyme type M2 (PKM2) is one of the four PK isoforms expressed in mammalian cells and is generally accepted to be the embryonic isoform, also expressed in cancer and normal proliferating cells such as lymphocytes and intestinal epithelial cells (6–8). PKM1 is the alternatively spliced product of the same *Pkm* gene (9–11). PKM1 has high PK enzymatic activity and is expressed in tissues with increased catabolic demands such as heart, muscle, and brain. The remaining isoforms PKL and PKR are expressed in the liver and red blood cells, respectively.

PKM1 and PKM2 are generated by exclusive alternative splicing of a pair of mutually exclusive exons of the *Pkm* pre-mRNA. The full open reading frame is composed of 12 exons where inclusion of exon 9 will generate PKM1 transcript, and exon 10 is specific for expression of PKM2 (9, 11). Although only different by a small number of amino acids, the two gene products display distinct function and characteristics due to the isoform specific exons giving rise to structural differences in the fructose-1,6-bisphosphate (FBP)-binding site (discussed below) and dimer–dimer interface.

Two regulatory events have been identified resulting in reciprocal effects on the mutually exclusive exons 9 and 10, such that exon 9 is repressed and exon 10 is activated. First, three heterogeneous nuclear ribonucleoproteins (hnRNPs) polypyrimidine tract-binding protein (PTB, also known as hnRNPI), hnRNPA1 and hnRNPA2, have been shown to bind specifically and repressively to sequences flanking exon 9 resulting in exon 10 inclusion (12). These hnRNP proteins are in turn controlled by c-Myc, contributing to deregulated PK mRNA splicing in cancer. Second, the serine/arginine-rich splicing factor 3 (SRSF3) will, through binding within exon 10, promote its inclusion, resulting in increased transcript for PKM2 (13).

Evidence supports a switch in the expression of PKM1 in favor of PKM2 during malignant transformation such that expression of PKM1 decreases proportionally as the expression of PKM2 increases. However, this has recently been reevaluated, suggesting that upregulation of PKM2 is primarily due to the elevated transcriptional levels of the entire *Pkm* gene, where no decrease in PKM1 expression is observed, rather than due to a switch in isoform expression (7, 14, 15).

In addition, efforts to identify specific micro-RNAs (miRs) that target PKM2 expression have revealed a possible role for miR-let-7a, miR-122, miR-326, miR-133a, and miR133b (16–19); however further validation will be required.

REGULATION OF PKM2 ACTIVITY

Since PKM2 plays a critical role in the metabolic changes observed in cancer and inflammation, discovering the mechanism of the regulation of PKM2 activity is important to our understanding of how alterations in cellular metabolism are controlled.

The enzymatic activity of PK is, in part, determined by the configuration of the enzyme into a tetramer, dimer, or monomer. PKM1 naturally exists as a stable tetramer, which allows for optimal binding of the substrate PEP. Experiments using partially denatured PKM1 demonstrate that the monomeric and dimeric forms retain only a fraction of the PK activity observed with PKM1 as a tetramer (20).

On the other hand, PKM2 requires binding of an activator in order to trigger high enzymatic PK activity (**Figure 1**). PKM2 can be allosterically activated by multiple endogenous regulators that affect binding affinity of PEP to the active site on the enzyme. One such example is FBP, an upstream glycolytic intermediate (21). In the absence of FBP, PKM2 even as a tetramer has a low affinity for PEP. Binding of FBP to PKM2, at a site distinct to the active PEP binding site, will promote and stabilize tetramer formation of PKM2 as well as increase PEP binding affinity, making the kinetic parameters of PKM2 almost identical to those of PKM1.

In addition to FBP, other non-glycolytic metabolites, amino acids, and small molecules also affect PKM2 activity. The small-molecules DASA 58 and TEPP 46 are highly specific activators of PKM2 (22–24). They bind to PKM2, at a site distinct from the FBP binding site, resulting in PKM2 forming a tight tetramer with PKM1-like kinetic properties, an event that is resistant to inhibition by tyrosine phosphorylation (see below). In cancer cells, as well as in activated macrophages, the increase in PKM2 expression and the decrease in overall PK activity will allow for glycolytic intermediates to be channeled into production of, for example, serine and glycine (23). This increased metabolic flux into serine and glycine biosynthetic pathways is critical for cancer cell survival [for review, see Ref. (25)]. It is, therefore, not surprising that a link between serine abundance and PKM2 activity has been reported, where serine is shown to act as a natural ligand and allosteric activator of PKM2 (26) (**Figure 1**). In a similar manner, cellular accumulation of the *de novo* purine nucleotide synthesis intermediate SAICAR promotes cancer cell survival through interaction of SAICAR with PKM2 (27). Since SAICAR is synthesized as a by-product of glutaminolysis and can be cleaved to provide the TCA cycle with fumarate, this interaction allows for a potential mechanism to convey cellular metabolic demands to PKM2.

Death-associated protein kinase (DAPk) is a serine/threonine kinase with tumor suppressor properties that was identified as binding to PKM2 in a yeast-two-hybrid screen (28). The direct

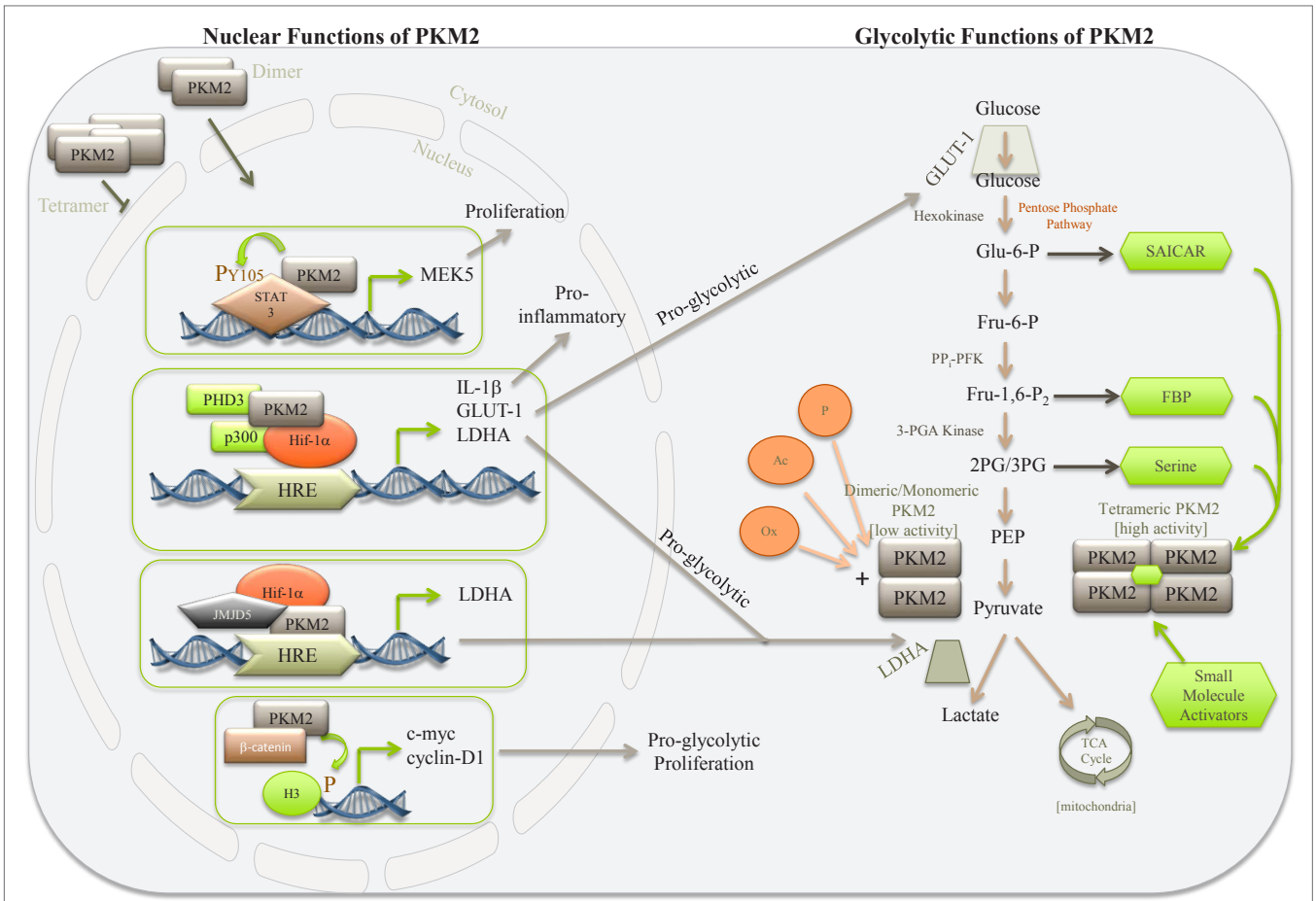

FIGURE 1 | Simplified diagram depicting some of the nuclear and glycolytic functions and regulation of PKM2. PKM2 is the major PK isoform expressed in cancer, proliferating cells, and populations of activated immune cells. The activity of PKM2 can be controlled by stabilizing or destabilizing the formation of PKM2 tetramers. Allosteric activation of PKM2 by, for example, succinylaminoimidazolecarboxamide ribose-5′-phosphate (SAICAR), serine, FBP, or small-molecule activators, such as TEPP 46 and DASA 58, encourages tetramer formation of PKM2, thereby promoting the last rate-limiting step of glycolysis, converting PEP to pyruvate. Pyruvate will enter the TCA cycle of the mitochondria where it is used to generate ATP through oxidative phosphorylation. In the absence of allosteric activators PKM2 primarily takes on a dimeric or monomeric form, which through lacking enzymatic activity will give rise to accumulation of glycolytic intermediates, thereby meeting the requirements for biosynthetic precursors of the activated or proliferating cell. Dimeric PKM2 can translocate to the nucleus where it further promotes aerobic glycolysis through Hif-1α co-activation, aiding expression of proglycolytic genes, such as *ldha* and *glut-1*, as well as proinflammatory IL-1β. Furthermore, PKM2 can interact with other transcription factors, such as STAT3, as well as histone H3 and JMJD5, thereby further regulating genes important for proliferation and glycolysis.

binding of DAPk to PKM2 increases the PK activity of PKM2 and provides another means of regulating the cellular glycolytic rate.

PKM2 enzymatic activity can also be allosterically inhibited. Binding of phenylalanine to a site distinct from both the active site and the FBP binding site will decrease the affinity of PEP to PKM2 through stabilizing PKM2 in an inactive tetrameric form (29, 30). Alternatively, the same site can be occupied by alanine, a scenario that promotes dissociation of PKM2 into a less-active dimeric form. Moreover, the thyroid hormone triiodo-L-thyronine (T3) stabilizes an inactive monomeric form of PKM2, an inhibitory event that can be overcome by binding of PKM2 to FBP (31, 32). Furthermore, tyrosine phosphorylation has been reported as a mechanism for negatively regulating PKM2, thereby promoting tumor growth (33). This phosphorylation event on tyrosine 105 (Y105) disrupts tetramer formation of PKM2 by releasing FBP, thereby regulating the switch from oxidative phosphorylation to aerobic glycolysis. In addition, Y105 phosphorylation of PKM2 by nucleophosmin–anaplastic lymphoma kinase (NPM–ALK) results in decreased enzymatic activity of PKM2 in anaplasic large-cell lymphoma, supporting a role for NPM–ALK in the regulation of metabolism (34). Another kinase important in regulating PKM2 activity in hepatocellular carcinoma is the proapoptotic enzyme JNK-1, which phosphorylates PKM2 at threonine 365. JNK-1 activity in turn is negatively regulated by poly(ADP-ribose) polymerase (PARP)14, which thereby regulates Warburg metabolism, and promotes cell survival and tumor growth (35). A role for *O*-GlcNAc transferase regulating serine phosphorylation and *O*-GlcNAcylation levels of PKM2 in colorectal cancer has also been reported (36).

Shikonin and its derivatives are also inhibitors of PKM2 activity (37, 38). Shikonin is a naturally occurring naphthoquinone isolated from the herb *Lithospermum erythrorhizon* and has been investigated as a potential anticancer drug. Shikonin and its analog alkannin showed promising selectivity toward PKM2, since they did not inhibit PKM1 and PKL activity at IC_{50} to PKM2 (38).

Superfluous production of reactive oxygen species (ROS), commonly associated with cancer cells, requires detoxification by the tripeptide glutathione (GSH). GSH in turn is maintained in the cell by the reduced form of NADPH, which is provided by the PPP. This increase in intracellular ROS has been shown to be alleviated by the inhibition of PKM2 through oxidation of Cys^{358} (39). This inhibitory event will promote glucose flux into the PPP, providing the reducing power required for ROS detoxification and promoting cancer cell survival during conditions of oxidative stress.

NON-GLYCOLYTIC PROCESSES

In addition to being an important control point in glycolysis, PKM2, upon mitogenic, oncogenic, and LPS stimulation, also translocates to the nucleus where it regulates the expression of numerous proglycolytic enzymes (**Figure 1**). In cells activated with EGRF, ERK2 binds directly to PKM2 and phosphorylates Ser37 on PKM2, leading to recruitment of peptidyl-prolyl *cis–trans* isomerase NIMA-interacting 1 (PIN1). PIN1 aids binding of PKM2 to importin α5, thereby facilitating translocation of PKM2 to the nucleus (40, 41). Modification of PKM2 through sumoylation by the SUMO–E3 ligase as well as acetylation by p300 acetyltransferase will prevent binding of PKM2 to FBP and promote nuclear translocation (42, 43). A recent study proposes a role for sirtuin 6 (Sirt6) in regulating nuclear localization of PKM2. Sirt6 will bind and deacetylate PKM2 at lysine 433, thereby promoting nuclear export resulting in reduced cell proliferation and oncogenic properties of PKM2 (44). Furthermore, enhanced tetramer formation of PKM2 using TEPP 46 and DASA 58 will prevent nuclear localization of PKM2 (23, 45). In addition, nuclear PKM2 has been linked to caspase-independent programed cell death (46).

In cancer cells, PKM2 has been shown to function as a coactivator of hypoxia-inducible factor 1-alpha (HIF-1α) (**Figure 1**). HIF-1α is a key mediator of the Warburg effect and was originally identified as part of a family of transcription factors responsive under conditions of low oxygen or hypoxia. HIF-1α plays a critical role in the induction and maintenance of aerobic glycolysis, partly through inducing expression of glycolytic enzymes. Prolyl hydroxylase 3 (PHD3) acts as a cofactor to PKM2, promoting HIF-1α transactivation of target genes including lactate dehydrogenase (LDH), the glucose transporter GLUT-1, and pyruvate dehydrogenase kinase-1 (PDK-1) (47, 48).

Expression of Jumonji C domain-containing dioxygenase 5 (JMJD5) has been linked to carcinogenesis and regulates PKM2 activity by binding and preventing PKM2 tetramers to form, thereby blocking the enzymatic activity and promoting nuclear translocation (45). PKM2 together with HIF-1α and JMJD5 are then recruited to the HRE element of LDHA (**Figure 1**). PKM2 can also bind and regulate the activity of octamer-binding transcription factor 4 (Oct-4), a protein important for the maintenance and regulation of undifferentiated stem cells (49).

Recent findings propose a role for nuclear PKM2 as a transcriptional coactivator of c-Src-phosphorylated β-catenin as well as in promoting phosphorylation of histone H3 by PKM2 in EGFR-activated cells (50, 51) (**Figure 1**). Numerous other reports have confirmed the protein kinase function of PKM2, where PKM2 catalyzes transfer of phosphate from PEP to serine, threonine, or tyrosine residues on target substrates. Phosphorylation of histone H3 suggests a critical role for PKM2 in the epigenetic regulation of gene transcription in the metabolic switch observed during Warburg metabolism, as well as in G1–S phase transition of the cell cycle (51). Furthermore, PKM2 may also regulate the cell cycle through phosphorylation of important cell cycle regulators, including Bub3 and myosin light chain 2 (MLC2), to initiate cytokinesis (52). Nuclear PKM2 directly phosphorylates STAT3 on tyrosine 107-promoting transcription of MEK-5 (53). However, recent data failed to demonstrate PKM2-dependent phosphorylation *in vitro* using either PEP or ATP as phosphate donors, questioning the role of PKM2 as a protein kinase (54).

PKM2 AS A NEW PLAYER IN INFLAMMATION

Understanding the intricate interplay between cell signaling and metabolic pathways has emerged as an important focus of research in the field of cancer and, most recently, in inflammation.

Inflammation is a well-controlled process triggered by signals from damaged tissue or infection aiming to re-establish tissue homeostasis. It is a complex reaction that starts with activation of the "*front-line*" resident leukocytes (i.e., macrophages and dendritic cells) that leads to activation of surrounding microcirculation, and recruitment of neutrophils and other leukocytes to infected/damaged foci (55). Therefore, the inflammatory response is an energy-intensive process that involves a dramatic switch from a resting to a highly active metabolic state. This metabolic reprograming thereby directs nutrients to the efficient generation of ATP and synthesis of macromolecules that are required for the production of proinflammatory mediators, cytoskeleton rearrangement, and proliferation by immune cells. In this realm, it is not surprising that such highly active inflammatory cells undergo a metabolic shift from oxidative phosphorylation to aerobic glycolysis, resembling the well-described Warburg effect found in tumor cells. Indeed, it is becoming increasingly clear that metabolic enzymes and their regulators, initially implicated in the control of cellular metabolism, also display critical roles in regulating immune cell functions. Thus, immune cell metabolism has become a new attractive target area for the development of potential therapies for inflammatory diseases.

Although the full picture in cancer progression still needs to be resolved, increased expression of PKM2 has been reported in a wide range of tumors. Accumulating evidence suggests a central role of this protein in regulating the Warburg effect and many biological processes in cancer cells, including proliferation and survival [for review, see Ref. (56, 57)]. Emerging evidence

has also implicated PKM2 as critical regulator of immune cell metabolism and functions *via* regulating the Warburg effect, supporting its potential role in the genesis of inflammation. It has been shown that the expression of PKM2 is strongly increased in LPS-activated macrophages, mainly in a less-active monomeric/dimeric conformation and phosphorylated state (23, 58, 59). As mentioned above, the less active monomeric/dimeric form of PKM2 drives aerobic glycolysis, while the active PKM2 tetramer provides pyruvate for the TCA cycle. Thus, the expression PKM2 in LPS-activated macrophages adds another piece to the puzzle of metabolic reprograming toward aerobic glycolysis in activated macrophages. Meanwhile, LPS-induced PKM2 translocates into the nucleus and forms a transcriptional complex with HIF-1α that directly binds to the IL-1β promoter gene and activates its transcription. This highlights the interplay between metabolic reprograming and control of gene expression in activated macrophages induced by PKM2. Driving PKM2 into tetramer conformation with DASA-58 and TEPP-46 inhibited LPS-induced nuclear translocation and, subsequent LPS-induced expression of IL-1β and a range of other HIF-1α-dependent genes. Accordingly, macrophages lacking PKM2 also showed reduced expression of the HIF-1α-responsive genes *Il1*β and *Ldha* in response to LPS (23). Moreover, it was also demonstrated that PKM2 functions as a regulator of high mobility group box-1 (HMGB1) release by activated macrophages through interaction and activation of HIF-1α (58). HMGB1 is a ubiquitous nuclear protein that can be released by activated macrophages and act as a potent proinflammatory cytokine (60). The knockdown or inhibition of PKM2 using shRNA or shikonin, respectively, markedly reduces the release of HMGB1 by activated macrophages (58). Additionally, activation of colorectal carcinoma cells with LPS results in an increased production of TNF-α and IL-1β in a PKM2/STAT3-dependent manner. Mechanistically, LPS induces PKM2 nuclear translocation and binding to the STAT3 promoter, enhancing its transcription and subsequent activation (61). A recent report has also directly implicated a critical role for dimeric PKM2 in the hyper-inflammatory behavior of macrophages from coronary artery disease (CAD) patients (59). It was shown that nuclear translocation of dimeric PKM2 results in phosphorylation of STAT3 in LPS-activated CAD macrophages, boosting IL-1β and IL-6 transcription. Forcing PKM2 into tetramer conformation with ML265 prevented its LPS-induced nuclear translocation and STAT3 phosphorylation. Thus, PKM2 seems to be a critical regulator of expression and secretion of proinflammatory mediators, highlighting the possibility of targeting this protein in the treatment of inflammatory and infectious diseases.

Indeed, inhibition of dimeric PKM2 by shikonin conferred significant protection of mice against LPS-induced endotoxemia (58). Furthermore, mice treated with TEPP-46 showed reduced produced of IL-1β in response to LPS and *Salmonella typhimurium*-induced production *in vivo* (23). In line with these observations, studies in recent years have reported increased expression of PKM2 in different inflammatory disorders. The expression of PKM2 in intestinal tissue was found at high levels in patients with Crohn's disease and positively correlated with disease activity scores or serum inflammatory markers (62). Moreover, elevated levels of PKM2 were found in stool samples from patients with active Crohn's disease, suggesting that this protein can be a useful non-invasive marker for inflammatory bowel disease (63, 64). In accordance with this, expression of PKM2 was progressively increased in intestinal tissue of mice undergoing TNBS-induced colitis (62, 65). Finally, proteomic analysis revealed that PKM2 was one of the 33 over-expressed proteins found in synovial tissue from patients with rheumatoid arthritis (66). These findings indicate that PKM2 expression is upregulated in a multitude of inflammatory disorders. However, further studies are warranted to understand the regulatory functions of PKM2 on different inflammatory conditions.

PERSPECTIVES AND CONCLUSION

During the past years, metabolism and immunology have existed as two distinct fields of investigation, but there is now a general consensus that they intersect at several points. The concept of metabolic reprograming as a mechanism to drive an inflammatory response has mainly focused on how an immune cell's metabolic status can directly influence its activity and function. In recent years, PKM2 has emerged not just as a key regulator of metabolic reprograming but also as a key player in controlling the transcription of critical genes in cancer cells, and most recently, in immune cells.

The current strategy for the treatment of inflammatory diseases is fundamentally based on interrupting the production or action of mediators that orchestrate the host's response to tissue injury. An ideal drug to treat inflammatory disease would be able to both turn off the inflammatory response as well as activate the resolution program, including the induction of neutrophil apoptosis and polarization of macrophages into M2 (alternatively activated or pro-resolution) phenotype. Notably, recent studies show that PKM2 regulates the expression of proinflammatory mediators, prevents apoptosis, and drives macrophage polarization toward M1 phenotype (23, 58, 61, 67–69), indicating the potential of this enzyme as a target for the development of anti-inflammatory and proresolutive therapies.

Furthermore, recent studies have unraveled a notable involvement of PKM2 in controlling the transcriptional activity of HIF-1α and STAT3 pathways during inflammation. The expression and enzymatic activity of PKM2 can be regulated at multiple levels, including transcription, posttranslational modifications, and allosteric regulation of conformational stability. Therefore, PKM2 represents a novel potential target for the development of anti-inflammatory drugs.

AUTHOR CONTRIBUTIONS

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

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Modulation of Chemokine Responses: Synergy and Cooperativity

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Chemokine biology is mediated by more complex interactions than simple monomolecular ligand–receptor interactions, as chemokines can form higher order quaternary structures, which can also be formed after binding to glycosaminoglycans (GAGs) on endothelial cells, and their receptors are found as dimers and/or oligomers at the cell surface. Due to the complexity of the chemokine binding and signaling system, several mechanisms have been proposed to provide an explanation for the synergy observed between chemokines in leukocyte migration. Pioneering studies on interactions between different chemokines have revealed that they can act as antagonists, or synergize with other chemokines. The synergism can occur at different levels, involving either two chemokine receptors triggered simultaneously or sequentially exposed to their agonists, or the activation of one type of chemokine receptor triggered by chemokine heterocomplexes. In addition to the several chemokines that, by forming a heterocomplex with chemokine receptor agonists, act as enhancers of molecules of the same family, we have recently identified HMGB1, an endogenous damage-associated molecular patterns (DAMPs) molecule, as an enhancer of the activity of CXCL12. It is now evident that synergism between chemokines is crucial at the very early stage of inflammation. In addition, the low-affinity interaction with GAGs has recently been shown to induce cooperativity allowing synergy or inhibition of activity by displacement of other ligands.

Keywords: chemokines, cell migration, synergy, oligomerization, glycosaminoglycan

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CHEMOKINES AND THEIR RECEPTORS

Chemokines are key regulators of leukocyte migration and function, playing fundamental roles both in physiological and pathological immune responses, such as inflammatory diseases (1). The chemokine system includes ~50 ligands, which engage a panel of over 20 chemokine receptors in a promiscuous fashion, which are differentially expressed by all leukocytes and many non-hematopoietic cells (2). Proper tissue distribution of distinct leukocyte subsets, under normal and pathological conditions, is guaranteed by the resulting combinatorial diversity in cell responsiveness to chemokines.

To mediate their activity chemokines bind to cell surface receptors which belong to the largest branch of the γ subfamily of rhodopsin-like G protein-coupled receptors (GPCRs) (3), a receptor superfamily which represents the most successful target of small molecule inhibitors for treating diseases affecting different systems in modern pharmacology. All chemokine receptors couple to

heterotrimeric $G\alpha_i$-proteins and accordingly most responses can be fully inhibited by treatment of cells with *Bordetella pertussis* toxin. Today, a total of 19 signaling receptors have been identified: 7 CXCRs (CXCR1–6 and CXCR8), 10 CCRs (CCR1–10), CX3R3, and CKR1. In addition, there are four "atypical" receptors that use alternative signaling pathways, and act mainly by sequestering and degrading the chemokines present in the microenvironment (4). Thus, the ~50 chemokines outnumber their receptors indicating that a receptor can bind more than one chemokine. In addition, several chemokines can also bind to multiple receptors (2, 5). Novel findings indicate that polysialylation of CCR7, the central chemokine receptor controlling immune cell trafficking to secondary lymphatic organs, is essential for the recognition of the CCR7 ligand CCL21 (6), and that the glycosylation pattern of this receptor shapes receptor signaling (7), suggesting that this further level of control might be shared with other chemokine receptors.

CHEMOKINE SYNERGY AND COOPERATION

A vast range of *in situ* experiments, aimed at understanding which chemokines are produced under specific circumstances, has revealed that a variety of chemokines can be concomitantly produced at the target sites of leukocyte trafficking and homing (8–12). This renders the chemokine system a good target for therapy and has promoted the search by pharmaceutical companies for small molecule chemokine antagonists (13–16). While we understand the effects of different chemokines singly, much less is known about the potential consequences of the concomitant expression of multiple chemokines and their interaction with other inflammatory molecules (17, 18).

The suggestion that chemokines might have additional regulatory mechanisms started with the identification of natural chemokine antagonists. Many reports have demonstrated that certain chemokines can also antagonize non-cognate chemokine receptors, by altering agonist-induced signaling and abrogating cellular responses *via* several mechanisms, including occupancy of the chemokine receptor-binding pocket or signaling through Rac-2 (19–24).

The studies on possible regulatory mechanisms continued when three reports showed that chemokines can synergize to enhance leukocyte functions in response to chemoattractants. The first described a bovine chemokine, regakine 1 that induces enhanced neutrophil migration when combined with CXCL7, CXCL8, and C5a. The receptor or the mechanism of regakine-1-induced synergism is not known. Competition with labeled C5a for binding to neutrophils or receptor-transfected cell lines demonstrated that regakine 1 does not alter receptor recognition. The protein kinase inhibitors 2′ amino 3′ methoxyflavone (PD98059), wortmannin, and staurosporine had no effect on the synergy between C5a and regakine 1 (25). The second study showed that migration of natural IFN-producing cells, a subpopulation of murine and human lymphocytes, to the CXCR3 agonists requires stimulation of CXCR4 by CXCL12. The mechanism by which CXCL12 induces enhanced migration in response to CXCR3 agonists is yet unknown. CXCL12 does not upregulate the expression of CXCR3 and does not increase the affinity of CXCR3 for its agonists (26). The third report (27) showed the same enhanced migration, on human plasmacytoid dendritic cells, in response to CXCR3 agonists induced by stimulation with CXCL12 as observed by Krug et al. (26). These reports undoubtedly indicate, as for the natural antagonist chemokines, that it is necessary to carefully analyze the effects that the concomitant expression of chemokines can have on cell functions and to elucidate the molecular mechanisms governing cell activities at sites of inflammation. Synergism can thus occur at different levels, involving either two chemokine receptors triggered simultaneously or sequentially exposed to their agonists (26–30). We have identified a further mechanism by which chemokines, forming chemokine heteromeric complexes, can activate one type of chemokine receptor (**Figure 1A**) (31): (i) CXCL13 enhances CCL19 and CCL21 triggering of CCR7 (32); (ii) CXCL10 enhances CCL22 triggering of CCR4 (33); (iii) CCL19 and CCL21 enhance the activity of CCR2 ligands and protect them from degradation (34); and (iv) CXCL9 enhances migration induced by CXCL12 on $CXCR4^+/CXCR3^-$ malignant B cells (35). Other groups have also shown that the synergism between a chemokine agonist and a non-ligand chemokine can enhance the activity of selective chemokine receptors (36–40).

Chemokines have a second important interaction with cell surface expressed glycosaminoglycans (GAGs), which mediates their immobilization on the endothelial surface in order to provide their directional signal (41–43). This interaction was shown to be essential for their ability to recruit cells *in vivo* by the loss of activity of chemokine variants, which had abrogated GAG-binding capacity (44). Without the interaction with endothelial GAGs, most chemokines would be washed away from the local production site, especially under flow conditions, diluted to a concentration below the threshold required for binding, and distributed uniformly throughout the vasculature such that no localized chemotactic signal is generated for leukocytes to allow directional mobilization. Furthermore, differential binding to GAGs plays an important role in localization. Neutrophil recruitment to the lung is greater in response to chemokines that bind GAGs less strongly. This was demonstrated both by mutants of CXCL8 with abrogated GAG binding as well as comparison of another neutrophil chemoattractant, CXCL1. Although increased recruitment was postulated to be mediated by the stronger GAG binder, lower binding capacity resulted in enhanced recruitment, demonstrating that the tissue microenvironment plays a pivotal role in the spatial formation of chemokine gradients and defining GAGs functions (45, 46).

Recently, binding to cell surface GAGs has identified more subtle roles in chemokine biology, where competitive binding of chemokines to GAGs can either induce cooperative enhancement of activity or inhibition of activity by displacement of certain chemokines. Cooperative enhancement has been demonstrated for both classical receptors as well as atypical or non-signaling receptors such as CCX-CKR/ACKR4 (47). In both cases, competitive displacement of the chemokines from GAGs was shown to be responsible for the effects, using modified chemokines lacking the GAG-binding sequence. The competitive displacement is limited to chemokines which bind GAGs strongly such as CCL11,

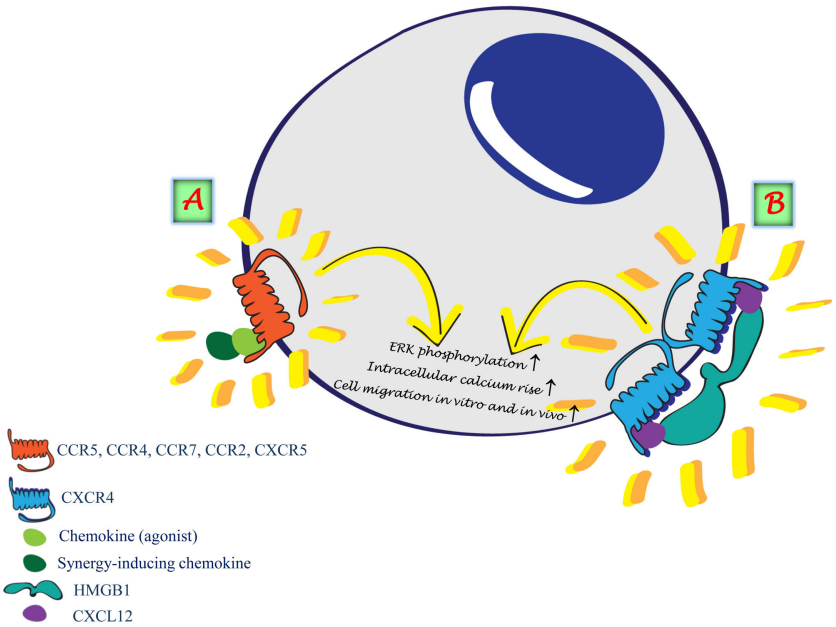

FIGURE 1 | Synergism induced by the formation of heterocomplexes. (A) Heterocomplex formed between two chemokines renders the agonist more potent on the selective receptor. **(B)** HMGB1 forms a heterocomplex with CXCL12 enhancing CXCL12 potency on CXCR4.

CXCL12, and CXCL13, compared with low-affinity binders, such as CCL3 and CCL4, being unable to induce this synergy.

A similar phenomenon was observed for CCL18, an interesting chemokine in that is has been shown to be unregulated in many pathological conditions, yet its receptor remained elusive until it was shown recently to activate CCR8 (48). Moreover, CCL18 is always present at considerably higher concentrations in the circulation than most chemokines, and it was shown to displace certain chemokines bound to heparin (49). This property led to the hypothesis that it could prevent the recruitment of leukocytes by these chemokines by removing them from the endothelial surface.

Since chemokine cooperativity *via* GAG binding would allow chemokines to activate their cognate receptors at lower chemokine concentrations, it is likely that *in vivo*, this phenomenon would extend the range from which chemokines can induce recruitment of leukocytes (50). GAG binding and/or formation of heterocomplexes can definitively contribute to the fine-tuning modulation of chemokine activities occurring *in vivo*.

It is well established that many chemokines exist in equilibrium between the monomeric and dimeric state, and even as higher order oligomers (51–53). It is therefore clear that chemokine biology is more complex than simple monomolecular ligand–receptor interactions. It has been shown *in vitro* that the quaternary structure of chemokines influences the affinity of binding to GAGs (54, 55). Moreover, *in vitro* studies have suggested that dimerization may also occur after binding to GAGs on endothelial cells (56). In fact, this phenomenon is essential for certain chemokines *in vivo* since obligate monomers of the proinflammatory chemokines, CCL2, CCL4, and CCL5, are unable to recruit cells when injected into the peritoneal cavity (44).

It is however important to note that alterations in GAG composition can occur in several pathological conditions (57–59). In addition, chemokine receptors can be found as dimers and/or oligomers at the cell surface (60–62). Due to the complexity of chemokine binding and signaling (63), several mechanisms have been proposed to provide an explanation for synergy between chemokines in leukocyte migration. It is now evident that the synergism between chemokines is crucial at the very early stage of inflammation, as *in vivo* disruption of pro-atherogenic heteromers of CCL5 and CXCL4 resulted in a significant decrease in atherosclerotic lesion formation (38, 64). Moreover, disruption of the heteromers, formed between CCL5 and the α-defensin HNP1, attenuated monocyte and macrophage recruitment in a mouse model of myocardial infarction (65). On the contrary, the study of the role of synergy-inducing chemokines in the tumor microenvironment is at its infancy, as it has been shown *in vitro* that the distinct co-expression of B and T cell attractant chemokines, present in the tumor microenvironment, control cell trafficking of both tumor-infiltrating lymphocytes and malignant B cells (35).

CHEMOKINES AND DAMPs

Under inflammatory conditions, the cross talk between different molecules plays a crucial role in reaching the balance in tissue regeneration. A complete system for the detection, containment, and repair of damage caused to cells in the organism requires warning signals for the cells to respond. These warning signals are called endogenous damage-associated molecular patterns (DAMPs) or alarmins. In addition to the several chemokines that act as enhancers of molecules of the same family, by forming a

heterocomplex with chemokine receptor agonists, we have recently identified HMGB1, an alarmin, as an enhancer of the activity of CXCL12 (**Figure 1B**) (66–68). The heterocomplex HMGB1/CXCL12 can be disrupted with a specific molecule, glycyrrhizin, which inhibits cell influx into the injured tissue. This indicates that a number of components, in addition to the direct activation of the receptor *via* a selective agonist, can regulate chemokine functions *via* a direct interaction with chemokines or chemokine receptors. Multiple chemokines within inflamed tissues selectively enhance each other's migratory functions, depending on their concentrations, proximity, and simultaneous exposure to leukocytes. The mechanisms underlying the involvement of endogenous DAMPs in chronic diseases are still largely unexplored, and the interaction with other molecules might be a possible approach to understand their targets and functions. The interaction between chemokines and inflammatory molecules needs to be taken into account when chemokine cleavage by proteolysis, or chemokine degradation by atypical chemokine receptors, would be beneficial to achieve a resolving microenvironment favorable for resolution of inflammation by abrogating chemokine signals and the recruitment of inflammatory cells (69). The heterocomplex HMGB1/CXCL12 was demonstrated to prevent CXCL12 degradation (70), similarly to the observation that the complex CCL19/CCL7 prevents CCL7 degradation by the atypical receptor ACKR2 (34).

FUTURE PERSPECTIVES

The chemokine system remains a promising biological target for the development of new therapeutic tools for the treatment of immunological disorders. Nevertheless, drug discovery programs have not yet produced successful drugs targeting the chemokine system for the treatment of inflammatory diseases. Most of the competitive chemokine receptor antagonists developed by all major pharma companies have been disappointingly unsuccessful when tested in clinical trials (71), and as a matter of fact, the only two small molecule inhibitors approved by the FDA do not target inflammation. Taking into account GAGs-binding properties, synergy induced by heterocomplexes formed with non-ligand chemokines or inflammatory molecules, and the possibility that the heterocomplexes might induce differential signaling pathways, will certainly help in elaborating the biology involved in this family and will surely contribute to the successful development of inhibitors of the chemokine system as therapeutics.

AUTHOR CONTRIBUTIONS

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

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Paradoxical Roles of the Neutrophil in Sepsis: Protective and Deleterious

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Sepsis, an overwhelming inflammatory response syndrome secondary to infection, is one of the costliest and deadliest medical conditions worldwide. Neutrophils are classically considered to be essential players in the host defense against invading pathogens. However, several investigations have shown that impairment of neutrophil migration to the site of infection, also referred to as neutrophil paralysis, occurs during severe sepsis, resulting in an inability of the host to contain and eliminate the infection. On the other hand, the neutrophil antibacterial arsenal contributes to tissue damage and the development of organ dysfunction during sepsis. In this review, we provide an overview of the main events in which neutrophils play a beneficial or deleterious role in the outcome of sepsis.

Keywords: sepsis, neutrophil migration, organ dysfunction, toll-like receptors, chemotactic receptors

INTRODUCTION

Sepsis represents a challenging health care and economical problem worldwide with lingering aftereffects (1). The incidence of sepsis has increased over the last decades (2). In terms of diagnosis, sepsis is a systemic response to infection, with increasing severity recognized as severe sepsis or septic shock. Severe sepsis is defined as sepsis in the presence of organ dysfunction and septic shock as the presence of hypotension unresponsive to vasoconstrictors (3). Intensive preclinical studies performed in the last decades have contributed greatly to the understanding the pathophysiology of sepsis, though it is not yet fully understood. Neutrophils are important players in the outcome of sepsis. Therefore, we will review the involvement of neutrophils in the pathophysiology of sepsis in this work.

CONTROL OF INFECTIONS BY NEUTROPHILS

Neutrophils are leukocytes with multi-lobed nuclei that form in the bone marrow and are released in their mature form to the blood. Neutrophils have a short life span and do not show proliferative properties (4, 5).

Classically recognized as phagocytic cells, neutrophils are associated with the innate immune response. These cells are recruited to the site of the infection in response to chemotactic mediators, where they play antimicrobial roles (5, 6).

The presence of neutrophils at the site of infection has been demonstrated to be essential for controlling the bacterial and fungal burden and avoiding the systemic spread of the infection (7). Indeed, depletion of neutrophils in mice infected with *Staphylococcus aureus* markedly reduced the clearance of the bacteria and also survival (8). Similarly, depletion of neutrophils in mice infected with *Candida albicans* induced dissemination of the fungus and led to a higher mortality rate. Likewise, neutropenic patients are more susceptible to bacterial and fungal infections (9–11).

Neutrophils induce killing of pathogens *via* phagocytosis, degranulation, or even the release of intracellular components such as DNA, histones, and lytic proteins, which form neutrophil extracellular traps (NETs) (12, 13). Nitric oxide (NO), a mediator produced by the enzyme inducible nitric oxide synthase (iNOS), is one crucial mediator of the microbicidal activity of neutrophils. Deletion of *iNOS* induces a high mortality rate due to impaired control of the infection, despite the presence of neutrophils in the locale of the infection (14).

Additionally, neutrophils are equipped with receptors that recognize pathogen-associated molecular patterns or damage-associated molecular patterns, initiating signaling cascades and leading to the production of inflammatory mediators to establish an appropriate response against the pathogen. This results in amplification of the inflammatory process, including emigration of the new waves of neutrophils to the site of infection (15).

Chemokines are a family of small cytokines that are divided into small subfamilies based on variations of a conserved cysteine motif and play an important role in neutrophil recruitment (16). Most chemokines belong to the CC and CXC chemokine subfamilies (17), which exhibit two juxtaposed cysteine residues or one amino acid between the first two cysteine residues, respectively (18). Under physiological conditions, lymphocytes, monocytes, and macrophages express CC receptors (CCR) and respond to CC chemokines, whereas neutrophils express CXC receptors (CXCR)1 (IL-8R in humans) and CXCR2 and respond to CXC chemokines (19).

NEUTROPHIL MIGRATION IS IMPAIRED DURING SEVERE SEPSIS

As mentioned above, the control of an infection depends on the efficient migration of neutrophils to the site of infection as well as appropriate microbicidal activity (20). Our group and others have demonstrated that mice subjected to severe sepsis show inadequate migration of neutrophils to the site of infection, despite the high levels of chemokines at the site. The insufficient number of neutrophils recruited to the site of infection does not control the infection locally, contributing to the systemic spread of the pathogen. As consequence, a marked systemic inflammatory response is established, which is associated with high mortality rates (21).

Among the mechanisms leading to the failure of neutrophil migration, it has been shown that CXCR2 is internalized in circulating neutrophils from mice or patients with severe sepsis (22–24). Accordingly, neutrophils isolated from septic patients show reduced migration toward chemotactic mediators *ex vivo*, which is associated with patient survival: survivors show higher neutrophil migration compared with non-survivors (25).

In recent years, several studies have described the mechanisms underlying CXCR2 internalization in circulating neutrophils during sepsis, resulting in failure of migration to the infectious focus (**Figure 1**) (24, 26–29). It has been reported that chemokine receptors belong to the G protein-coupled receptors (GPCRs), and their expression is precisely regulated (30). Prolonged or repeated exposure to agonists induces desensitization and

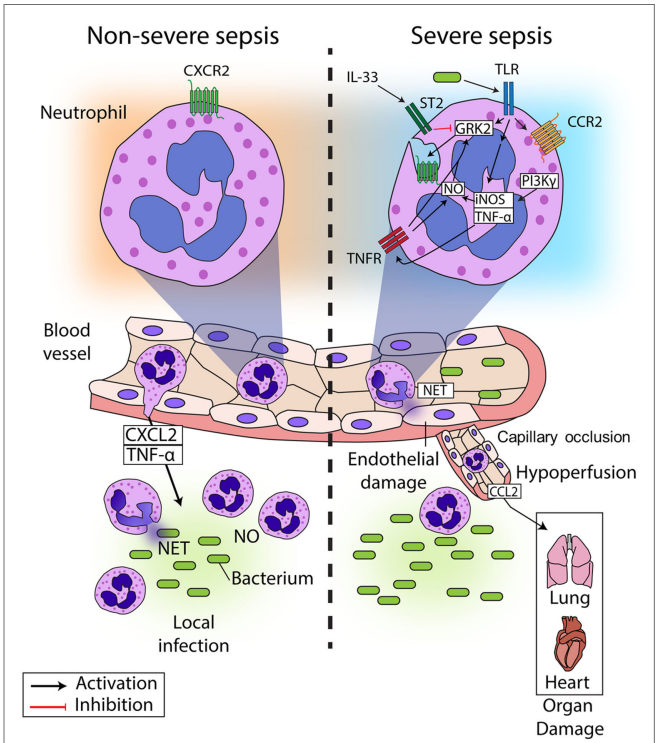

FIGURE 1 | Neutrophil migration in sepsis. During non-severe sepsis, neutrophils expressing CXCR2 are recruited from the blood to the site of infection in response to CXCL2 and other chemoattractants. Neutrophils migrate to the locale of the infection, where they release NETs and produce reactive oxygen and nitrogen intermediates (such as NO) to kill the pathogens and avoid its spreading. By contrast, neutrophils are systemically stimulated during severe sepsis, which leads to impaired neutrophil migration to the infection focus. Bacterial components present in the blood activate TLRs expressed on neutrophils, leading to the up-regulation of GRK2, which induces desensitization of CXCR2 on the neutrophil surface. Additionally, TLR activation induces the expression of TNF-α and iNOS, the latter of which might also be activated by PI3K. Both TNF-α and NO can lead to upregulation of GRK2, exacerbating the down-regulation of CXCR2 on the neutrophil surface. As a consequence, neutrophil migration fails, and bacterial growth is not controlled. Furthermore, activation of TLRs also induces the expression of CCR2 on the surface of neutrophils, favoring the recruitment of these cells to distant organs producing CCL2, which contributes to organ damage in association with the capillary occlusion and the hypoperfusion observed in sepsis. The systemic activation of neutrophils also induces the release of NETs in the blood vessels, which causes endothelial damage, culminating in the aggravation of sepsis and possible death. Interestingly, it has been demonstrated that IL-33 can prevent the upregulation of GRK2 expression induced by TLR overactivation and consequently prevent the failure of neutrophil migration to the site of infection.

internalization of GPCRs in a process dependent on the activation of GPCR kinases (GRKs) (31). GRKs phosphorylate the intracellular domains of the activated GPCR, leading to the recruitment of arrestin, which decouples the G protein from the receptor and trigger its internalization (32). Our group and others have demonstrated that ligands of Toll-like receptor (TLR)2, TLR4, and TLR9 (lipoteichoic acid (LTA), lipopolysaccharide (LPS), and CpG-oligodeoxynucleotide, respectively) induce GRK2 upregulation in circulating neutrophils, which in turn, leads to CXCR2 internalization (26, 29, 33–35). Indeed, pretreatment of neutrophils with a GRK2 inhibitor prevented the effect of TLR4 and TLR9 activation on CXCR2 internalization (33, 34). Corroborating these data, *tlr*2-, 4-, and 9-deficient mice show an increase in CXCR2 expression on circulating neutrophils, compared with WT mice subjected to severe sepsis (26, 34, 36). Additionally, IL-33, a member of the IL-1 family that binds to the heterodimeric receptor complex ST2, has been shown to prevent the up-regulation of GRK2 mediated by TLR signaling. IL-33 treatment improved the recruitment of neutrophils to the site of infection in mice and prevented LPS-induced chemotaxis reduction in human neutrophils (33, 37).

It is noteworthy that in contrast to the harmful TLR overactivation in circulating neutrophils, adequate activation of TLRs in migrated neutrophils is crucial for establishing the local immune response. Indeed, *tlr4*-mutant mice fail to control a low-dose infection with the Gram-negative bacterium *Salmonella typhimurium* (36). Moreover, *myd88*-deficient mice are highly susceptible to polymicrobial sepsis because the lack of the adaptor protein involved in most of TLR signaling prevents the establishment of the local inflammatory response. In contrast to the TLRs, the pattern recognition receptors Nod-like receptors 1 and 2 are not involved in neutrophil migration to the site of infection or in the establishment of the inflammatory response locale in mice subjected to CLP-induced polymicrobial sepsis (38).

Further investigation of how TLRs modulate the expression of CXCR2 on the neutrophil surface suggested the involvement of tumor necrosis factor (TNF)-α and NO. Neutrophils isolated from *tnf receptor*-deficient mice activated with LPS do not show internalization of CXCR2 or impaired chemotaxis to CXCL2. Additionally, neutrophils treated with TNF-α exhibit reduced chemotaxis toward CXCL2 (27). Moreover, NO confers a similar effect in LPS- or IL-8-stimulated neutrophils. Indeed, inhibition of iNOS reduces the effect of the LPS or IL-8 on the internalization of CXCR2 and the chemotactic activity of CXCR2 agonists (24, 29). NO triggers the activation of soluble guanylate cyclases (GCs) as well as cyclic-GMP formation and protein kinase G (PKG) phosphorylation (39). As expected, inhibition of sGC or PKG had the same effect as iNOS inhibition after LPS stimulation of neutrophils. Interestingly, inhibition of sGC and PKG during experimental sepsis protected mice from death, and this effect was associated with reduced expression of GRK2 in neutrophils, increased expression of CXCR2 and, consequently, increased neutrophil migration to the infectious focus compared with non-treated animals (29).

Based on these observations, we could suggest that TNF-α production and/or release in neutrophils is important to the effect of TLRs on the CXCR2 expression on these cells. In addition, both TLR- and TNF-dependent pathways upregulate inducible NO synthase, which could in turn induce GRK2 expression, leading to reduced CXCR2 expression on the neutrophil surface (40).

In addition to TNF-α and iNOS, it has been demonstrated that phosphoinositide-3 kinase gamma (PI3Kγ) plays an important role in this process (28). Interestingly, PI3K may be involved in the dimerization of iNOS, an essential process for the activity of this enzyme (41). GRK2 upregulation and CXCR2 internalization were shown to be inhibited in *PI3Kγ*$^{-/-}$ neutrophils incubated with CXCL2. Additionally, *PI3Kγ*$^{-/-}$ mice subjected to CLP present reduced GRK2 expression and increased CXCR2 expression on the neutrophil surface, resulting in higher survival rates (28). Altogether, these data provide substantial evidence of the links between all pathways discussed above and highlight new potential targets for sepsis treatment.

In contrast to the deleterious role of the pathways described above, there are also mediators that protect the organism against sepsis. One example is hydrogen sulfide (H_2S), a gas produced by the organism that is synthesized from L-cysteine, mainly *via* the cystathionine b-synthase and cystathionine g-lyase (CSE) enzymes (42). It has been demonstrated that CSE activity is increased during sepsis, and inhibition of CSE reduces CLP-induced leukocyte–endothelial interactions in mesenteric venules, decreases neutrophil migration to the site of infectious, and consequently decreases the survival rate of animals subjected to non-severe sepsis (43). In contrast, treatment of mice subjected to severe sepsis with an H_2S donor has the opposite effect, resulting in increased CXCR2 expression on circulating neutrophils, increased neutrophil migration to the infection focus, and improvement survival (43). Thus, H_2S donors could be considered for use in sepsis treatment.

Additionally, neutrophil migration events during sepsis have been demonstrated to be regulated by several other mediators, such as lectin-like oxidized low-density lipoprotein receptor (LOX)-1, peroxynitrite, and the acute-phase alpha-1 acid protein, which contribute to the failure of neutrophil migration to the site of infection (44–46). Conversely, the cytokine IL-17 has been shown to be crucial for recruiting neutrophils to the site of infection during sepsis (47). In contrast, the role of the peroxisome proliferator-activated receptor in neutrophil migration during sepsis remains to be confirmed, as both protective and deleterious role have been described (48, 49). The effects of these mediators on the neutrophil migration have been reviewed elsewhere (20, 50, 51) and will not be further addressed here.

NEUTROPHIL-INDUCED ORGAN DAMAGE

In addition to the host-protective role of neutrophils in sepsis *via* the killing of microorganisms, these cells have been described as exhibiting deleterious functions (6). During sepsis, it has been shown that the systemic inflammatory response leads to the activation of circulating neutrophils sequestered in capillary beds, occluding the lumen, and inducing tissue ischemia. Additionally, neutrophils can migrate to vital organs and release lytic factors and pro-inflammatory cytokines, contributing to organ damage and subsequent multiple organ dysfunction (52, 53). Chemokines

and chemokine receptors are also involved in the process of neutrophil infiltration into vital organs during sepsis. In contrast to the observation that CXCR2 is internalized in circulating neutrophils during severe sepsis (24), this receptor has been implicated in neutrophil infiltration into the lungs, due to the release of CXC chemokines in this organ during sepsis (54, 55). This apparent contradiction could be explained by the differences in the severity of sepsis induced in each study.

Furthermore, our group and others have demonstrated that CCR receptors, which are not expressed on neutrophils under physiological conditions, are induced in this cell type in various inflammatory processes (56–60). It was demonstrated that CCR2 is induced on the neutrophil surface in mice and patients with sepsis in a TLR2- or TLR4-dependent manner. Importantly, CCR2 does not mediate neutrophil recruitment to the site of infection, but it does mediate neutrophil infiltration in vital organs, such as the lungs, kidneys, and heart. Blockage of CCR2 decreases organ damage and death in animals subjected to severe CLP. Moreover, CCR2 expression is positively correlated with the severity of the disease, as measured using the Sepsis-related Organ Failure Assessment (SOFA) score. Accordingly, human neutrophils isolated from non-surviving septic patients express more CCR2 than neutrophils from surviving patients (59).

Another important feature of neutrophils is the formation of NETs, a network of chromatin fibers associated with granules of antimicrobial peptides and enzymes such as myeloperoxidase, elastase, and cathepsin G, which immobilize and kill invading microorganisms to prevent their spreading (61). The role of NETs in the control of bacterial spreading in sepsis is controversial. Similar bacterial loads were observed in animals lacking an important enzyme (peptidylarginine deiminase 4) for NET formation and in animals treated with rhDNAse compared with control mice (62, 63). However, our group and others (64, 65) have observed an important role of NETs in the control of bacterial spreading during sepsis.

In addition to the role of the NETs in bacterial control during infection, excessive formation of NETs has been observed in many pathological conditions, which is related to organ damage (66). Activated endothelial cells induce the formation of NETs by neutrophils *in vitro* (67, 68). Moreover, in an LPS-induced endotoxic shock model, NETs have adhered and activated the vascular endothelium (69). Additionally, the interaction between neutrophils and activated platelets during sepsis induces NET formation, which contributes to endothelial cell damage and organ injuries (70). Moreover, it has been reported that histones and myeloperoxidase could be responsible for NET-induced endothelial dysfunction, and histones can also interact with TLR2 and TLR4 to induce cytokine production *via* MyD88 signaling, contributing to the systemic inflammatory response observed in sepsis (68, 71–74).

Surprisingly, the survival rate was not found to differ between rhDNAse-treated and non-treated mice after CLP (65). Further investigations revealed that this lack of a difference was due to the deleterious role of NETs in organ damage, as discussed above. Thus, when antibiotic therapy and rhDNAse treatment or inhibition of the enzyme peptidylarginine deiminase 4 were used in combination to control a bacterial infection, a marked increase in the survival rate of the animals was observed, which was associated with decreased organ damage (63, 65). In addition, pretreatment with rh-DNAse in animals challenged with LPS decreased the organ damage and increased the survival rate during endotoxemia (65).

The observations from mouse models confirm the human ones. Notably, autopsy examinations of tissues from septic patients with multiple organ dysfunction syndrome indicate the presence of neutrophils sequestered into the kidneys and lungs (75). Furthermore, severity of acute respiratory distress syndrome in septic patients is proportional to the intensity of the inflammatory infiltrate and proteolytic enzymes in the bronchoalveolar lavage (76).

During sepsis, organ failure is associated with hypoperfusion and tissue hypoxia, both of which are attributed to hypotension and occlusion of neutrophils in the microcirculation (77, 78). The cytokines secreted by neutrophils attached to a vessel wall can also cause endothelial dysfunction, establishing a thrombogenic profile and favoring intravascular coagulation (79). Additionally, neutrophil products can also induce increased NO production by various cell types, which can contribute to lowering blood pressure (80) and favors the generation of peroxynitrite, a potent oxidant agent. In the heart, peroxynitrite can cause changes in the structure and function of proteins that may be related to sepsis-associated myocardial failure (81). Thus, the adhesion of neutrophil to the endothelium and their sequestration to the heart may have multiple deleterious cardiovascular effects.

CONCLUSION AND PERSPECTIVES

It is clear that sepsis continues to represent a challenge for basic and clinical researchers. Despite the massive amount of basic and clinical results related to this syndrome that has been published in the literature in the last several decades, there has been an absence of effective new treatments. The high mortality associated with sepsis together with its increased incidence, points to the importance of re-evaluation of the literature as well as the new translational studies addressing the disease. Together, these approaches will help to identify new effective targets for the development of new therapies. In this context, the present review described the dual roles of neutrophils in the evolution of sepsis. These cells are key players in the innate immune response in the early phase of sepsis and their recruitment to sites of infection is crucial for controlling microorganism growth. Aggravation of sepsis is associated with failure of neutrophil migration to the site of infection. The molecular mechanism involved in this phenomenon was described, and several potential targets for the development of new therapies were identified. By contrast, neutrophils can be harmful and induce secondary organ damage during infection. Neutrophil recruitment to organs far from the site of infection is mediated by the expression of CCR2 under septic conditions. The mechanism involved in the harmful effect of the neutrophils was also described in this review, noting potential targets for the development of new therapies. In this context, new therapies targeting the harmful activity of neutrophils, such as blocking

NET formation or CCR2 activity, might be more helpful than targeting the general inflammatory response.

AUTHOR CONTRIBUTIONS

FS, FC, AK, CL, RF, DN, DC, VB, JA-F, and FC wrote and approved the text. CL draws the figure.

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REVIEW
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Allosteric Modulation of Chemoattractant Receptors

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Chemoattractants control selective leukocyte homing *via* interactions with a dedicated family of related G protein-coupled receptor (GPCR). Emerging evidence indicates that the signaling activity of these receptors, as for other GPCR, is influenced by allosteric modulators, which interact with the receptor in a binding site distinct from the binding site of the agonist and modulate the receptor signaling activity in response to the orthosteric ligand. Allosteric modulators have a number of potential advantages over orthosteric agonists/antagonists as therapeutic agents and offer unprecedented opportunities to identify extremely selective drug leads. Here, we resume evidence of allosterism in the context of chemoattractant receptors, discussing in particular its functional impact on functional selectivity and probe/concentration dependence of orthosteric ligands activities.

Keywords: biased signaling, functional selectivity, chemoattractant, chemokine receptor, leukocyte recruitment

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INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest family of cell-surface receptors encoded by the human genome and are involved in most pathophysiological aspects (1, 2). GPCRs fulfill the vital biological function of transducing effects of extracellular signals (photons, lipids, neurotransmitters, proteins, etc.) across the cellular membrane into the cytosolic space *via* the activation of dedicated signaling pathways. Physiologically, when the extracellular signal interacts with the so-called "orthosteric binding site" of a GPCR, a conformational change occurs that conveys the signal through the plasma membrane, thus triggering intracellular signaling cascades *via* heterotrimeric G proteins and other signal transducers (3). Because of the involvement of GPCRs in a plethora of physiological and pathological processes, this receptor family includes most of the targets of actual and potential drugs (1, 4, 5), thus making GPCRs the largest class of targets for drug discovery.

Selective leukocyte homing *via* chemoattractant/receptor interactions is pivotal for the organization of the immune system and for protection against infectious diseases. Chemoattractants are also key players in the development and exacerbation of immunomediated pathological conditions, such as allergic responses, autoimmune diseases, and other acute and chronic inflammatory disorders, and their fine regulation plays a crucial role for the development of an appropriate immune

Abbreviations: ACKRs, atypical chemokine receptors; ANCA, anti-neutrophil cytoplasmic antibodies; C5aR, activated complement component 5a receptor; FPR, formyl peptide receptor; GPCRs, G protein-coupled receptors; LTB4R, leukotriene B4 receptor; NAMs, negative allosteric modulators; NOD, non-obese diabetic; PAFR, platelet-activating factor receptor; PAMs, positive allosteric modulators; SAMs, silent allosteric modulators; TM, transmembrane helices; WHIM, warts, hypogammaglobulinemia, infections, and myelokathexis.

response (6). Leukocyte chemoattractant ligands include a structurally diverse collection of bioactive molecules, including lipids (leukotrienes, prostaglandins, and platelet-activating factor), peptides (formyl peptides), and proteins (chemokines, non-chemokine cytokines, and defensins). Chemoattractant ligands are recognized by a distinct GPCR family categorized into classical chemoattractant and chemokine GPCRs on the basis of their ligands. Classical chemoattractant GPCRs include formyl peptide receptors (FPR and its variants), the platelet-activating factor receptor (PAFR), activated complement component 5a receptor (C5aR), and leukotriene B4 receptors (LTB4R and its variants). Chemokine GPCRs are subcategorized in four families termed CCR, CXCR, CX3CR, and XCR based on the relative positioning of conserved cysteine residues in the N-terminal domain of their mature ligands. So far, roughly 50 chemokines and at least 18 chemokine GPCRs have been identified in humans (7). Beyond chemokine GPCRs, a group of atypical chemokine receptors (ACKRs), which appear to shape chemokine gradients and dampen inflammation by scavenging chemokines in a G protein-independent β-arrestin-dependent manner, has also recently been recognized (8).

G protein-coupled receptors are integral membrane proteins in constant equilibrium between various functionally distinct conformational states, and this equilibrium is influenced by their exogenous and endogenous ligands (9). Exogenous GPCR ligands can bind to their receptor either competitively (orthosterically) by interacting with the same receptor binding site as the endogenous agonist and are classified as agonists, antagonists, and/or inverse agonists, based on their effects on G protein signaling. Allosteric modulators induce biological responses through interaction with a distinct binding site and can directly modulate binding of orthosteric ligands and their signaling activity. Allosteric modulators have a number of potential advantages over orthosteric agonists/antagonists as therapeutic agents, including greater selectivity for receptor subtypes and the opportunity to identify synthetic ligands for a receptor whose orthosteric binding site has been proven to be chemically intractable, as for glucagon-like peptide 1 receptor agonists (10, 11). However, implications and potentials of allosteric modulation in chemoattractant GPCR biology are far to be fully elucidated, and this review aims at highlighting emerging concepts and open questions.

ALLOSTERISM AND GPCR SIGNALING

The ternary complex model for GPCRs activation, which describes a receptor that moves laterally in the cell membrane to physically couple to a trimeric G protein after activation by an agonist, only accounts for part of the complexity of GPCR-signaling system (12). Ligand binding in the extracellular compartment activates intracellular signals propagated not only through G proteins, but also through β-arrestin and accessory proteins binding, and literature (13) proposes more complex models for receptor activation accounting for multiple signaling states with several conformations stabilized by both different ligands and by single ligand in different conditions. Functional selectivity, probe dependence, and concentration dependence are all properties of chemoattactant receptors' signaling unraveling aspects of the complex processes underlying receptor activation.

Concentration-dependence signaling accounts for different concentrations of the same ligand inducing different receptor responses (14). The typical bell-shaped dose–response curve of chemoattractant-dependent cell migration represents a clear example of this behavior and is particularly relevant in the biology of chemoattractant receptors as they are sensitive to ligand gradients. As an example, high concentrations of a chemokine ligand, such as CXCL12, have been reported to induce inverse migration of CXCR4 expressing cells in several *in vitro* and *ex vivo* models (15, 16). The biological significance of this phenomenon, defined as chemorepulsion or fugitaxis, has been defined in the specific context of T-cell trafficking during thymic migration (17). A number of explanations have been proposed, including the existence of high- and low-affinity-binding sites for the same ligand and the concentration-dependent dimerization/oligomerization of the cognate receptor (18, 19).

A second property of GPCR signaling is functional selectivity or "biased signaling," an effect mainly observed for class A and C GPCRs (20), which refers to the ability of different ligands to activate a certain intracellular signaling pathway over another on a given receptor (21). At least three elements contribute to make functional selectivity a key element for chemoattractants: (i) spatiotemporal and tissue-specific expression of chemoattractant receptors and their ligands; (ii) modulation of receptor activity by proteins interacting with the receptor (or receptor oligomers) or making heterocomplexes with the ligand; (iii) receptor-intrinsic biased signaling triggered by different chemokines binding to the same receptor (22). Indeed, several chemoattractant receptors are activated by multiple endogenous ligands, which may activate distinct signaling pathways through the same receptor, thus suggesting the existence of different "active" conformations of the receptor associated with a particular repertoire of intracellular proteins (23). Consistent with this, several examples of chemoattractant receptors with biased signaling have been reported. This is particularly prominent in receptors with a large number of ligands, such as CCR1, which has partial agonists (CCL14, CCL15, and CCL23) becoming fully active after processing of their extended N-terminal domain (24, 25), β-arrestin-biased ligands (CCL5, CC15, and CCL23) (26), and G protein-biased agonists (CCL5) (26). CCR7 is activated by CCL19 and CCL21, which are equivalent for G protein signaling but differ in their GRK and β-arrestin engagement (27, 28), while all CCR2 ligands have balanced G protein/β-arrestin signaling but interestingly CCL8 is biased for signaling to β-arrestin2 vs. β-arrestin1 (29). Most importantly, there is also clear evidence of the biological relevance of biased signaling in chemokine receptors, as the warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome is caused by mutations deleting the CXCR4 C-terminal domain which generate receptor variants acting as G protein-biased receptors because compromised in their ability to engage β-arrestin for the absence of relevant phosphorylation residues (30). Finally, in the chemokine field, ACKR represents a striking evidence of β-arrestin-biased signaling receptors (31–33). Thus, not only chemokine receptors are complex

signaling molecules able to engage different signaling pathways, but different ligands have biased signaling effects, and this has become of particular relevance considering the chemokine system promiscuity. In this context, the property of allosteric ligands of interacting with ligand-bound receptors introduces a further element of complexity and, not surprisingly, the action of an allosteric modulator may differentially affect receptor functions depending on which agonist is used as activating probe. Probe dependence, a phenomenon widely reported for chemokine receptors (6), is therefore a clear consequence of the cooperativity between orthosteric and allosteric sites. An interesting example of the probe-dependent behavior of allosteric modulators has been reported for a series of CCR1 ligands showing opposite effect on the affinity of two endogenous receptor ligands with not overlapping binding sites. In fact, these metal ion chelating compounds originally selected as full CCR1 agonists were found to act as allosteric enhancers of CCL3 binding while displacing CCL5 binding at the orthosteric site (34). Similarly, AMD3100 acts as a potent allosteric inhibitor of CXCL12-induced CXCR4 activation but does not affect receptor-mediated response to CXCL12 peptide fragments with agonistic properties (35). Allosteric-biased modulation on GPCRs can also occur between G proteins and other signaling effectors, such as β-arrestins, as demonstrated in the case of a CXCR4 allosteric modulator (36). If, on the one hand, probe dependence gives a very high hurdle to the characterization of allosteric ligands; on the other hand, it offers unprecedented opportunities to identify extremely selective drug leads, allowing a fine modulation of receptor-activated signals in complex biological systems.

As discussed, GPCRs are allosteric proteins and G proteins behave as natural endogenous allosteric modulators of this class of receptors. The progressive characterization and identification of functionally conserved allosteric sites in different GPCRs unavoidably raise the question whether these sites may represent binding motifs for unknown ligands, physiologically behaving as allosteric receptor modulators (37). In this perspective, a huge number of natural substances belonging to diverse chemical classes (ions, lipids, and peptides) have been reported as putative endogenous allosteric modulators of GPCRs. Our studies have highlighted the functional relevance of a minor pocket conserved in both classical chemoattractant and chemokine GPCRs accounting for the fine regulation of receptors activation and not involved in the orthosteric ligand binding (38) (see below). The existence of specific endogenous ligands behaving as non-competitive allosteric modulators interacting at this minor pocket represents an attractive work hypothesis.

While offering unprecedented opportunities for the design of highly selective pharmacological tools, the allosterism concept implies a profound revision of the entire drug discovery process having impact on the design and characterization of novel lead candidates targeting the GPCR family. From the structural point of view, biased allosteric modulation, probe dependence, and ligand cooperativity require the ability to model multiple conformational states in the presence of different ligands that still represents a major hurdle for the rational design of drugs. Several independent studies have shown how subtle structural and electronic modifications in a class of allosteric GPCRs modulators may result in dramatic changes of the biological activity, thus limiting the possibilities to conduct large and efficient lead optimization programs (39–41). In this context, the synthesis of focused iterative libraries with limited structural variability is often more efficient than the classical high-throughput screening of large diverse chemical libraries. Furthermore, the biological characterization of a new class necessitates a multistep approach that carefully takes into account the multifaceted characteristics of the allosteric modulation mechanisms. When multiple endogenous ligands for the target receptor are reported, as for chemokine receptors, several *in vitro* assays using different probes are recommended for a correct evaluation of probe-dependent effects. The complexity further increases when biased signal is considered, in fact the development of several functional assays in relevant cellular systems is crucial to assess the effect of selected leads on the different signaling pathways including non G protein-mediated signaling. In many cases, the ideal drug profile for the treatment of a specific pathological condition may be scarcely predicted *a priori*; thus, the ultimate goal of a lead optimization program should be the selection of several chemical classes with distinct *in vitro* (probe dependence and functional selectivity) profiles to be in parallel evaluated in relevant *in vivo* models.

NEGATIVE AND POSITIVE ALLOSTERISM

Receptor allosteric sites are normally devoted to bind endogenous mineral cations, such as sodium, calcium, zinc, and magnesium, or synthetic drugs (42). From a structural point of view, allosteric modulators can be unrelated to the structure of competitive agonists or antagonists. Within the A class GPCR family, orthosteric binding sites are highly conserved and amino acid sequences necessary for the binding of endogenous ligands are retained, while allosteric modulator binding sites show a great structural diversity, thus displaying a high selectivity for a receptor subtype (43). Allosteric modulators can promote or reduce the binding affinity of orthosteric ligands *via* conformational coupling between the orthosteric and allosteric binding sites, or modulate efficacy by altering the functional response of the receptor to orthosteric ligand binding, thus resulting in positive, negative, or neutral effects on receptor activation (**Figure 1**). Negative allosteric modulators (NAMs) bind at the allosteric site to inhibit the efficacy or affinity of agonists to the orthosteric site and do not have any intrinsic agonist efficacy. This effect occurs either by stabilizing an inactive conformation of the receptor or by raising the energy barrier requested to activate the receptor (44). NAMs produce rightward and/or downward shifts in agonist concentration–response curves. This can result from the NAM decreasing agonist affinity (at equilibrium) by stabilizing a lower affinity receptor conformation, from the NAM increasing the energy barrier for transition to the active state, or both. The degree of shift is finite and reaches a maximum as the allosteric site is fully occupied by the NAM, differently from what occurs with competitive orthosteric antagonists which produce ever greater shifts at increasing concentrations with no theoretical limit, because of the direct competition for the agonist-binding site (44). Positive allosteric modulators (PAMs) bind to their

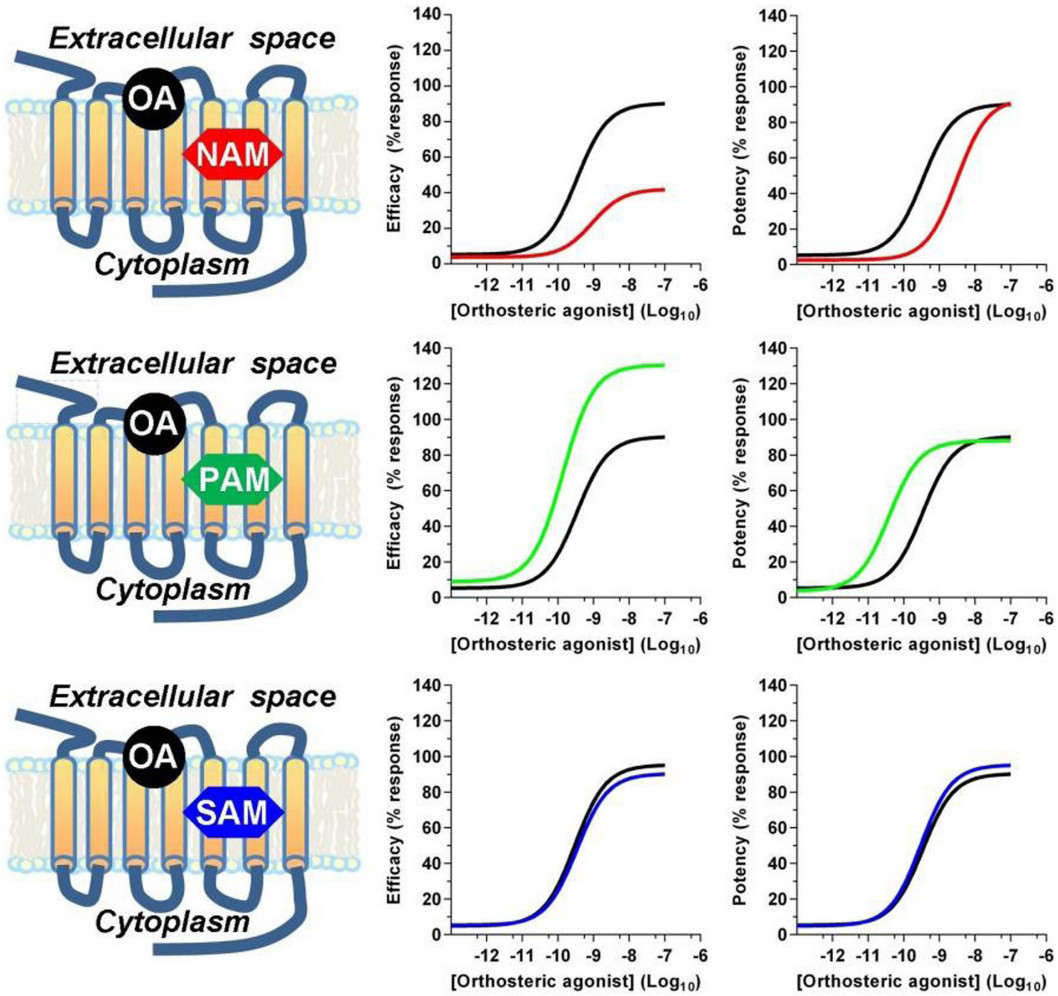


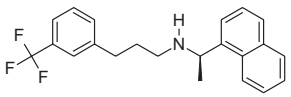
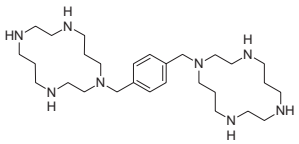
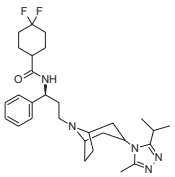
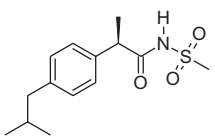
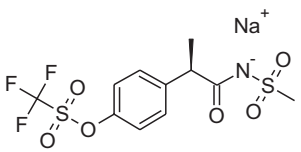
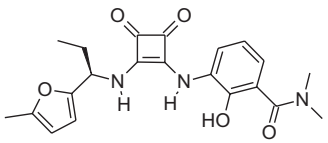
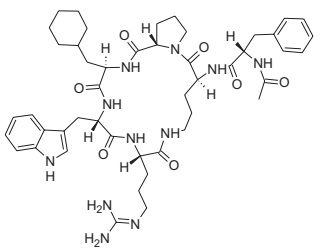
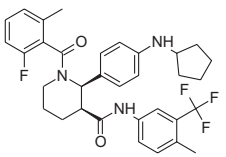
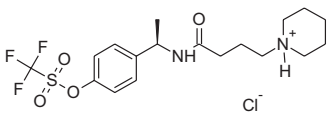
FIGURE 1 | **Allosteric modulators effects on orthosteric agonist efficacy and potency**. Positive (PAM) and negative (NAM) allosteric modulators modulate the affinity and/or the efficacy of orthosteric agonists, while silent allosteric modulators (SAM) have no effect on the affinity and/or efficacy mediated by the orthosteric agonist. Abbreviations used: OA, orthosteric agonist; NAM, negative allosteric modulator; PAM, positive allosteric modulator; SAM, silent allosteric modulator.

allosteric site and either promote the binding of the agonists at the orthosteric site or lower the energy barrier necessary to shift the receptor to the active conformation. PAMs do not display any activity or pharmacological effect in the absence of the endo/exogenous agonists, but when combined with an orthosteric agonist, they increase its efficacy, thus improving the overall side-effect profile of the agonist. From the mechanistic perspective, NAMs and PAMs can exert their effects either by altering the binding affinity of the orthosteric ligand or by inducing a conformational change that affects the ability of the ligand/receptor complex to propagate the stimulus to intracellular proteins. Finally, neutral allosteric ligands, previously referred to as silent allosteric modulators (SAMs), have no effect on orthosteric agonists affinity or efficacy but are able to act as competitive antagonists at the same allosteric site and block PAM or NAM activity, and are often used to confirm the receptor engagement by NAMs or PAMs (45).

ALLOSTERIC MODULATION OF CHEMOKINE RECEPTORS

The most relevant efforts to develop chemokine receptor inhibitors have been focused on drugs blocking HIV infection (see **Table 1**). This effort led to the registration as anti-HIV drug of the CCR5 antagonist Maraviroc (Celsentri/Selzentry; Pfizer), while the CXCR4 antagonist Plerixafor (Mozobil/AMD-3100; Genzyme), originally developed as a second anti-HIV drug, was subsequently assessed as an hematopoietic stem cell mobilizer and is now indicated in combination with G-CSF to mobilize stem cells to the peripheral blood in autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma. Conversely, a number of clinical trials of chemokine receptor antagonists for immunomediated diseases have been disappointingly unsuccessful, generally due to a lack of efficacy in Phase II for inappropriate target selection and/or insufficient receptor

TABLE 1 | Selected inhibitors of chemokine and chemoattractant receptors.

Name	Structure	MoA	Company	Stage	Indication
Cinacalcet		CaSR PAM	Amgen	M	End-stage renal disease
Plerixafor (AMD3100)		CXCR4 NAM	Genzyme	M	Bone marrow transplantation
Maraviroc		CCR5 NAM	Pfizer	M	HIV
Reparixin (DF1681Y)		CXCR1 NAM	Dompé farmaceutici	III	β-cell transplantation
Ladarixin (DF2156A)		CXCR2 CXCR2 NAM	Dompé farmaceutici	II	Onset type 1 diabetes
Navarixin		CXCR2 NAM	Pharmacopeia	D	COPD
PMX-53		C5aR antagonist	Cephalon (now Arana)	D	Immunity inflammation
Avacopan (CCX-168)		C5aR antagonist	ChemoCentrix	III	ANCA vasculitis
DF2593A		C5aR NAM	Dompé farmaceutici	P	Pain

CaSR, calcium-sensing receptor; HIV, human immunodeficiency virus; COPD, chronic obstructive pulmonary disease; ANCA, anti-neutrophil cytoplasmic antibodies; M, marketed; P, preclinical; D, discontinued.

coverage. However, new clinical programs in focused indications are ongoing, setting the premises for a better understanding of the therapeutic potential of these important targets. Among these, 29 drugs are reported as "allosteric modulators of chemokine receptors," three of them being in Phase II and two in Phase III of development (Thomson Reuters Cortellis Business Intelligence; https://cortellis.thomsonreuterslifesciences.com).

Reparixin (formerly known as repertaxin) and ladarixin represent the first examples of non-competitive allosteric modulators of chemokine receptors, showing the ability to behave as NAMs of CXCR1/CXCR2 without affecting the cognate ligand binding affinity. Interleukin-8 (IL-8; CXCL8) and related ELR^+ CXC chemokines are able to interact with CXCR1 and CXCR2 at a different degree, with IL-8 and CXCL6 being potent agonists for both CXCR1 and CXCR2, whereas the other chemokines show a higher selectivity degree toward the CXCR2 subtype. CXCR1 and CXCR2 are largely expressed on PMNs but also T lymphocytes and natural killer cells, and play a key role in leukocyte trafficking in inflammatory conditions (46–49). The contribution of IL-8 and its CXCR1/CXCR2 receptors to the physiopathology of several acute and chronic inflammatory conditions, from ischemia/reperfusion injury to chronic obstructive pulmonary disease and fibrosis, is well assessed by the scientific literature (50–53). Modulators of CXCR1 and CXCR2 function may be useful to treat chronic inflammatory conditions in humans (46). Reparixin was the first known non-competitive allosteric inhibitor of IL-8 receptors, with a 400-fold higher efficacy in inhibiting CXCR1 activity than CXCR2. Its molecular mechanism of action was thoroughly investigated showing that the molecule binds CXCR1 in an allosteric site spanning between transmembrane helices (TM) 1, 3, 6, and 7 and inhibits the signaling triggered by IL-8 without affecting its binding to the receptor (54). The efficacy of the molecule in preventing PMN recruitment and associated tissue damage was demonstrated in experimental models of ischemia/reperfusion injury (2, 55, 56) and organ transplantation (57), thus paving the way to clinical development. The molecule recently completed the first Phase III trial aimed at demonstrating its efficacy in the prevention of graft loss in allogeneic pancreatic islet transplantation, thus confirming the validity of the approach. The knowledge of reparixin molecular mechanism of action paved the way to a rational design approach to identify potent dual CXCR1/CXCR2 inhibitors with improved pharmacokinetic properties suitable for long term administration (41). Ladarixin (DF 2156A), the second clinical candidate in this class, is a highly potent CXCR1 and CXCR2 inhibitor (IC_{50} = 0.1 nM) that is able to block in a probe-independent manner the receptor activation process. Interestingly, mechanistic studies support the rationally derived binding mode hypothesis, thus confirming that the allosteric site is conserved among the two receptor subtypes. The binding mode of the molecule with CXCR1 and CXCR2 is in keeping with the concept that allosteric sites in the TM domains of GPCRs could represent valuable targets for selective allosteric inhibitors able to finely modulate receptor signaling, and suggests their therapeutic investigation in inflammatory disorders. Pharmacological studies were conducted to investigate the potency of CXCR1/CXCR2 inhibition for the prevention of inflammation- and autoimmunity-mediated damage of pancreatic islets. Blockade of CXCR1/CXCR2 was associated with inhibition of insulitis and modification of leukocytes distribution in blood, spleen, bone marrow, and lymph nodes, and was effective in preventing diabetes in an inflammation-mediated model based on multiple low dose injections of streptozotocin and in preventing diabetes in NOD mice (58). Pharmacokinetic, toxicological, and pharmacodynamic data have reinforced the therapeutic clinical potential of Ladarixin, and a Phase II clinical study to test ladarixin at the onset of type 1 diabetes has been recently activated with the aim to confirm this strategy and further investigate its potential in preserving residual β-cell function.

ALLOSTERIC MODULATION OF CLASSICAL CHEMOATTRACTANT RECEPTORS

The complement has long been recognized as a potentially useful therapeutic target, and a number of strategic approaches and therapeutic agents have been developed during the last years (see **Table 1**) (59, 60). Inhibition of complement activation has been approached with low molecular weight natural and synthetic compounds, polypeptides, and macromolecules; nevertheless, to place in the market, a complement-directed drug resulted more challenging than expected. Eculizumab, a humanized mAb against C5, was approved for the treatment of rare disorders only in 2007, then followed in 2008 by the approval of nanofiltered C1 inhibitor, and by the orphan drug designation for the human mAb OMS721 targeting mannan-binding lectin-associated serine protease-2. C5 and its GPCR C5aR have been the main targets for the inhibition of complement activation, with two molecules that have reached clinical stage. In Phase I clinical trials in rheumatoid arthritis and psoriasis, the C5aR cyclic peptidomimetic antagonist PMX-53 (Cephalon, now Arana) was found safe and well tolerated, and able to block C5aR at a stage in immune and inflammatory processes earlier than other current anti-inflammatory drugs, but has been discontinued in 2012 due to poor pharmacokinetic profile and off-target side effects. CCX-168 (now avacopan) ChemoCentrix is an orally administered C5aR inhibitor under development for various autoimmune disorders, including ANCA-associated vasculitis, atypical hemolytic uremic syndrome, and IgA nephropathy. Recently, positive top-line data from CCX-168 Phase II CLEAR trial have been announced in patients with ANCA-associated vasculitis, paving the way for a Phase III trial announced to start within 2016. At the same time, other Phase II trials in rare and orphan indications are ongoing (Thomson Reuters Cortellis Business Intelligence; https://cortellis.thomsonreuterslifesciences.com).

Among molecules still at a preclinical stage, DF2593A represents an interesting case of study on the topic of GPCR allosteric/regulatory sites. Our studies on C5aR were guided by the hypothesis that a minor allosteric pocket conserved across the TM region of the chemoattractant receptor family could represent a "triggering domain" crucial for the fine tuning of receptor activation (61). This pocket spanning between TM 1, 3, 6, and 7 was the same reported as the binding site of reparixin and ladarixin. Despite the low homology between C5aR, CXCR1, and CXCR2, the key

features of the minor pocket were found conserved, thus allowing the rational design of DF2593A as a putative high affinity selective ligand of C5aR. Extensive mutagenesis studies confirmed the mechanistic hypothesis showing that this region, as observed for CXCR1 and CXCR2, is not involved in orthosteric ligand biding but essential for intracellular signal transduction and receptor function (61). DF2593A was shown effective in several animal models of acute and chronic inflammatory and neuropathic pain (61), and is currently under evaluation as a potential clinical candidate in these indications. These studies further confirm the great potential of allosteric modulation as a promising strategy to generate potent and selective modulators of chemoattractant receptors.

ALLOSTERIC MODULATORS: NEW OPPORTUNITIES FOR DRUG DISCOVERY

Studies on allosteric ligands with different binding properties at cognate GPCRs have led to a substantial increase in our understanding of GPCR pharmacology, thus smoothing the way to the design of safer, better-tolerated, and more efficacious drugs. The recent advances in GPCR structure biology, with the elucidation of several high resolution crystal structures of GPCRs [references in Ref. (62)], will give a further significant boost to this complex and stimulating research field (62), offering new tools for the rational design of allosteric modulators.

As thoroughly discussed, allosteric GPCR modulators present unique advantages as compared to orthosteric ligands, mostly by virtue of their high receptor subtype selectivity and functional selectivity. The first characteristic relies on the greater divergence in the amino acid sequence of allosteric sites between receptor subtypes. While functionally conserved, allosteric sites apparently evolved under a lower evolutionary pressure as compared to the orthosteric sites involved in the recognition of the endogenous ligand. The functional selectivity and probe-dependence properties of many allosteric modulators are intrinsically associated with ability of these molecules to fine tune the dynamic conformational rearrangement of the receptor and ligand/receptor complexes. This second level of selectivity may offer unprecedented opportunities for the design of tailored pharmacological tools but also implicates a profound evolution of the drug discovery process demanding for a deep *in vitro* characterization of the new lead candidates for a correct interpretation of *in vivo* studies results. A fascinating aspect of this research field originates from the continuous mutual feedback between drug discovery and receptor biology research: in a virtuous circle, new lead candidates stemmed from medicinal chemistry programs become important research tools useful to improve the knowledge of GPCR structure and function that fundamentally influence the drug design and development process.

From the drug discovery point of view, allosteric modulators may provide functional advantages over classical orthosteric agonists and antagonists (63). First, as allosteric modulators do not compete with endogenous ligands, their effect on GPCRs is saturable, meaning that when all allosteric sites are occupied no more effects are achieved. Second, as allosteric ligands modulate activities of endogenous ligands engaging the orthosteric site, their influence on receptors' conformation and signaling will be evident only when the endogenous ligand is present. Third, NAMs often show only partial antagonist activity without exhibiting any agonist activity (64), thus suggesting that a partial NAM could have a greater safety index than a full antagonist. Also for PAMs, the above described ligand-dependent activity may improve safety profile, due to the fact that normal physiological regulation of signaling, including temporal regulation, remains unchanged (65).

The multifactorial interactions implicated in chemoattractant biology make very difficult to predict the *in vivo* behavior of allosteric ligands in pathological conditions, and a deep *in vitro* characterization in different conditions is absolutely required for the interpretation of the results of pharmacological studies. Antagonist affinities can vary depending on the agonist, the presence or absence of allosteric ligands, the specific site through which the effect is exerted, and the specific signaling under consideration. Since most GPCRs can engage different downstream signaling pathways, which are often cell-, tissue-, and/or context-specific, it is crucial to take into account the entire signaling repertoire for the drugs of reference in normal vs. pathological conditions. Future research efforts should be oriented toward the development of approaches aiming to elucidate the full spectrum of ligand signaling in different cell models and able to integrate new screening and quantitative analytical methods, with the aim to link these signaling signatures to preclinical or clinical data.

CONCLUSION

The discovery of allosteric modulators has represented a profound advance in the research of drugs acting on chemoattractant receptors, and over the last years, several NAMs and PAMs of both chemoattractant receptors belong to both classical chemoattractant (Cinacalcet, PAM of CaSR of PTH) and chemokine GPCRs (Reparixin, NAM of CXCR1; Ladarixin NAM of CXCR1/CXCR2; and Navarixin, NAM of CXCR2) entered clinical trials, and in some cases, successfully reached the market (Maraviroc, NAM of CCR5; Plerixafor, NAM of CXCR4). The pharmacological implications potentially deriving from the availability of multiplicity of molecules that, acting through a single receptor, differentially regulate its signaling activity are still far from being fully exploited and will offer opportunities for the development of new drugs targeting chemoattractant receptors in the near future.

AUTHOR CONTRIBUTIONS

MA, MC, and ML contributed equally to writing and critically revised the paper.

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Evasins: Therapeutic Potential of a New Family of Chemokine-Binding Proteins from Ticks

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Blood-sucking parasites, such as ticks, remain attached to their hosts for relatively long periods of time in order to obtain their blood meal without eliciting an immune response. One mechanism used to avoid rejection is the inhibition of the recruitment of immune cells, which can be achieved by a class of chemokine-binding proteins (CKBPs) known as Evasins. We have identified three distinct Evasins produced by the salivary glands of the common brown dog tick, *Rhipicephalus sanguineus*. They display different selectivities for chemokines, the first two identified show a narrow selectivity profile, while the third has a broader binding spectrum. The Evasins showed efficacy in animal models of inflammatory disease. Here, we will discuss the potential of their development for therapeutic use, addressing both the advantages and disadvantages that this entails.

Keywords: chemokines, binding proteins, antagonists, pathogens, ticks, protein therapeutics

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INTRODUCTION

The recruitment of immune cells is essential for the establishment of an immune response that, if uncontrolled, can lead to an unwanted inflammatory situation. The pharmaceutical industry has, for several decades, sought to inhibit excessive leukocyte recruitment by interference with the chemokine system, unfortunately with only limited success to date. Therefore, we investigated the manner in which pathogens and parasites avoid rejection by an immune response. It has been known for some time that pathogens, such as viruses, have pirated many molecules of the mammalian immune system, including molecules that either mimic or inhibit chemokines and their receptors to subvert the immune system. Among these molecules, there are a number of chemokine-binding proteins (CKBPs) that directly interact with chemokines to inhibit their activity (1). The vast majority of CKBPs have been identified in viruses, and these proteins are often able to recognize a large number of chemokines. As an example, M3, a protein encoded by the murine gammaherpesvirus-68, binds to chemokines from the four different subfamilies (CC, CXC, C, and CX_3C) (2), whereas gG (from herpes simplex virus) and Crm (encoded by smallpox virus) interact with chemokines from at least three subfamilies (3, 4). In 2005, the first eukaryotic CKBP was identified in the worm *Schistosoma mansoni* and was shown to bind promiscuously to some CC chemokines, notably CCL3 and CCL5, CXCL8, and CX3CL1 *in vitro* (5).

Following a report describing anti-CXCL8 activity in the salivary glands of several Ixodid tick (or hard tick) species (6), we extended this observation by identifying the molecule responsible for this activity. To do this, we first tested the ability of the saliva from the hard tick *Rhipicephalus sanguineus* to inhibit the binding of three chemokines, CCL3, CCL5, and CXCL8 to their receptors, and were also able to identify the presence of these molecules in the saliva by surface plasmon

resonance (SPR) and mass spectrometry (7). Using cDNA expression libraries constructed from the tick salivary glands, we used a cross-linking approach to analyze the proteins secreted into culture supernatants after transient expression of pools of cDNAs in HEK293 cells. We successfully identified three CKBPs that we named the Evasins (7, 8).

CHARACTERISTICS

Evasins have been quite extensively characterized in terms of their chemokine-binding profile and inhibitory potency *in vitro* and *in vivo* activity. On the one hand, Evasin-1 demonstrated the highest specificity as it binds only to three closely related chemokines, CCL3, CCL4, and CCL18. On the other hand, Evasin-3 recognizes a subset of CXC chemokines, the family of the so-called ELR$^+$ chemokines, i.e., CXCL1, -2, -3, -5, -6, and -8. Both of these CKBPs efficiently inhibit the activity of their ligands, preventing cell migration *in vitro*. Evasin-4, which was initially identified using I^{125}-labeled CCL5 and CCL11 as bait, displayed a broader selectivity profile than the other two Evasins, being able to interact with at least 18 chemokines, yet it is still highly specific as it recognizes only members of the CC subfamily (9). Although minor discrepancies were observed between the binding and the inhibitory profile of Evasin-4, it blocks the activity of the majority of its ligands, including the proinflammatory chemokines CCL3, 5, 8, and 11.

Based on their binding profile, Evasin-1 and -3 would be expected to inhibit the migration of neutrophils in mice, which is crucial in the first steps of the immune response. Here, it should be noted that one of the major differences in the leukocyte recruitment profiles in the chemokine system is that of the neutrophil. In mice, neutrophils express both CCR1 as well as CXCR2, which results in the recruitment of this leukocyte by both the CCL ligands and the CXCL ligands activating these receptors, respectively. Regarding Evasin-4, its ability to prevent the interaction of CC chemokines with their receptors may also lead to the inhibition of eosinophil recruitment, an essential cellular component of the response against parasites.

Vancova et al. have reported the presence of anti-CXCL8 activity and anti-CXCL1 activity in salivary gland extracts from males and females of several other species of ticks: *Amblyomma variegatum*, *Rhipicephalus appendiculatus*, and *Dermacentor reticulatus*, suggesting that Evasin-3-like activity is common among metastriate ixodid tick species (10). In a separate study, the same group also reported activity against human CXCL8, CCL2, CCL3, CCL5, and CCL11 in adult *R. appendiculatus* ticks (11), suggesting that Evasin-1 and Evasin-4 orthologs probably exist in this species too. Also in this study, the authors showed that anti-chemokine activity differed significantly at different times during feeding and also differed between males and females supporting the concept of "mate guarding," in which males help their mates to engorge by controlling their host's immune response, and the possibility that ticks benefit from feeding together in close proximity by exploiting molecular individuality. Interestingly, in this species, anti-CCL11 activity was high in unfed ticks, initially declined, and then increased in both males and females as feeding progressed (11).

As previously mentioned, the existence of viral CKBPs was reported before the identification of Evasins. However, although they probably share similar functions *in vivo*, several differences have been highlighted between these two families of CKBP. First, as mentioned above, the large majority of viral CKBPs display broad-spectrum chemokine-binding profiles, whereas the Evasins are much more selective. It is noteworthy that the Evasin-4-binding profile closely mimics that of the viral CKBP vCCI. These two CKBPs recognize between 13 and 18 chemokines, yet are still highly selective in that they bind only to CC chemokines (9, 12). Therefore, among CKBPs, they form a unique class of chemokine binders with "semi-broad" selectivity.

Another key difference between tick and viral CKBPs is the size of these proteins. Viral CKBPs are large proteins, which might even form dimers as reported for M3 (13). On the contrary, Evasins are small proteins of around 80–100 amino acids, indicating that the two partners of the chemokine:Evasin interaction have similar sizes.

DO EVASINS EXIST IN OTHER SPECIES?

We were intrigued to know whether these molecules formed a family of CKBPs in both ticks and in other species, particularly man. Blasting their sequences against the human genome, and all other mammalian genomes available, did not produce any significant hits.

However, at least five putative Evasin-1 and Evasin-3 homologs have been identified following in-depth sequence analysis of the *R. sanguineus* sialotranscriptome (14). Expressed sequence tags that are Evasin-3-like have been identified in *Ixodes scapularis*, *Ixodes ricinus*, and *Dermacentor andersoni* (10), and the sequences of potential Evasin homologs have also been identified in Genbank for *Boophilus microplus* and *I. scapularis* (Power, unpublished analysis). At least 18 Evasin homologs have been identified for *Amblyomma maculatum* (15), and Radulovic et al. recently reported a sequence homologous to Evasin-1 in *Amblyomma americanum* (16). Considering there are over 700 Ixodidae species, and about 200 soft tick or Argasidae species, with recent developments in next generation sequencing and proteomics, it is likely that many more homologous sequences will be identified in the coming years. Yet whether any of the homologs described above encode a CKBP has not yet been confirmed by functional analysis.

Even though blasting the primary sequences of the Evasins did not reveal homologs in mammals, we hypothesized that proteins with similar folds might exist in eukaryotics. While the structures of Evasin-1 and Evasin-3 were found to be totally different from each other, blasting the PDB database of three-dimensional protein structures, once again did not produce any hits. The third CKBP, Evasin-4, has a disulfide bridge pattern that aligned with that of Evasin-1, indicating that it would probably have the same three-dimensional fold (**Figure 1**) (17), so it was unlikely to possess a different structural motif. Thus, it appears that while this tick species has unique CKBPs, one cannot rule out that other ticks, particularly hard ticks that feed for extended periods, will have their own unique CKBP(s).

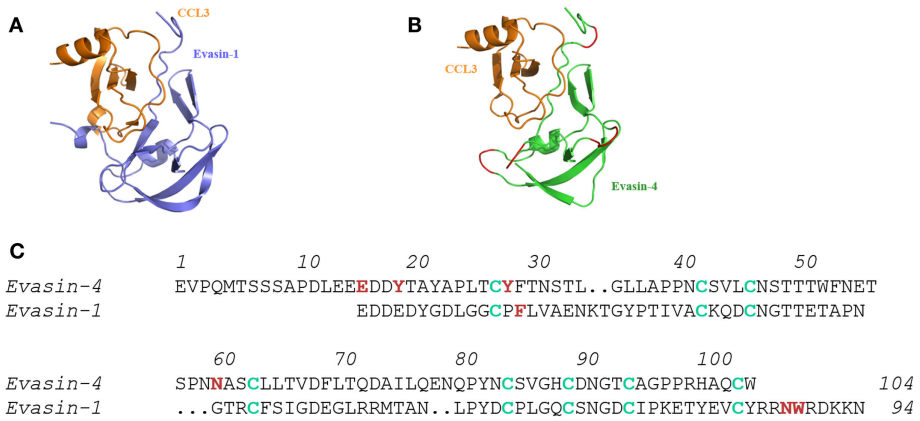

FIGURE 1 | Molecular interactions of Evasin-1 and -4 with CCL3. **(A)** Structure of the complex of Evasin-1 and CCL3 determined by X-ray crystallography. **(B)** Evasin-4 in complex with CCL3 by *in silico* modeling using **(A)** (17). **(C)** Alignment of the primary amino acid sequences of Evasin-1 and -4. Cys residues are shown in green and amino acids identified to play a role in chemokine binding are shown in red (17), demonstrating that the selective CHBP, Evasin-1 predominantly uses the carboxy terminal region, whereas Evasin-4 that binds many CC chemokines predominantly uses the amino terminal region.

ACTIVITY *IN VIVO*

The Evasins have all shown efficacy *in vivo* in several disease models. As predicted from its binding profile, Evasin-1 reduced neutrophil recruitment induced by CCL3 in a peritoneal cell recruitment assay in a dose-dependent manner (8). This highlights one of the anomalies of translating *in vivo* data from mice to man as described above. In humans, CCL3 is not a principal mediator of neutrophil recruitment since its receptors CCR1 and CCR5 are primarily expressed on monocytes/macrophages, although they can be induced, for example, by IFNγ (18). However, in mice, CCR1 is highly expressed on neutrophils, resulting in strong recruitment in response to CCL3. On the other hand, neutrophil recruitment in mice is also mediated by CXCR2 ligands, as it is in the human system. Thus, in accordance with its ability to inhibit neutrophil infiltration, Evasin-1 showed good efficacy in reducing the fibrosis, which follows neutrophil infiltration into the lung after bleomycin administration, and also reduced the mortality observed in this model (19).

Evasin-3 was effective in several neutrophil-dependent disease models as expected from its *in vitro* selectivity profile, showing that it binds and inhibits ELR$^+$ chemokines that bind to CXCR2. Again, dose-dependent inhibition of leukocyte infiltration into the peritoneal cavity, this time in response to CXCL8, was inhibited by Evasin-3. Antigen-induced arthritis (AIA), induced by intradermal administration of mouse BSA, is highly neutrophil dependent. In AIA, disease symptoms were significantly decreased by the administration of Evasin-3. In another neutrophil-mediated scenario, ischemic reperfusion injury, both Evasin-1 and Evasin-3 were effective, but Evasin-3 was shown to be more efficacious, indicating that the CXCR2 ligands play a predominant role in this model. On the contrary, only Evasin-1 and not Evasin-3 was effective in inhibiting the first wave of dendritic cell recruitment to the site of infection with *Leishmania major*, since it is mediated by neutrophil-secreted CCL3 (20). Intriguingly, despite the fact that Evasin-1 has only been shown to bind to three chemokines *in vitro*, it was able to reduce the skin inflammation observed in the D6$^{-/-}$ mice in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (8), a model which had previously been shown to depend on several inflammatory ligands (21).

Studies with Evasin-4 produced some puzzling observations. In line with its broad selectivity profile and inhibitory activity against several CC chemokines known to have proinflammatory activity, it was shown to be effective in reducing post-infarction myocardial injury and remodeling (22) and DSS-induced colitis (23), yet Evasin-3 that only binds ELR$^+$ CXC chemokines *in vitro* was also effective in the myocardial injury model. This highlights the problem in the translation of agents inhibiting neutrophil-mediated inflammation in mice to the human setting.

Because of its broad CC chemokine-binding spectrum, Evasin-4 was considered the most suitable Evasin for development as a possible therapeutic candidate. However, it is well known that small proteins have a very short half-life *in vivo* and are not orally available, which means that for chronic indications, they would have to be injected with a frequency that is not convenient for patients. In order to prolong the serum half-life of therapeutic proteins, the strategy of making Fc fusions is often employed. Therefore, we created fusions of Evasin-4 with the Fc portion of human IgG1, making both N- and C-terminal versions (9). Having characterized the Fc fusion proteins *in vitro* and selected the format that had activity closest to wild-type Evasin-4, we compared their activity in a simple disease model, fluorescein isothiocyanate (FITC)-induced contact hypersensitivity. While Evasin-4 had dose-dependent efficacy in reducing the disease symptoms, molar equivalents of the fusion protein were totally ineffective (**Figure 2**).

The negative result obtained with Evasin 4 fused to the C-terminal of Fc (Fc-Evasin-4) was unexpected as we predicted that the Fc domain would increase the therapeutic potential of Evasin-4 and not abrogate its anti-inflammatory activity. In the hypersensitivity model, the treatment schedule was based

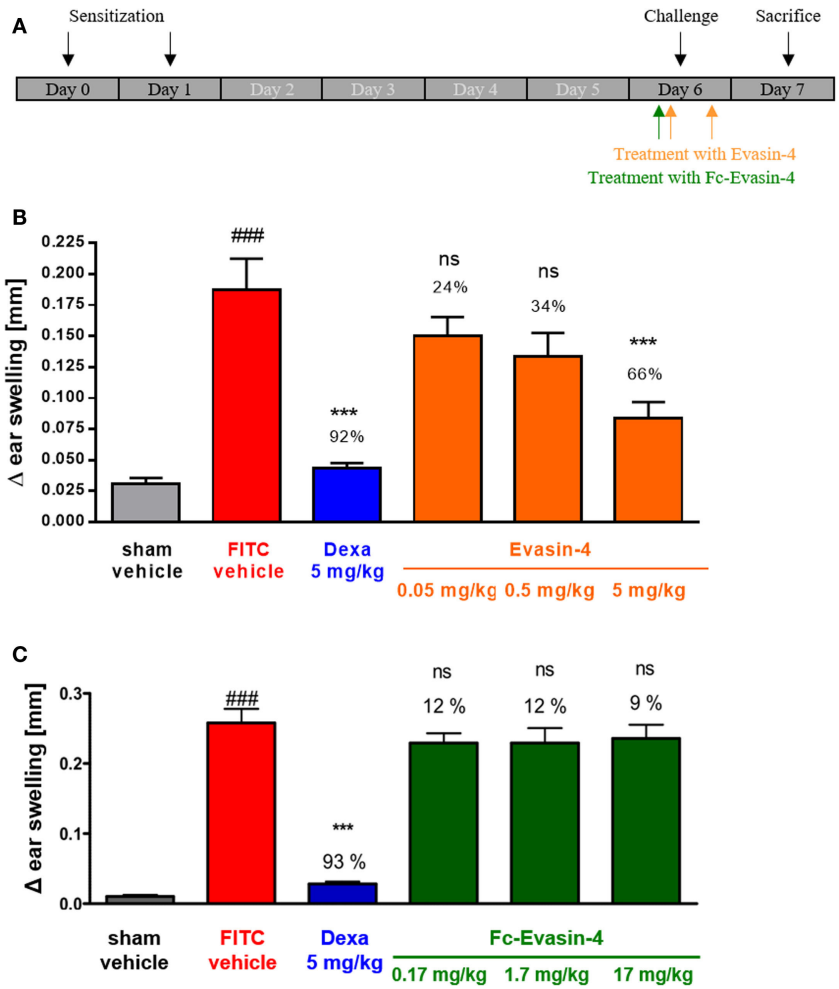

FIGURE 2 | **FITC-induced contact hypersensitivity**. **(A)** Schematic design of the experiment. **(B)** Treatment with Evasin-4 reduces ear swelling (n = 5–9 mice per group). **(C)** Treatment with Fc-Evasin-4 does not prevent ear inflammation (n = 8 mice per group). Data are presented as mean ± SEM and were analyzed with one-way ANOVA and Dunnett's post-test. The percentage of ear swelling inhibition is reported for each treatment. $^{###}p < 0.001$ compared with sham, $^{**}p < 0.01$ or $^{***}p < 0.001$ compared with vehicle-treated FITC group, ns not significant.

on the half-life of the protein, and as Fc-Evasin-4 exhibited a much longer half-life than Evasin-4, Evasin-4 was injected twice, whereas Fc-Evasin-4 was injected only once, before the challenge with FITC. These different treatment schedules may explain the lack of activity of Fc-Evasin-4 in comparison to Evasin-4. Our current hypothesis is based on the fact that during inflammation, a large amount of chemokines is produced and that the administration of one single dose of the Fc fusion protein might not have been sufficient to saturate the system.

This was confirmed by the measurement of chemokine levels in serum samples from contact hypersensitivity models, which demonstrated a significant accumulation of MCP-1 (the murine equivalent to CCL2), MIP-1α (equivalent to CCL3), and RANTES (equivalent to CCL5) following treatment with Fc-Evasin-4, as has been reported for administration of an anti-CCL2 antibody (24). Therefore, although the extended half-life of Fc-Evasin-4 allows it to remain in the circulation for more than 1 week, the drug is probably rapidly saturated and no unbound Fc-Evasin-4 molecules are available to inhibit newly produced chemokines and to reduce inflammation. In the case of Evasin-4, CKBP:chemokine complexes are probably more rapidly degraded, preventing their accumulation in circulation. Furthermore, the second bolus of drug provides an additional amount of free Evasin-4 molecules available to inhibit chemokine activity. Therefore, although theoretically equimolar, the doses of Evasin-4 and Fc-Evasin-4 used are probably not equivalent *in vivo*. Thus, the Fc fusion would only be useful if the chemokine is released and degraded in the endosome allowing the fusion protein to recycle back into the circulation to pick up more chemokines, necessitating engineering the Evasin to bind chemokine at pH 7.2 and release it at pH 6.0, as has been reported for mAbs (25, 26).

THERAPEUTIC POTENTIAL

A major consideration for the development of protein therapeutics is immunogenicity – where the body elicits an immune

response (production of antibodies) against the therapeutic entity. Antibodies against a therapeutic protein may elicit a wide range of consequences, from no detectable change to life-threatening conditions. One of the main concerns is altered drug safety or compromised efficacy. Antibody formation may attenuate the efficacy even to the extent that higher doses cannot overcome the clinical resistance induced by the antibody response. Deleterious effects can occur when antibodies against a therapeutic agent cross-react with endogenous proteins. Neutralizing the endogenous protein can be particularly dangerous, especially if it is a unique protein with non-redundant function. Such was the case for erythropoietin a few years ago. An immune response that neutralized the activity of both the administered recombinant protein Eprex® and that of the endogenous protein in patients had dramatic consequences, resulting in an acute anemia called pure red-cell aplasia (PRCA) that was fatal in a few patients [reviewed in Ref. (27)]. In the case of non-human proteins, especially those with no homology to any known human protein, this type of reaction would not be relevant. Nevertheless, nearly all therapeutic proteins – be they native human proteins, monoclonal antibodies, antibody drug conjugates, or fusion proteins – can elicit an immune response. Another potential danger of antibody formation against therapeutic proteins is the elicitation of immunoglobulin (Ig) E-mediated hypersensitivity reactions ranging from local skin reactions to more severe systemic reactions such as anaphylaxis. However, cases of anaphylaxis have been seen with almost every substance administered to man, ranging from peanuts to recombinant interferon-β, but fortunately are not common.

Nevertheless, there are already examples of non-human proteins in the clinic. Hirudin, a small protein produced by leeches, is an inhibitor of thrombin and is used extensively for the retreatment of heparin-induced thrombocytopenia for patients undergoing hip replacement surgery (28). A second example is the GLP-1 receptor agonist exenatide (synthetic exendin-4), a 39 amino acid peptide, marketed as Byetta®, which was originally identified in the salivary secretions of a poisonous lizard known as the Gila monster (*Heloderma suspectum*). Exenatide was developed as a first-in-class type 2 diabetes therapy (29). In a recent report, it was shown that low-titer anti-exenatide antibodies were common with exenatide treatment but had no apparent effect on efficacy. Higher titer antibodies were less common, and increasing antibody titer was associated with reduced average efficacy, but other than injection-site reactions there were no safety issues (30).

A number of factors are now known to influence the immunogenicity of therapeutic proteins, but in general, the less "human" a protein is, the more likely it is to elicit an immune response, particularly following repeated administrations. However, predicting immunogenicity remains a subject of much debate. We used both proprietary (Antipred) and publicly available software (TEPITOPE) for *in silico* prediction of potential $CD4^+$ T-cell epitopes in the Evasins. Interestingly, interferon-β, one of the most widely used treatments for multiple sclerosis, was predicted to contain more antigenic sites than the Evasins (unpublished data).

It should also be noted that the Evasins produced by the tick are highly glycosylated proteins. Their apparent molecular weights as estimated by SDS-PAGE analysis during their expression cloning was about five times their actual protein mass. This could be hypothesized to render them less susceptible to a host immune response. The counter argument to their status of immune-silent is that they are injected into the host in saliva containing a plethora of other proteins, which could equally play a role in preventing an immune reaction. This is obviously not the case for a therapeutic protein, where in fact the route of administration and relatively large amounts that would be administered systemically (compared to the miniscule amounts injected locally in tick saliva) would be influential on the ensuing immunogenicity.

In view of the potential of the Evasins as therapeutic modalities, we produced Evasin-3 and -4 as Fc fusion proteins. This was to counteract their rapid elimination as is the case for all small proteins. Both fusion proteins retained neutralizing activity *in vitro* comparable to the wild-type proteins. However, surprisingly, Evasin-4 lost its neutralizing activity when administered as an Fc fusion. The WT protein showed dose-related activity in inhibiting the clinical symptoms in a contact hypersensitivity model, but administration of equivalent molar amounts of the Fc fusion had no effect whatsoever. The reason for this remains unexplained, but these results prompt us to wonder whether simultaneous inhibition of chemokines with a multispecific chemokine-binding protein may be an efficient strategy to clinically improve chemokine-driven diseases. As pan-specific chemokine inhibitors bind to multiple targets, and as the amount of chemokines present in the body may be very large, due to immobilization on cell surfaces, as well as to rapid turnover and production rates, multispecific inhibitors might be saturated *in vivo*, and very high doses may be required to observe an anti-inflammatory activity. This hypothesis may also explain the lack of long-term efficacy reported with the fusion protein vCCI-Fc (31). As discussed above, a solution could be the engineering of the CKBPs to render them pH dependent. Alternatively, if simultaneous inhibition of several chemokines is required, a more successful strategy may be the broad inhibition of chemokine-induced cell migration without direct interaction with chemokines or their receptors. This strategy is exemplified by the broad-spectrum chemokine inhibitors known as somatotaxins, such as NR58-3.14.3, which is effective in a range of inflammatory disease models, including atherosclerosis and graft-versus-host disease (32–34). Another example of broad-spectrum chemokine inhibition would be by interfering with chemokine signaling as demonstrated by PI3K inhibitors. These results suggest that indirect interference with cell migration may be a promising strategy to prevent excessive recruitment to inflamed sites.

It is clear from the above examples that certain pathologies may be driven by the action of several chemokines acting on distinct receptors, thus arguing that the use of broad-spectrum chemokine antagonists or at least multispecific antagonists would be beneficial. However, a recent report demonstrating biased agonism (35) has provided rationale for targeting individual ligands with biologicals, such as mAbs, to avoid off-target effects. A good example is the receptor CXCR3, whose ligands CXL10 and CXCL11 play opposing roles – the former having a proinflammatory activity while the latter is anti-inflammatory (36). To date,

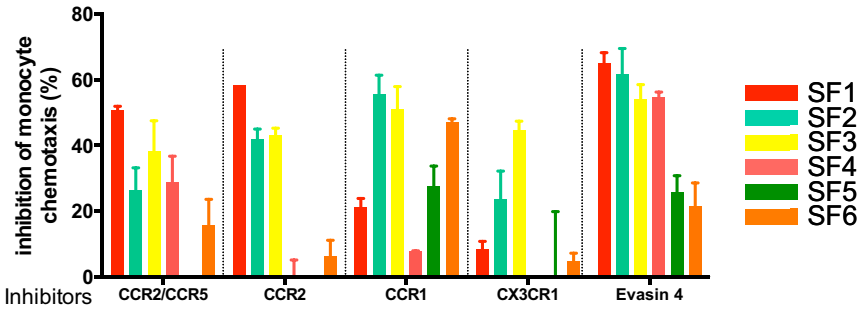

FIGURE 3 | **Inhibition of synovial fluid-induced monocyte recruitment by selective chemokine receptor inhibitors and Evasin-4**.

most of the therapeutic approaches taken by the pharmaceutical industry have been to inhibit individual chemokine receptors. Nevertheless, there are still gaps in our understanding of their precise roles. An interesting example is CCR2 that binds several CC chemokines: CCL7, CCL8, and CCL14, which also bind to other receptors, but the CCL2/CCR2 interaction is non-redundant. CCR2 has been targeted in three separate clinical trials without much success. Is this due to a problem with the target or the drug? The importance of the CCL2/CCR2 interaction in monocyte recruitment is compelling, yet animal models suggest that CCR1 may also play role (37). How does this relate to monocyte recruitment in human disease? In a recent *in vitro* study, we looked at the ability of specific chemokine receptor antagonists and Evasins to block monocyte chemotaxis in response to synovial fluid harvested from six rheumatoid arthritis patients (Proudfoot et al., unpublished data).[1] We observed that only Evasin-4 could inhibit monocyte migration in all samples (**Figure 3**). In this system, Evasin-4 acts as a soluble chemokine receptor with specificity for multiple monocyte-directed ligands, providing a much more simple approach to chemokine antagonism than targeting one, two, or even multiple chemokine receptors with small-molecule antagonists or antibodies.

Thus, the Evasins present therapeutic potential in pathologies where several chemokines are involved. However, there are certain aspects that must be addressed for future development of these molecules. In view of the observed lack of activity with the Evasin-Fc fusion, treatment of acute indications, where short half-life is not a problem, would be first choice. Moreover, the administration of such proteins for an acute regimen would circumvent the potential issues of immunogenicity. The development of the Evasins for more chronic diseases would require optimization of the potential biologic modality, for example, half-life extension. Production of a pH-dependent chemokine-binding molecule could also solve the problem of the large amount of target protein(s) to be neutralized. With their small size and unique structure, Evasins are also very attractive targets for protein engineering to introduce exquisite specificity, as more and more information becomes available on the role of specific chemokines in human disease. However, we believe that future directions in the search for novel innovative approaches to the treatment of inflammatory diseases should include the study of how nature deals with the immune system – there is still a lot to be learnt from pathogens and parasites that have evolved elegant mechanisms to avoid rejection by their hosts.

[1] A. Proudfoot, C. Gabay, A. Garin. 2016, unpublished work

AUTHOR CONTRIBUTIONS

AP initiated and directed the project and wrote the manuscript; CP supervised the cloning and characterization of Evasins and wrote the manuscript; and PB performed experiments and wrote the manuscript.

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frontiers in Immunology

MINI REVIEW
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STAT3, a Key Parameter of Cytokine-Driven Tissue Protection during Sterile Inflammation – the Case of Experimental Acetaminophen (Paracetamol)-Induced Liver Damage

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Acetaminophen (APAP, *N*-acetyl-*p*-aminophenol, or paracetamol) overdosing is a prevalent cause of acute liver injury. While clinical disease is initiated by overt parenchymal hepatocyte necrosis in response to the analgetic, course of intoxication is substantially influenced by associated activation of innate immunity. This process is supposed to be set in motion by release of danger-associated molecular patterns (DAMPs) from dying hepatocytes and is accompanied by an inflammatory cytokine response. Murine models of APAP-induced liver injury emphasize the complex role that DAMPs and cytokines play in promoting either hepatic pathogenesis or resolution and recovery from intoxication. Whereas the function of key inflammatory cytokines is controversially discussed, a subclass of specific cytokines capable of efficiently activating the hepatocyte signal transducer and activator of transcription (STAT)-3 pathway stands out as being consistently protective in murine models of APAP intoxication. Those include foremost interleukin (IL)-6, IL-11, IL-13, and IL-22. Above all, activation of STAT3 under the influence of these cytokines has the capability to drive hepatocyte compensatory proliferation, a key principle of the regenerating liver. Herein, the role of these specific cytokines during experimental APAP-induced liver injury is highlighted and discussed in a broader perspective. In hard-to-treat or at-risk patients, standard therapy may fail and APAP intoxication can proceed toward a fatal condition. Focused administration of recombinant STAT3-activating cytokines may evolve as novel therapeutic approach under those ill-fated conditions.

Keywords: acetaminophen, acute liver injury, hepatocytes, STAT3, IL-6, IL-11, IL-13, IL-22

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INTRODUCTION

Acute liver injury (ALI) is a major burden of health care systems worldwide. Viral infections and side effects of pharmacotherapy stand out among pathological challenges provoking ALI. Specifically, overdosing of the weak-to-moderate analgesic acetaminophen (APAP; *N*-acetyl-*p*-aminophenol; or paracetamol) is regarded as one major cause of ALI in the developed countries. Notably, over-the-counter availability, underrated toxicity, and a narrow therapeutic margin further APAP

misuse/intoxication which, if not timely treated with its antidote *N*-acetylcysteine, can proceed toward a fulminant condition requiring transplantation for patient survival (1–3).

Specifically, APAP is hold responsible for up to 80,000 emergency visits, 2500 hospitalizations and 500 fatal intoxications in the United States annually (2, 4). A recent study analyzing between 2005 and 2007 patients from selected European countries documented 114 drug overdose-related cases of ALI demanding transplantation (of 600 totals). Ninety-seven percent (111 cases) of those concerned APAP (5). In Germany, 850 hospitalizations due to APAP intake were recorded 2012 for patients with statutory health insurance. However, only four fatalities were documented (6). Altogether, epidemiological studies indicate noticeable variations in the incidence of severe APAP-induced ALI in different populations within Europe (5) and the developed countries altogether.

On a cellular level, liver injury by APAP is regarded a two-hit process involving initial direct induction of hepatocyte cell death and, subsequent to that, activation of innate immunity that triggers an inflammatory response having the complex potential to either aggravate disease or to actually support tissue repair and hepatic regeneration (7–12).

Hepatocyte cell death, being at the root of APAP toxicity, is dependent on drug metabolizing cytochrome P450 enzymes (Cyp), particularly Cyp2e1 and Cyp1a2 (8, 13). These enzymes generate from APAP poisonous *N*-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive metabolite capable of coupling to protein sulfhydryl groups thereby disturbing hepatocyte cell physiology. Specifically, NAPQI mediates mitochondrial oxidative stress, drop in ATP generation, c-jun N-terminal kinase (JNK) activation, and eventually cell death (8, 14). Although apoptosis and necrosis as well as necrosis-related necroptosis have all been detected during experimental APAP-induced ALI, the latter two types of cell demise prevail in the context of pathological intoxication. Notably, as opposed to immune-deactivating apoptosis, necrosis and necroptosis connect to activation of innate immunity and inflammation (7, 8, 14, 15) whereby the leukocytic cell compartment becomes involved into course and outcome of APAP-induced ALI.

THE COMPLEX ROLE OF INNATE IMMUNITY AND INFLAMMATORY CYTOKINES IN EXPERIMENTAL MURINE APAP-INDUCED LIVER INJURY

Key to sterile necro-inflammation, as detected in APAP-induced ALI, is release of danger-associated molecular patterns (DAMPs) from cells undergoing necrosis. Once increasingly present in the extracellular compartment or later on taken up by leukocytes, those are detected by sensors of innate immunity, e.g., toll-like receptors (TLR), setting in motion inflammatory processes that can drive pathology but also setting the stage for parenchymal tissue repair and regeneration (9–12, 16, 17).

DAMPs reported to mediate pathological immunoactivation during APAP-induced ALI include high-mobility group box 1 protein (HMGB1) (18–20) and histones (21). Both couple to TLR4 on hepatic monocytes/macrophages, including resident Kupffer cells. Besides that, HMGB1 was found to activate the receptor for advanced glycation end product (RAGE) on neutrophils, whereas histones may mediate pathological effects also *via* TLR2. Nucleic acids released from necrotic hepatocytes likewise display a strong potential to aggravate APAP intoxication by action on TLR sensors. Specifically, DNA targeting TLR9 (22, 23) and RNA targeting leukocytic or hepatocyte TLR3 (24) contribute to hepatic injury. A pivotal role for TLR9 was confirmed by pharmacological application of a small-molecule TLR9 antagonist to mice undergoing APAP intoxication (25). Among DAMPs sensed independently from the TLR system, ATP and uric acid stand out. Both can aggravate APAP-induced ALI (26–28) supposedly by action on the inflammasome, a multiprotein complex consisting of interleukin (IL)-1β/IL-18-activating caspase-1. In this scenario, ATP binds to purinergic $P2X_7$ receptors on monocytes/macrophages (including Kupffer cells) at the hepatic microenvironment connecting to cellular K^+-efflux and subsequent inflammasome activation. After being released by dying cells or derived from degradation of nucleic acids, uric acid, on the other hand, is taken up in the form of crystals that directly activate inflammasomes and, thus, IL-1β/IL-18-dependent inflammation (29, 30).

Although, at first sight, it appears obvious that innate immunity and sterile inflammation amplify pathogenesis of APAP-induced ALI, this topic in fact is controversially discussed. For example, while several studies indicate a pathological role of TLR4 (21, 31, 32), a recent report did not endorse a disease-promoting but rather a protective function of myeloid TLR4 signaling in APAP-related liver damage. Interestingly, deleterious action of RAGE and TLR9 was confirmed in this same study (33). Another recent report disputed a pathogenic role for $P2X_7$ receptors in APAP intoxication (34). While some parameters, such as mice characteristics, including their microbiome (35), or APAP dosage cannot be fully ruled out as sources of discrepancies, those divergent observations may actually reflect janus-faced functions of innate immunity and sterile inflammation in APAP-induced ALI – aggravating tissue damage, likely at an early phase of disease, but simultaneously displaying the strong potential to initiate and perpetuate hepatic repair and regeneration (36). The unique ability of the liver to, upon injury, most efficiently initiate processes aiming at preservation of organ function is driven by initial hepatocyte hypertrophy (increase in size) and an adjacent proliferative phase enabling compensatory hyperplasia (37, 38). Notably, if hepatic damage stays below a pathological threshold, the regenerative capacity of the liver can fully restore organ function in response to APAP (39, 40).

The remarkable fact of quite divergent observations concerning the role of sterile inflammation in APAP-induced ALI particularly applies to the function of nuclear factor-κB (NF-κB)-activating inflammatory cytokines that are induced adjacent–distal to innate sensing. This specifically holds true for prototypic IL-1 and tumor necrosis factor (TNF)-α (41), both produced during APAP-induced ALI (22, 24, 42–46). Whereas aggravation of disease by pretreatment of mice with recombinant TNFα is undisputed (47), modulation of endogenous TNFα biological activity, as achieved by administering neutralizing

antibodies or by investigating TNF receptor-1-deficient mice, resulted in quite heterogeneous outcome. Reports, on the one hand, demonstrate amelioration of APAP-induced toxicity by application of anti-TNFα antibodies (48, 49) or by using TNF receptor-1-deficient mice (49). By contrast, other reports observed either no effect of TNFα-neutralization (50, 51) or even aggravation of disease as detected using TNF receptor-1-deficient mice (52, 53). Those latter two studies actually indicate a tissue-protective function of endogenous TNFα in APAP-induced ALI that coincides with enhanced hepatocyte proliferation and activation of the key pro-regenerative transcription factor signal transducer and activator of transcription (STAT)-3 (54). To assess the role of IL-1 in APAP-toxicity is likewise puzzling. Either pathogenic functions (22, 55), no major role (42), or protection (56) by IL-1 has been put on record. The view that inflammatory cytokines, such as IL-1 and TNFα, have the potential to actually promote liver regeneration was recently extended to the IL-1 family member IL-36γ (57, 58). In fact, administration of IL-36 receptor antagonist and thus blockage of IL-36 biological activity during APAP-induced ALI impairs recovery in the late phase of intoxication (58). Interestingly, IL-36 mediating tissue protection likewise applies to intestinal healing (59, 60).

Altogether, current data support the concept that sterile inflammation and accompanied NF-κB-activating cytokines may promote hepatic repair and regeneration particularly in the later phase of APAP toxicity thereby affecting disease outcome (12). It is tempting to speculate that secondary induction of STAT3-activating cytokines, alike IL-6 (61), by NF-κB-activating cytokines essentially contributes to the vital process of restoring liver homeostasis in response to APAP.

STAT3 IN HEPATIC REPAIR AND REGENERATION

STAT-3 is a member of the STAT family of transcription factors, which exerts decisive and context-dependent functions in inflammation, tissue survival, and carcinogenesis. Those characteristically include promotion of anti-apoptosis, proliferation, and stress resistance. Efficient activation of STAT3 is achieved under the influence of specific cytokines displaying janus-kinase signaling but also by selected growth factors, among others epidermal and platelet-derived growth factor. Phosporylation at Tyr705 is regarded a hallmark of STAT3 activation that couples to protein dimerization, nuclear translocation, and regulation of gene expression (62–65). In addition, phosphorylation at Ser727 (63, 65) and/or protein acetylation (66) amplify, whereas S-nitrosylation at Cys259 (67) and/or protein sumoylation (68) curb STAT3 activity. As already alluded to, enforcing hepatocyte anti-apoptosis and proliferation is key to liver protection by STAT3. Those functions are achieved by upregulation of gene products pivotally involved in cell fate decisions, among others, B-cell lymphoma-extra large (*bclxL*), myeloid cell leukemia-1 (*mcl1*), or *survivin* mediating anti-apoptosis as well as c-myc (*myc*), cyclin B1/D1 (*ccnb1*/*ccnd1*), or cyclin-dependent kinase-2 (*cdc2*) mediating proliferation (62, 63).

The albumin-promoter was used to generate hepatocyte-specific conditional STAT3 knockout mice in order to address the role of STAT3 in this cell type. Experiments revealed that hepatocyte STAT3 is, to a substantial part, accountable for hepatocyte proliferation and liver regeneration after murine partial hepatectomy. Notably, hepatocyte c-myc expression is aberrant and its inducibility retarded in aforementioned conditional STAT3-deficient mice undergoing this procedure (69). In a study using a similar approach, hepatocyte STAT3 was functionless regarding parameters of liver injury evaluated in early APAP-induced ALI. However, analysis in that study was performed only 6 h after APAP administration and, thus, in the initial phase of intoxication (70) – leaving open the question of STAT3 functions during the later repair/regeneration phase. Notably, increased STAT3 activation in murine liver is still detectable 24 h after APAP application (71); the same holds true for expression of STAT3-activating IL-6 (72, 73).

TISSUE PROTECTION BY STAT3-ACTIVATING CYTOKINES AS DETECTED IN APAP-INDUCED ALI: IL-6, IL-11, IL-13, AND IL-22 – AND IL-10

Whereas the role of NF-κB-activating cytokines in APAP-induced ALI appears complex and bewildering, STAT3-activating cytokines capable of directly targeting hepatocytes must be regarded as major drivers of liver regeneration. Those include IL-6, IL-11, IL-13, and IL-22.

Interleukin-6 is the flagship of a family of cytokines operating through transmembrane gp130 as signal transducing unit thereby coupling to activation of STAT transcription factors, in case of IL-6 foremost STAT3 (61). This also applies to its cytokine sibling IL-11 (74). Both, IL-6 and IL-11, are upregulated during initial hepatocyte injury and stay elevated, along with activated STAT3 (71), in the repair/regeneration phase at 24 h after APAP administration to mice (72, 73). In fact, protection by endogenous IL-6 was observed early on. Particularly in time-wise advanced disease 24 h (73) or 48 h (75) after APAP administration, IL-6-deficient mice endure aggravated toxicity associated with low production of hepatocyte-associated proliferating cell nuclear antigen (PCNA) and weakened liver macrophage inflammatory protein-2 (MIP-2) expression (75). Both, PCNA and MIP-2 (76), are key parameters of hepatocyte proliferation under the influence of APAP. Those observations suggest impaired recovery upon lack of IL-6. As expected, treatment of IL-6 deficient mice with recombinant IL-6 attenuated retardation of repair and regeneration (75). It is noteworthy that hepatocytes are among the few non-leukocytic cell types expressing functional IL-6 receptors and, thus, allow classical IL-6 signaling. Despite this fact, recent data indicate that trans-signaling by soluble IL-6R/IL-6 complexes (61, 77) is essential for the function of this cytokine during APAP-induced ALI (78). In fact, specific blockage of IL-6 trans-signaling by sgp130Fc (77) substantially exacerbated disease (78); whereas pretreatment of mice with hyper-IL-6 (77), a recombinant agent specifically activating trans-signaling, ameliorated APAP toxicity – albeit to a more moderate degree (78).

Interleukin-11 is a further STAT3-activating member of the IL-6 family directly targeting hepatocytes (74, 79) and, in stark contrast to IL-6, is efficiently expressed by inflamed/stressed hepatocytes under the influence of APAP (71). Autocrine or paracrine action may, thus, ensure high local IL-11 bioactivity that likely feeds back on the course of APAP-induced ALI. Notably, early data already revealed amelioration of murine APAP-toxicity by recombinant human IL-11 (80). This observation has been corroborated recently. A super-active modification of human IL-11 indeed enhanced protective hepatocyte compensatory proliferation in diseased mice. In female IL-11 receptor-deficient mice (*IL11Ra*$^{-/-}$) aggravated toxicity and diminished hepatocyte proliferation indicate a significant role for endogenous IL-11 during APAP-induced ALI. Interestingly, this observation does not apply to male *IL11Ra*$^{-/-}$ mice that actually display compensatory augmentation of supposedly protective IL-6 (71). It should be emphasized that female mice, compared to males, generally display reduced sensitivity toward APAP that is connected to an enhanced capability in females to restore hepatocyte mitochondrial glutathione levels (81).

Interleukin-13 is renowned as key Th2 cytokine that, however, is produced by various cell types of foremost leukocytic origin. By binding to its heterodimeric IL-4Rα/IL-13Rα1 receptor complex, IL-13 activates STAT3 (along with STAT6) in even more diverse cell types (82), including murine hepatocytes (83). Elevated systemic levels of IL-13 are well-detectable at 4, 12, and 24 h after APAP administration to mice (51, 84). Notably, exacerbated disease connecting to IL-13 blockage by neutralizing antibodies or lack of bioactivity in knockout mice firmly indicates protection by this cytokine during APAP intoxication (51). Activation of hepatocyte STAT3 by IL-13 (83) suggests direct protective action during APAP-induced ALI. However, upregulation of the supposedly detrimental IL-12/IFNγ-axis (72) during intoxication in IL-13-deficient mice (51) additionally implicates macrophage-addressing immunomodulatory functions of IL-13 (85). Whether administration of surplus recombinant IL-13 can ameliorate APAP-induced ALI has, to the best of our knowledge, not been investigated.

Interleukin-22 is mainly a lymphocyte-derived member of the IL-10 cytokine family that gained significant attention due to tissue-protective properties largely mediated by STAT3 activation specifically in epithelial (-like) cells, including hepatocytes. Accordingly, IL-22 mediates favorable effects in various preclinical disease models affecting biological barriers at the lung, intestine, and liver. Notably, IL-22 generally does not activate leukocytes (86–88). A single dosage of recombinant IL-22 is actually sufficient to ameliorate APAP toxicity in mice (43, 70). Protection by IL-22 is dependent on STAT3 (70), does not involve modulation of APAP-metabolizing cytochrome P450 enzymes but is associated with increased compensatory hepatocyte proliferation (43). The role of endogenous IL-22 during APAP-induced ALI has, to the best of our knowledge, not been investigated. Recently, a functionally relevant aspect of IL-22 biology attracted attention. A potent synergism between the IFN signaling system and IL-22 concerning activation of STAT1 was identified in human colon carcinoma cells, HepG2 hepatoma cells, and primary keratinocytes on a biochemical level (89). In contrast to STAT3, STAT1 (e.g., activated by IFNγ) promotes cell death, inhibits proliferation, is generally considered pro-inflammatory (90), and pathogenic in APAP-induced ALI (72). This regulatory path has recently been extended to murine *in vivo* pathology during viral infection (91) or graft-versus-host disease (92) and may affect the function of IL-22 not only under conditions of overt IFN production but likewise in the context of typ I IFN immunotherapy (90).

Interleukin-10 is a mainly leukocyte-derived protein that drops out of the list of aforementioned STAT3-activating cytokines because it is supposed to act foremost on leukocytic cells. IL-10 serves as principal deactivator of T cells and in particular of mononuclear phagocytes thereby modulating in STAT3-dependent manner inflammatory processes (93, 94) and holding in check potentially poisonous mediators, among others inducible nitric oxide (NO) synthase (95) -derived NO (84). During APAP intoxication systemic levels and hepatic expression of IL-10 increase. Notably, IL-10 deficient mice display enhanced sensitivity to APAP-induced ALI, which is unrelated to APAP metabolism but detectable on the level of serum ALT, morphologically, and by analysis of mortality rates (84). Since STAT3 can principally drive IL-10 expression (93, 96, 97), this regulatory path may possibly contribute to tissue protection by STAT3-activating cytokines, such as IL-6. However, the therapeutic potential of surplus exogenously applied IL-10 in APAP-induced ALI seems limited as administration of the recombinant cytokine failed to protect diseased mice (50).

Although this review focuses on cytokines efficiently targeting hepatocytes, it is important to note that modulation of murine APAP-induced ALI by endogenous IL-10 (and IL-13) unequivocally indicate a pivotal function of STAT3 also in myeloid cells (monocytes/macrophages/Kupffer cells) for determining course and outcome of APAP intoxication. Besides addressing STAT3 in hepatocytes, hepatic myeloid STAT3, thus, certainly is a further promising target for development of therapeutic strategies aiming at APAP-induced ALI.

TRANSLATIONAL/THERAPEUTIC IMPLICATIONS AND CONCLUSIONS

Administering hepatocyte STAT3-activating cytokines emerges from preclinical studies as encouraging pharmacological strategy that aims at hard-to-treat patients with APAP-induced ALI. Moreover, APAP intoxication may serve as paradigm for a whole group of injury-driven acute inflammatory liver diseases independent on the nature of the initiating insult (54). To translate preclinical knowledge to clinical application is, however, in some cases advantaged in others complicated by specific properties ascribed to aforementioned cytokines.

Although IL-6 displays significant tissue-protective characteristics, administration of the recombinant cytokine to patients is hampered by its pro-inflammatory effects especially on lymphocyte biology (61). Specifically, IL-6 promotes human IL-17 production and associated Th17 differentiation (98). Notably, IL-17 is pathogenic in murine APAP-induced ALI (19). As IL-6-induced Th17 associates with compromised Treg functions (99–101) and,

if applicable, pathological antibody production (102), current knowledge supports serious concerns that administration of IL-6 to patients may initiate or enhance autoimmune inflammation.

Mice undergoing APAP toxicity did not benefit from exogenously provided IL-10 (50), which may likewise apply to human intoxication. As chief deactivator of leukocytes (93, 94), recombinant IL-10 should actually interfere with desired production of potentially pro-regenerative factors. In fact, this has been demonstrated for IL-6 and TNFα production by human Kupffer cells under the influence of active TLR4 signaling (103).

Interleukin-11 and IL-22 are functionally related cytokines that efficiently activate hepatocyte STAT3 signaling and associated downstream gene expression. Both have been described to mediate tissue protection at host/environment interfaces, in particular at the digestive tract. For example, IL-11 (104, 105) and IL-22 (106, 107) display protective properties in *Citrobacter rodentium*-driven infectious as well as in trinitrobenzene sulfonic acid chemically induced experimental colitis. Accordingly, use of both cytokines is discussed, albeit with caution, for the treatment of inflammatory bowel diseases (108). Aforementioned liver protective properties of IL-11 and IL-22 are not restricted to APAP intoxication. Among others, experimental hepatic disorders mediated by reperfusion injury (109, 110) or administration of either carbon tetrachloride (111, 112) or concanavalin A (112, 113) likewise exposed beneficial effects of both cytokines. Although the role of IL-11 and IL-22 in liver repair/regeneration should primarily be mediated by STAT3, it must be stressed that activation of MAPK- and PI3K/Akt-pathways may support IL-11/IL-22 action in this context (74, 86, 114). The feasibility of recombinant IL-11 therapy for the treatment of ALI is emphasized by its relatively favorable compatibility in clinical trials (115). In fact, recombinant IL-11 has been approved for the treatment of severe thrombocytopenia by the US Food and Drug Administration (79). At dosages showing biological activity, F-652 [Generon (Shanghai) Corporation Ltd.], an IL-22-like biopharmaceutical agent consisting of a human IL-22-Fc-fusion protein (116), is likewise reported to have a good safety profile as determined in a phase I study in healthy volunteers (http://www.businesswire.com/news/home/20151123005647/en/Generon-Collaborating-Mayo-Clinic-Initiate-Phase-IIa).

Pharmacotherapy of APAP-induced ALI must obviously be successful when initiated hours after ingestion. Whereas most studies assessed prophylactic treatment, therapeutic application has been investigated in a translational setting for IL-22. Specifically, when administered 2 h after APAP together with suboptimal N-acetylcysteine dosing, recombinant IL-22 improved murine intoxication (43). Notably, IL-22 application 2 h post-APAP is after the drop of cellular glutathione as well as the onset of APAP-adduct formation and liver necrosis (43, 117). More studies on treatment timing, however, are needed before experimental models can be translated to clinical intoxication.

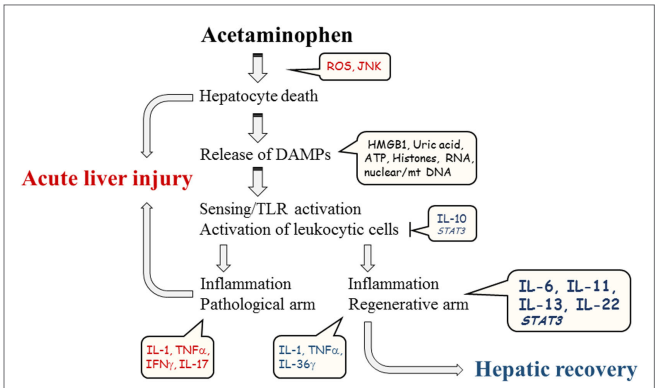

FIGURE 1 | Schematic illustration of major parameters contributing to course of experimental APAP-induced ALI.

Altogether, APAP-induced ALI is a complex disorder determined by the extent of initial hepatotoxicity, by the nature of adjacent sterile inflammation, and by the actual regenerative potential of the liver at the time of injury (**Figure 1**). Preclinical data suggest that providing recombinant STAT3-activating cytokines directly targeting hepatocytes, especially IL-11 and IL-22, may evolve as additional novel pro-regenerative therapeutic option in hard-to-treat patients where standard therapy with N-acetylcysteine alone falls short. Notably, the benefit of focused short-term application of IL-11 or IL-22 in acute disorders, such as APAP-induced ALI, should likely outweigh the inherent danger of these cytokines to promote in the long run tumor growth (74, 86, 118), which has been detected for IL-22 and hepatocellular carcinoma patients (118–120).

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The author confirms being the sole contributor of this work and approved it for publication.

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Potential of PEGylated Toll-Like Receptor 7 Ligands for Controlling Inflammation and Functional Changes in Mouse Models of Asthma and Silicosis

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Prior investigations show that signaling activation through pattern recognition receptors can directly impact a number of inflammatory lung diseases. While toll-like receptor (TLR) 7 agonists have raised interest for their ability to inhibit allergen-induced pathological changes in experimental asthma conditions, the putative benefit of this treatment is limited by adverse effects. Our aim was to evaluate the therapeutic potential of two PEGylated purine-like compounds, TMX-302 and TMX-306, characterized by TLR7 partial agonistic activity; therefore, the compounds are expected to induce lower local and systemic adverse reactions. *In vitro* approaches and translation to murine models of obstructive and restrictive lung diseases were explored. *In vitro* studies with human PBMCs showed that both TMX-302 and TMX-306 marginally affects cytokine production as compared with equivalent concentrations of the TLR7 full agonist, TMX-202. The PEGylated compounds did not induce monocyte-derived DC maturation or B cell proliferation, differently from what observed after stimulation with TMX-202. Impact of PEGylated ligands on lung function and inflammatory changes was studied in animal models of acute lung injury, asthma, and silicosis following Lipopolysaccharide (LPS), allergen (ovalbumin), and silica inhalation, respectively. Subcutaneous injection of TMX-302 prevented LPS- and allergen-induced airway hyper-reactivity (AHR), leukocyte infiltration, and production of pro-inflammatory cytokines in the lung. However, intranasal instillation of TMX-302 led to neutrophil infiltration and failed to prevent allergen-induced AHR, despite inhibiting leukocyte counts in the BAL. Aerosolized TMX-306 given prophylactically, but not therapeutically, inhibited pivotal asthma features. Interventional treatment with intranasal instillation of TMX-306 significantly reduced the pulmonary fibrogranulomatous response and the number of silica particles in lung interstitial space in silicotic mice. These findings highlight the potential of TMX-306, emphasizing its value in drug development for lung diseases, and particularly silicosis.

Keywords: TLR7, PEGylated ligands, asthma, ALI, silicosis

INTRODUCTION

Inhalation of environmental airborne substances in the form of aeroallergens and particulate matter can result in allergic respiratory dysfunctions and pneumoconiosis, such as asthma and silicosis, respectively (1–4). Moreover, air pollutants may impact on allergic airway-related morbidity and mortality (5, 6). While asthma is among those diseases with an obstructive pulmonary function pattern (7), silicosis is pathologically characterized as a fibrogranulomatous disease with a restrictive pulmonary function profile (8). Both asthma and silicosis are highly prevalent worldwide, cause elevated socioeconomic costs, and can be fatal (3). Steroidal anti-inflammatory agent combined to bronchodilators is the best way of controlling asthma currently, but glucocorticoid resistance and adverse effects limit the efficacy of this treatment (2). The situation is even more alarming in case of silicosis, since no proper therapy is available (9).

Pulmonary inflammation is central in these diseases. In asthma, inflammation is driven by the adaptive arm of host immunity and reflects an aberrant immune response specifically against otherwise harmless environmental factors in genetically predisposed individuals (2). Yet, the basis of the inflammatory response mounted following exposure to occupational air pollutants, such as crystalline silica particles, remains poorly understood (9). What is well established for both diseases, however, is the crucial role displayed by the airway wall as an immune-privileged innate barrier in which interdigitated dendritic cells (DCs), with the help of macrophages and epithelial cells, sense and respond to antigens, pollutant particles, and infectious microorganisms that traffic into the lung (10, 11). Upon intrusion, pathogens are recognized by pattern recognition receptors, and among them scavenger receptors (12–14) and Toll-like receptors (TLRs) play a pivotal role (15–18).

Toll-like receptors are located on the plasma membrane (TLR1, 2, 4, 5, 6, and 10) and endosomal/lisosomal vesicles (TLR3, 7, 8, 9, 11, 12, and 13) of immune cells (17). In humans, TLR1–10 are expressed by DCs, monocytes, macrophages, T cells, and B cells, and play important roles in their task of sensing and responding to "danger signals" presented by pathogens (19). All TLRs signal through the myeloid differentiation factor 88 (MyD88) adapter, with the exception of TLR3 that depends on TIR domain-containing adaptor inducing IFNβ (TRIF) (20–22). No detectable signaling occurs through TLRs in the absence of MyD88 and TRIF (20, 21). Within the airways, activation of TLR7 decreases adaptive response in a mechanism associated with upregulation of type 1 interferon (15, 16, 23). Moreover, TLR7 rapidly relaxes human airways (24).

Several synthetic small TLR7 agonists have been studied for their potential use to treat asthma (25, 26), but with limited benefits because of local and systemic inflammatory reactions (27–29). More recent investigations indicate that the conjugation of TLR7 ligands with a 6-unit oligo-ethylene glycol (PEG) moiety showed potential to inhibit the course of inflammatory diseases, such as diabetes, with retained TLR7 specificity and attenuated non-specific inflammation (30, 31). We hypothesize that PEGylated TLR7 partial agonists have potential to control not only allergic inflammatory lung diseases but also pneumoconiosis, with minimized adverse side effects. Hence, this study was undertaken in order to assess the impact of treatment with two PEGylated purine-like compounds, TMX-302 and TMX-306, upon pulmonary inflammation and function changes triggered by Lipopolysaccharide (LPS), allergen, or silica particles in mice. TMX-302 was previously planned by linking a specific TLR7 ligand, 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (1V136), to a six-unit polyethylene glycol (PEG) (30), whereas, TMX-306 resulted from a molecular simplification of TMX-302.

MATERIALS AND METHODS

Reagents

The PEGylated TLR7 ligands TMX-302 [3-(1-(1-(4-((6-amino-8-hydroxy-2-(2-methoxyethoxy)-9H-purin-9-yl)methyl) phenyl)-1-oxo-5,8,11,14,17,20-hexaoxa-2-azadocosan-22-yl)-1H-1,2,3-triazol-4-yl) propanoic acid] (MW = 791) and TMX-306 [1-(4-((6-amino-2-(2-methoxyethoxy)-8-oxo-7H-purin-9(8H)-yl)methyl)phenyl)-1-oxo-5,8,11,14,17,20-hexaoxa-2-azatricosan-23-oic acid] (MW = 695), as well as the reference compound TMX-202 [2-(4-((6-Amino-2-(2-methoxyethoxy)-8-oxo-7H-purin-9(8H)-yl) methyl) benzamido) ethyl 2,3-Bis (dodecanoyloxy) propyl phosphate] (MW = 920) were provided by Telormedix (Bioggio, CH). LPS (strain *Escherichia coli* O127:B8), ovalbumin (OVA) (grade V), and crystalline silica particles were purchased from Sigma Chemical, St. Louis, MO, USA. All the others were obtained as further indicated.

Animals

Male A/J and Swiss Webster mice (18–20 g) were obtained from the Oswaldo Cruz Foundation breeding colony and housed in standard laboratory cages at 22–25°C, on a 12 h light/dark cycle, and fed with food and water *ad libitum*. All the protocols involving animal care and use were approved by the Animal Ethics Committee of the Oswaldo Cruz Institute (License L-030/2015). C57BL/6 mice, purchased from Harlan (Italy), were maintained in the animal facility of the Institute for Research in Biomedicine, and all procedures were approved by the veterinarian authorities from the local committee (Comitato Etico Cantonale del Ticino, Switzerland) with the authorization number TI17/2010.

Human Cell Isolation and Stimulation

Human PBMCs were isolated from buffy-coats (Central Laboratory of the Swiss Red Cross, Basel, Switzerland) using Ficoll-hypaque density centrifugation. Monocytes were isolated from PBMCs using CD14-magnetic beads (Miltenyi Biotec) and monocyte-derived dendritic cells (mo-DCs) generated *in vitro*, as previously described (32). Briefly, $CD14^+$ cells were cultured for 4 days in complete medium supplemented with GM-CSF and IL-4 to induce mo-DCs differentiation. At day 4, medium was completely washed out, and cells were treated for 24 h with 10 μM of the indicated PEGylated TLR7 agonists or with vehicle only. At day 5, mo-DCs supernatant was collected to quantify the production of pro-inflammatory cytokines by stimulated cells, and mo-DCs were stained for maturation markers. Total PBMCs were cultured for 24 h in complete medium, supplemented with different concentrations of PEGylated TLR7 agonists (1 or 10 μM),

and the supernatant was collected to quantify pro-inflammatory cytokine release.

Cytokines Detection in Cell Supernatant

The concentration of IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF in the supernatant of human PBMCs and human mo-DCs was determined using the BD™ cytometric bead array (CBA) (human inflammatory cytokine kit – 551811, BD Biosciences), according to manufacturer's instructions.

Flow Cytometric Analysis

For surface staining of human specimens, cell suspensions were incubated with the appropriate combination of the following monoclonal antibodies: CD19-PC5 (J3-119, Beckman Coulter), CD80-Brilliant Violet 421™ (2D10, BioLegend®), CD83-APC (HB15e, BioLegend®), CD86-APC (IT2.2, BioLegend®), and HLA-DR-V500 (G46-6, BD Horizon™). For surface staining of mouse specimens, cell suspensions were incubated with Fc-blocking antibody (Bioxcell, 2.4G2) to avoid unspecific Fc-Receptor binding. After washing, the cells were incubated with the appropriate combination of the following antibodies: CD11b-PECy7 (M1/70, BioLegend®), Ly6G-PE (1A8, BD Biosciences), Ly6C-Biotin (AL-21, BD Biosciences), CD3-APC(17A2, BioLegend®), CD45R-B220-PerCP-Cy5.5 (RA3-6B2, eBiosciences). To detect anti-Ly6C-Biotin antibody binding, cells were subsequently stained with streptavidin-FITC (Dako). The samples were acquired with BD LSRFortessa (BD Biosciences), and results were analyzed with FlowJo software (Tree Star, Inc.).

B Lymphocytes Proliferation

Total human PBMCs were stained with 5 μM carboxyfluorescein succinimidyl ester (CFSE), using CellTrace™ CFSE Cell Proliferation kit (Invitrogen Molecular Probes, C34554), according to manufacturer's instructions, and cultured in complete medium supplemented with 10 μM of the indicated PEGylated TLR7 agonists for 4 days. At day 4, B lymphocytes were stained with an anti-CD19 antibody, and proliferation was assessed by CFSE dilution.

Leukocyte Mobilization from Bone Marrow

Age- and sex-matched mice (6–8 weeks) were randomly assigned to two groups, which were injected intraperitoneally with sterile saline ($n = 5$) or 200 nmoles of TMX-306 ($n = 5$). After 24 h, mice were sacrificed, and cellular suspension was obtained by blood, spleen, and bone marrow and analyzed by flow cytometry.

LPS-Induced Inflammation

A/J mice were exposed to a single dose of LPS (25 μg/mouse) or phosphate buffered solution (PBS) by intranasal route. The analyses were performed 24 h after stimulation. Treatment with TMX-302 (500 nmoles/mouse) was performed subcutaneously, 1 and 24 h prior to LPS exposure (**Figure 1A**).

Ovalbumin-Induced Inflammation

A/J mice were sensitized, subcutaneously, with 50 μg of OVA and 5 mg of aluminum hydroxide dissolved in 0.2 mL PBS. Two protocols to induce the allergic response were used. For the short-term protocol (**Figure 1B**), mice were sensitized at day 0, boosted on day 14, and then exposed to intranasal OVA (25 μg/mouse), or sterile PBS, at days 19 and 20 (33). Treated animals received TMX-302 either by subcutaneous (500 nmoles/mouse) or intranasal route (65 nmoles/mouse), 1 and 24 h before allergen challenge. In another set of experiments, mice were exposed to aerosol of TMX-306 (2 and 6 mg/mL) also following protocol B (**Figure 1B**). For the interventional treatment, mice were sensitized at day 0, boosted at day 7, and then challenged with OVA (50 μg/mouse), or PBS, days 14, 21, 28, and 35 post-sensitization (4). Treated animals received intranasal TMX-306 (70 nmoles/mouse) or oral dexamethasone (1 mg/kg), at days 26 and 33 postsensitization. In both protocols, the analyses were performed 24 h after the last OVA challenge (**Figure 1C**).

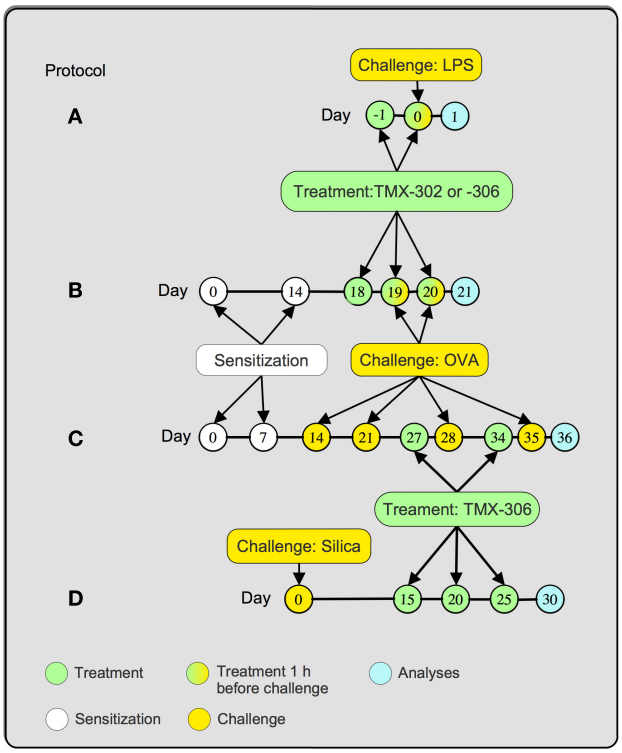

FIGURE 1 | Treatment protocols. Mice were treated 1 and 24 h before LPS (25 μg/25 μL), and analyses were made 24 h after provocation **(A)**. Mice were sensitized at days 0 and 14 and subjected to two consecutive daily injections of OVA (25 μg/25 μL) at days 19 and 20 postsensitization. TMX-302 was given subcutaneously (500 nmoles/mouse) or intranasally (65 nmoles/mouse), 1 and 24 h before OVA. TMX-306 was aerosolized (2 and 6 mg/mL, 30 min) under the same conditions. Analyses occurred 24 h after the last challenge **(B)**. Mice were sensitized at days 0 and 7 and subjected to a series of four provocations with OVA (50 μg/25 μL) at days 14, 21, 28, and 35 postsensitization. TMX-306 (70 nmoles/mouse) was intranasally injected at days 27 and 34 **(C)**. Mice were challenged with crystalline silica particles at day 0 and subjected to three treatments with TMX-306 (70 nmoles/mouse) at days 15, 20, and 25 **(D)**. Analyses were performed at day 30 post-silica.

Silica-Induced Chronic Inflammation

Swiss Webster mice were exposed to crystalline silica particles (10 mg/mouse) (size 0.5–10 μm) or sterile PBS as control (1).

The interventional treatment with TMX-306 (70 nmoles/mouse) was given at days 15, 20, and 25, and the analyses performed at day 30 after silica instillation (**Figure 1D**).

Invasive Assessment of Respiratory Mechanics

Mice were anesthetized with nembutal (60 mg/kg), and neuromuscular activity was blocked with bromide pancuronium (1 mg/kg). Lung resistance (cmH_2O s/mL) and elastance (mL/cmH_2O) were assessed in tracheostomized and mechanically ventilated mice using a FinePointe R/C Buxco Platform (Buxco® Electronics, Sharon, CT, USA) (1).

Bronchoalveolar Lavage

Airways were lavaged by a polyethylene cannula, inserted into the trachea, with two consecutive instillations of 0.75 mL of PBS containing 10 mM of EDTA. Bronchoalveolar Lavage (BAL) was centrifuged at 300 × *g* for 10 min at 4°C, and the cell pellet was resuspended in 0.25 mL of PBS for leukocyte enumeration. Total cells were counted in Neubauer chamber by means of light microscopy, after dilution of samples in Turk solution. The differential analysis was performed in cytocentrifuged smears stained for identification of mononuclear cells, neutrophils, and eosinophils by May-Grunwald-Giemsa under an oil immersion objective and light microscope (BX51, Olympus) (34).

Histology

The left lung was removed, fixed in Milloning buffer solution (pH 7.4) with 4% paraformaldehyde to preserve pulmonary architecture. Briefly, samples were embedded in paraffin (Sigma-Aldrich), and 4 μm-thick sections were cut and stained with hematoxylin and eosin for quantification of granuloma area, Picrosirius for collagen fibers and Sirius Red (pH 10.2) for neutrophils and eosinophils counted in the parenchyma and in peribronchiolar area, respectively. Slides were scanned with 3DHISTECH–Pannoramic MIDI whole slide scanner (capture with a 20× objective lens) and the resulting images analyzed with CaseViewer 3.3, Pannoramic Viewer 1.15.4, and HistoQuant softwares (3DHISTECH). Silica crystals were analyzed, in 15 independent fields, with a light microscope (Olympus BX50) equipped with polarizing attachment for detecting birefringent particles and Image-Pro Plus Version 4.

Immunohistochemistry

Left lung samples were examined for immunohistochemical localization of TGF-β using paraffin-embedded sections. Primary anti-TGF-β1/2/3 (sc-7892) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Secondary antibody HAF008 was conjugated with horseradish peroxidase (HRP) and obtained from R&D Systems (Minneapolis, MN, USA). In negative controls, primary antibody was omitted, and tissues were incubated with antibody diluent only. To improve visualization of the primary label, slides were counterstained with Mayer's Hematoxylin (Lillie's modification) as previously described (1). The slides were scanned with 3DHISTECH–Pannoramic MIDI and quantified as previously reported (1).

Cytokine Quantification

Murine TNF-α, MIP-1α/CXCL-3, MIP-2/CXCL-2, IL-6, and eotaxin-2 levels were measured in the right lung tissue samples, which were homogenized in PBS containing 0.05% Triton X-100 and a protease inhibitor cocktail (Hoffmann-La Roche, Basel, Switzerland). Samples were quantified using commercially available ELISA kits (DuoSet system, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The results were expressed as picograms of cytokine per right lung.

Protein Quantification

Total protein levels were measured by Bradford technique. Right lung tissue samples were homogenized in PBS 1 mL with Triton X-100 (0.1%), containing protease inhibitor COMPLETE (Hoffmann-La Roche Ltd., Switzerland). The results were expressed as micrograms of protein per right lung.

Statistical Analysis

Statistical analyzes were performed using GraphPad Prism Software, version 5.0 (USA). For *in vitro* experiments on human PBMCs, the analyses were performed with repeated measures two-way ANOVA followed by Tukey's multiple comparison. For *in vivo* experiments, the analyses were done with one-way ANOVA followed by the Student–Newman–Keuls test or two-way ANOVA with *post hoc* Bonferroni correction. Statistical differences were considered significant if p values were less than 0.05 (two-tailed tests).

RESULTS

PEGylated TLR7 Partial Agonists Activity of Human Leukocytes

The effects of the two 1V136 PEGylated derivatives, TMX-302 and TMX-306, on human PBMCs and on the maturation of mo-DCs, were assessed in cells from healthy donors. *In vitro*, none of the PEGylated compounds resulted to be toxic on PBMCs; with no relevant apoptosis induced after over night exposure to TMX-302 or TMX-306 (10 μM, data not shown). Both TMX-302 and TMX-306 induced a minimal cytokine production as compared to the TLR7 full agonist TMX-202 at 1 μM. When the compounds were used at high concentration (10 μM), we observed production only of IL–6 and IL–8 that was comparable to the one observed using TMX-202 (**Figure 2**). None of the tested PEGylated TLR7 partial agonists induced relevant cytokine production by mo-DCs (**Figure 3A**) or mo-DCs maturation (**Figure 3B**). Moreover, cytofluorimetric analysis on human PBMCs labeled with CFSE and incubated with the different compounds showed that the PEGylated partial agonists do not induce B cell proliferation (**Figure 3C**).

Effect of TMX-302 on LPS-Induced Inflammation and Airway Hyper-Reactivity

Subcutaneous pre-treatment with TMX-302 (500 nmoles/mouse given twice), 1 and 24 h before LPS (protocol A,

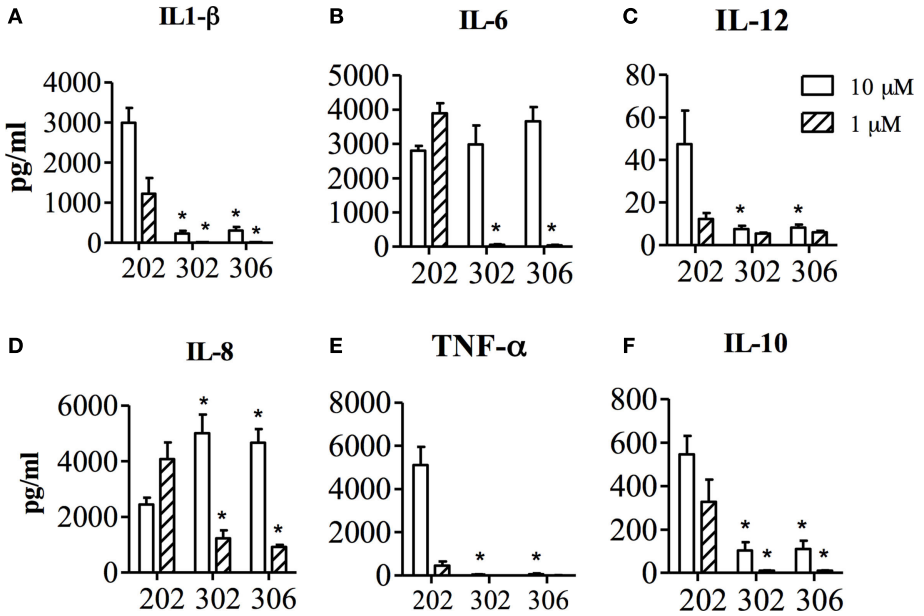

FIGURE 2 | Concentration of IL1-β **(A)**, IL-6 **(B)**, IL-12 **(C)**, IL-8 **(D)**, TNF-α **(E)** and IL-10 **(F)** in the supernatant of human PBMCs, after 24 h stimulation with TMX-202, TMX-302 or TMX-306 at 1 or 10 μM. Data are presented as mean ± SEM from $n = 6$.

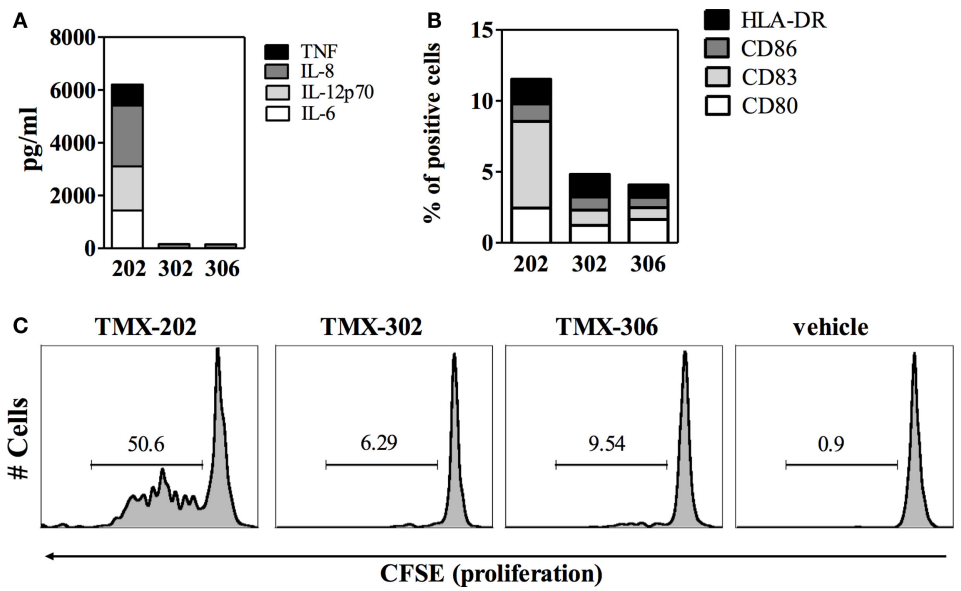

FIGURE 3 | **Effect of PEGylated TLR7-agonists on maturation of mo-DCs or proliferation of B cells**. **(A)** Concentration of the indicated inflammatory cytokines in the supernatant of mo-DCs ($n = 6$), after 24 h stimulation with TMX-202, TMX-302, or TMX-306 at 10 μM. Data are presented as mean values. **(B)** Frequency of mo-DCs expressing the maturation markers CD80, CD83, CD86, and HLA-DR, after 24 h stimulation with TMX-202, TMX-302, or TMX-306 at 10 μM. Data are presented as mean values of three independent experiments. Dotted lines in **(A,B)** represent the amount of cytokines or expression of maturation markers in the absence of TLR7 stimulation. **(C)** B cell proliferation measured by CFSE dilutions after 4 days in culture with TMX-202, TMX-302, or TMX-306 at 10 μM. One representative plot out of three experiments performed with cells from different donors is shown.

Figure 1), reduced the increased lung elastance response noted in mice challenged with LPS (**Figure 4A**). As expected, LPS also caused protein extravasation (**Figure 4B**) and augmentation in total leukocyte levels, as indicated by enumeration of these cells in BAL effluents (**Figure 4C**) and lung histological sections (**Figures 4D,G**), in comparison to the respective negative

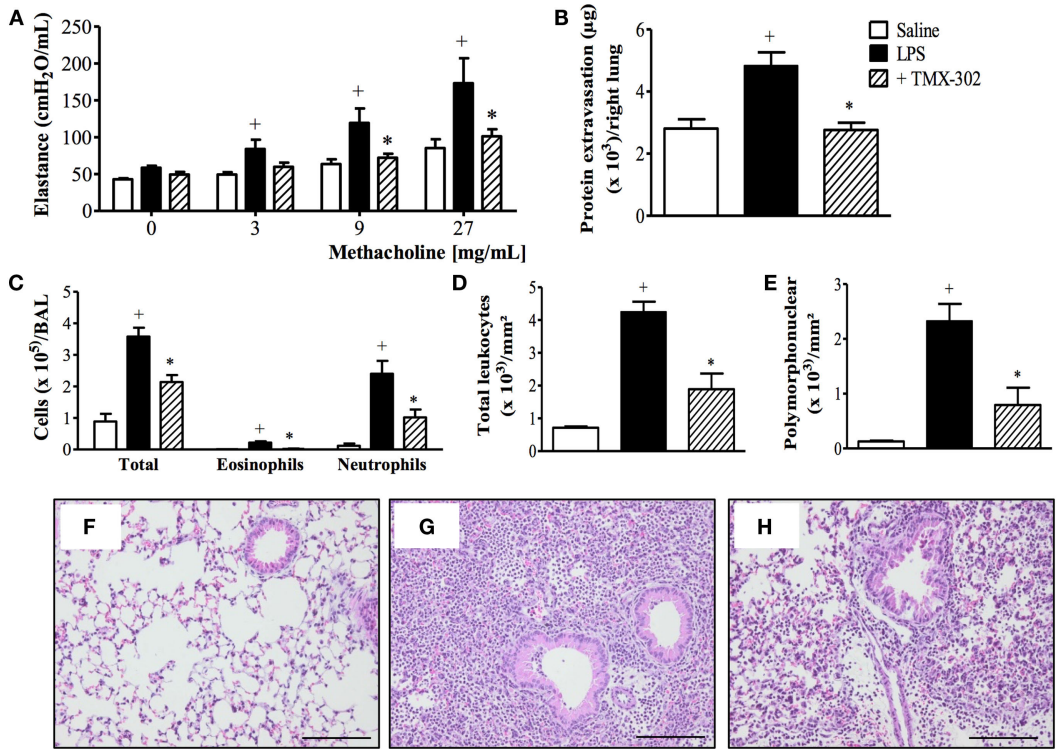

FIGURE 4 | Effect of subcutaneous treatment with TMX-302 on LPS-induced inflammation in the lung of mice. **(A)** Lung elastance; **(B)** protein exudation; **(C)** leukocytes in BAL; **(D)** total leukocytes; and **(E)** polymorphonuclear in the lung tissue. Histological sections of lungs from animals instilled with **(F)** PBS, **(G)** LPS (25 µg/mouse), and **(H)** LPS and treated with TMX-302 (500 nmoles/mouse). Slides were stained with H&E. Scale bar = 200 µm. All the analyses were made 24 h after LPS stimulation. Values represent mean ± SEM from at least six animals. $^{+}p < 0.05$ as compared to PBS-challenged group; $^{*}p < 0.05$ as compared to LPS-challenged group.

TABLE 1 | Effect of TMX-302 on cytokine/chemokine generation in the lung tissue of LPS-stimulated mice.

Cytokine (pg/lung tissue)	PBS	LPS	LPS + TMX-302
TNF-α	97.5 ± 8.7	290.2 ± 49.2^{+}	178.9 ± 29.1*
MIP-1α	314.5 ± 48.6	1276.1 ± 277.7^{+}	816.9 ± 120.6*
IL-6	280.2 ± 57.7	1131.1 ± 285.0^{+}	777.6 ± 122.2

TMX-302 (500 nmoles/mouse) was given 1 h before LPS (25 µg), and the analyses were performed 24 h after LPS. Values represent the mean ± SEM from at least six animals.

$^{+}p < 0.05$ vs. PBS-challenged group.

$^{*}p < 0.05$ vs. LPS-challenged group.

controls (**Figures 4C,F**). Neutrophils were the predominant leukocyte subtype found in the bronchoalveolar space (**Figure 4C**) and lung parenchyma (**Figures 4E,G**). All these changes were significantly inhibited by the subcutaneous pre-treatment with TMX-302 (**Figures 4B–E,H**). Quantification of pro-inflammatory cytokines and chemokines in lung homogenates in response to LPS revealed increased levels of TNF-α, MIP-1α, and IL-6, all of which appeared inhibited by TMX-302 though, in case of IL-6, the 40% blockade was not statistically significant (**Table 1**).

Effect of TMX-302 on Allergen-Induced Inflammation and Airway Hyper-Reactivity

Confirming previous reports (33), OVA intranasal challenge of sensitized mice exacerbated both airway resistance (**Figure 5A**) and lung elastance responses (**Figure 5B**) to inhaled methacholine (3–27 mg/mL). Increased levels of leukocytes, mainly eosinophils, were detected in the BAL fluid (**Figure 5C**) as compared to control mice challenged with PBS. The same figures show that the pre-treatment with TMX-302 (500 nmoles/mouse, subcutaneous) (protocol B, **Figure 1**) prevented allergen-induced airway hyper-reactivity (AHR) and eosinophilic leukocyte accumulation (**Figures 5A–C**).

When the systemic prophylactic was replaced by the local prophylactic treatment with TMX-302 (65 nmoles/mouse, intranasal instillation) (protocol B, **Figure 1**), no more protective effect was seen for allergen-induced increased airway resistance (**Figure 6A**) and lung elastance (**Figure 6B**) in response to methacholine. Moreover, TMX-302 itself caused AHR in naive mice (**Figures 6A,B**). A significant blockade of the OVA-induced eosinophilic, but not neutrophilic infiltration, was apparent following TMX-302, as observed in BAL samples (**Figure 6C**) and lung tissue samples (**Figures 6D,E**). Moreover, the nasal instillation of TMX-302 (65 nmoles/mouse) in naive mice led

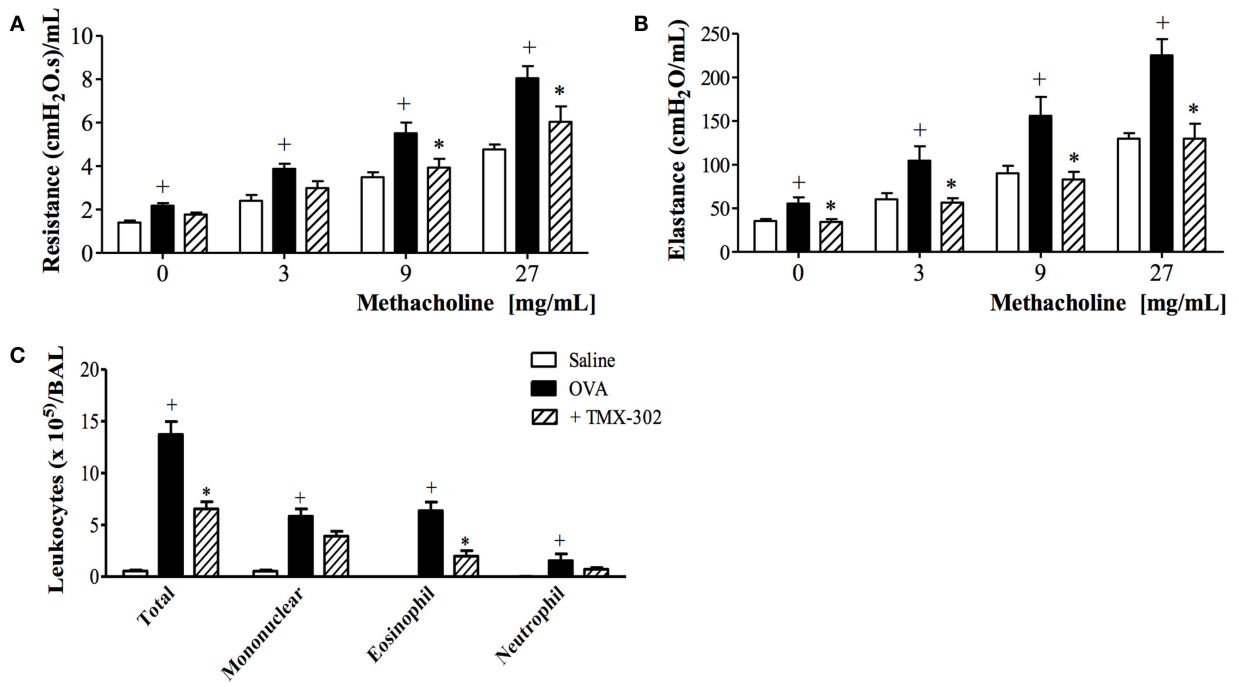

FIGURE 5 | Effect of subcutaneous treatment with TMX-302 on OVA-induced inflammation in the lung of mice. Lung function: **(A)** resistance and **(B)** elastance **(C)** total leukocytes in BAL. Animals were sensitized on days 0 and 7 and then challenged with OVA (25 μg/mouse) or PBS, on days 19 and 20. Treatment with TMX-302 (500 nmoles/mouse, subcutaneous) was given 1 h before each OVA challenge, and analyses were performed 24 h after the last stimulation. Values represent mean ± SEM from at least six animals. $^{+}p < 0.05$ as compared to PBS-challenged group; $^{*}p < 0.05$ as compared to OVA-challenged group.

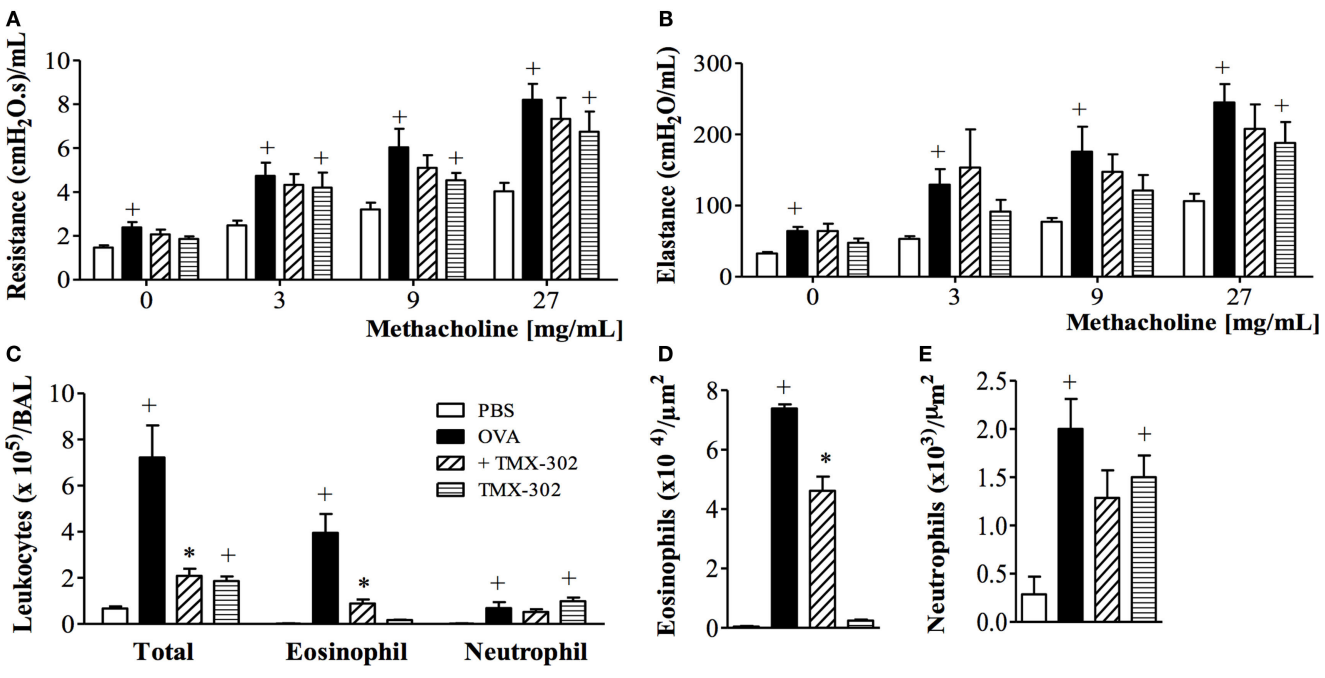

FIGURE 6 | Effect of intranasal treatment with TMX-302 on OVA-induced inflammation in the lung of mice. Lung function: **(A)** resistance; **(B)** elastance, **(C)** total leukocytes in BAL, **(D)** tissue eosinophil numbers and **(E)** tissue neutrophil numbers. Animals were sensitized on days 0 and 7 and then challenged with OVA (25 μg/mouse) or PBS on days 19 and 20. Treatment with TMX-302 (65 nmoles/mouse, intranasal) was given 1 h before each OVA challenge, and analyses were performed 24 h after the last stimulation. Values represent mean ± SEM from at least six animals. $^{+}p < 0.05$ as compared to PBS-challenged group; $^{*}p < 0.05$ as compared to OVA-challenged group.

to neutrophil accumulations in the BAL fluid (**Figure 6C**) and, particularly, in the lung parenchyma (**Figure 6E**). As shown in **Table 2**, the intranasal instillation of TMX-302, despite inhibiting OVA-induced TNF-α and MIP-2, clearly up-regulated OVA-induced MIP-1-α, and caused itself significant elevation of MIP-1α, MIP-2, and TNF-α levels in lung homogenates.

Effect of TMX-306 on Allergen-Induced Inflammation and Airway Hyper-Reactivity

Once the topical administration, through intranasal instillation of TMX-302, was ineffective upon asthmatic changes and caused adverse events, the effects of the parent compound TMX-306 were investigated. As shown in **Figure 7**, the prophylactic treatment with aerosolized TMX-306 (6 mg/mL) (protocol B, **Figure 1**) prevented allergen-induced AHR, in respect to airway resistance (**Figure 7A**) and lung elastance (**Figure 7B**), as well as the infiltration of eosinophils in the peribronchiolar zone (**Figure 7C**). When aerosolized at 2 mg/mL, TMX-306 prevented allergen-induced AHR but not eosinophilic infiltration (**Figures 7A–C**).

We next assessed the effectiveness of TMX-306 (70 nmoles/mouse, intranasal) on ongoing asthmatic changes according to the protocol C (**Figure 1**). Contrarily to dexamethasone, TMX-306 failed to reduce AHR (**Figures 8A,B**) and peribronchiolar eosinophilic infiltration (**Figure 8C**) caused by OVA challenge, suggesting the lack of beneficial effects for the therapeutic treatment with TMX-306 on allergen-induced pathological changes, under the conditions applied.

TABLE 2 | Effect of TMX-302 on cytokine/chemokine generation in the lung tissue of allergen-stimulated mice.

Cytokine (pg/lung tissue)	PBS	OVA	OVA + TMX-302	TMX-302
MIP-1-α	117.2 ± 76.4	937.5 ± 169^{+}	1672.7 ± 295.1*	605.4 ± 84.9^{+}
MIP-2	275.6 ± 70.6	665.6 ± 66.8^{+}	243.9 ± 68.7*	934.9 ± 126.8^{+}
TNF-α	88.7 ± 7.6	138.9 ± 11.0^{+}	110.1 ± 8.7*	132.1 ± 11.2^{+}

The analyses were performed 24 h after ovalbumin provocation, and values represent the mean ± SEM from at least six animals.
$^{+}p < 0.05$ vs. PBS-challenge group.
$^{*}p < 0.05$ vs. OVA-challenge group.

Effect of TMX-306 on Silica-Induced Inflammatory, Fibrotic, and Respiratory Changes

Initially, in order to assess if the PEGylated compound TMX-306 would impact *per se* cell mobilization from the bone marrow as well as subsequent distribution in blood circulation and spleen, TMX-306 was injected intraperitoneally in mice. The results obtained indicate that this PEGylated analogue, at the dose of 200 nmoles/mouse, does not affect cell mobilization and compartmentalization in wild type mice (Figure S1 in Supplementary Material).

The next step was the evaluation of the effect of TMX-306 on experimental silicosis, which was performed in accordance to protocol D (**Figure 1**) and prior investigations (1, 35). Based

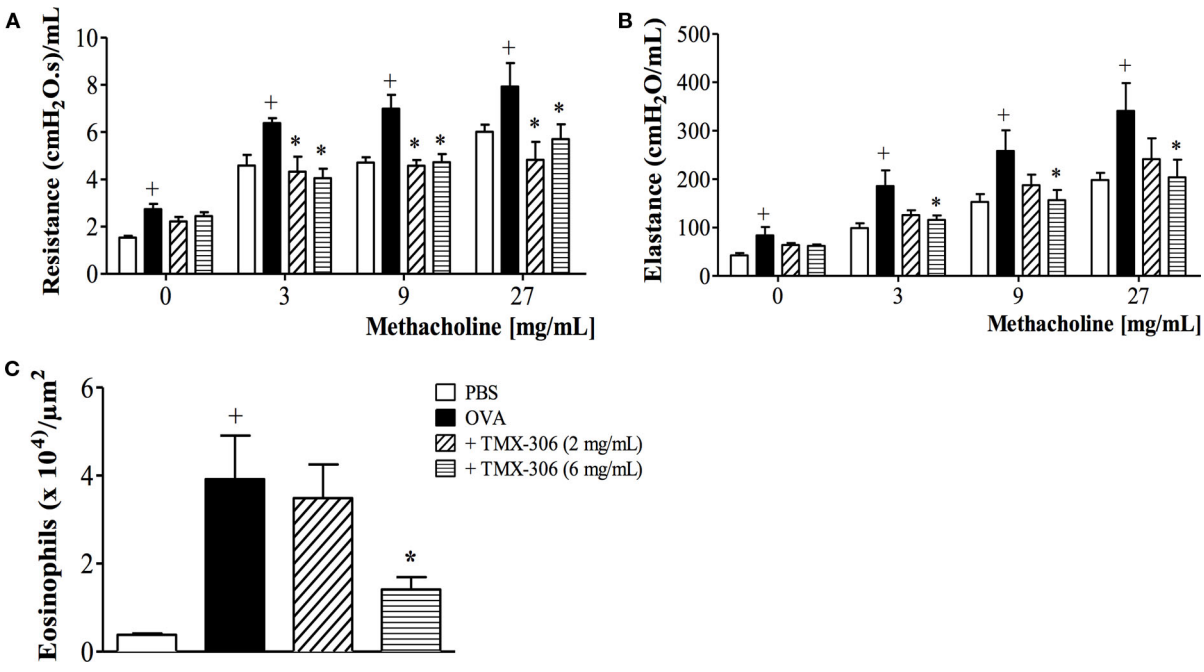

FIGURE 7 | Effect of aerosol treatment with TMX-306 on OVA-induced inflammation in the lung of mice. Lung function: **(A)** resistance, **(B)** elastance, and **(C)** peribronchiolar eosinophil infiltration. Animals were sensitized on days 0 and 7 and then challenged with OVA (25 μg/mouse) or PBS on days 19 and 20. Treatment with TMX-306 (2 and 6 mg/mL, aerosol) was given 1 h before each OVA challenge, and analyses were performed 24 h after the last stimulation. Values represent mean ± SEM from at least six animals. $^{+}p < 0.05$ as compared to PBS-challenged group; $^{*}p < 0.05$ as compared to OVA-challenged group.

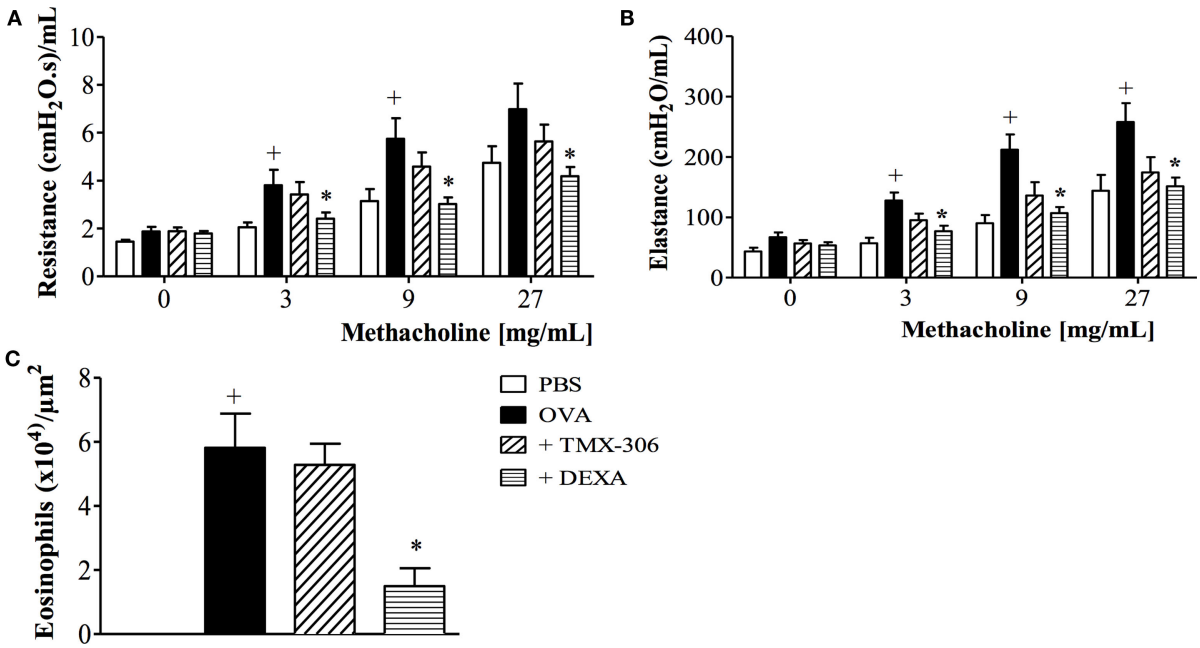

FIGURE 8 | Effect of intranasal treatment with TMX-306 on OVA-induced inflammation in the lung of mice. Lung function: **(A)** resistance; **(B)** elastance, and **(C)** peribronchiolar eosinophil infiltration. Animals were sensitized on days 0 and 7 and then challenged with OVA (25 µg/mouse) or PBS on days 14, 21, 28, and 35. Animals were treated with TMX-306 (70 nmoles/mouse, intranasal) or dexamethasone (1 mg/Kg, oral) on days 26 and 22, 1 h before OVA challenge, and analyses performed 24 h after the last challenge. Values represent mean ± SEM from at least six animals. $^{+}p < 0.05$ as compared to PBS-challenged group; $^{*}p < 0.05$ as compared to OVA-challenged group.

on the histologic analyzes of lung sections stained with H&E for assessment of granuloma (**Figure 9**, upper panels) and Picrosirius red for evaluation of fibrotic lesions (**Figure 9**, lower panels), it became clear that, compared to mice exposed to PBS (**Figure 9A**), those exposed to silica particles (**Figure 9B**) reacted with an intense granulomatous response, which occupied about 40% of the left pulmonary lobe 30 days postprovocation (**Figure 9G**). Moreover, a dense area of collagen fiber deposition appeared distributed in those spaces occupied by granuloma in silicotic mice (**Figure 9E**). Remarkably, both granuloma and fibrotic lesions caused by silica inhalation were clearly inhibited by the treatment with TMX-306 (70 nmoles/mouse), carried out at days 15, 20, and 25 after silica provocation (**Figures 9G,H**, respectively).

Using immunohistochemistry technique based on anti-TGF-β staining, the quantitative assessment of expression of TGF-β under conditions of exposure to PBS, silica particles, or silica plus TMX-306 revealed significant increase in lung tissue levels of IL-TGF-β in samples recovered from mice exposed to silica particles (**Figure 10B**), as compared to those exposed to PBS (**Figure 10A**). Given through nasal instillation in the regime mentioned before, TMX-306 inhibited silica-induced production of TGFβ (**Figure 10C**). The quantitative data are shown in **Figure 10D**.

Effect of TMX-306 on Silica Particle Diffusion in Lung Parenchyma

In this study, we used a light microscope equipped with polarizing filters when examining lung tissue sections from mice exposed to silica particles. Having lung section from mice exposed to PBS as reference (**Figure 11A**), our findings confirmed the presence of numerous crystals of silica in sections from mice exposed to the particles, seen as small bluish bright specks, distributed throughout lung areas mainly those occupied by granuloma (**Figure 11B**). The amount of silica particles present in the interstitial space appeared significantly reduced in mice treated with TMX-306 (70 nmoles/mouse) (**Figure 11C**). Quantitative data are shown in **Figure 11D**.

DISCUSSION

Toll-like receptors play a crucial role in sensing and responding to respirable "dangerous triggers" including allergens and ambient pollutant particles, which may lead to asthma and pneumoconiosis (13, 36). Synthetic low molecular weight TLR7 agonists, including 1V136 and others, have been shown to down-regulate immune responses during inflammatory conditions (25, 26, 37, 38). Additionally, PEGylation improves bioavailability and safety of these ligands (30, 39). The overall purpose of this study was to access the effects of 1V136 PEGylated derivatives on pulmonary inflammatory and functional changes caused by three distinct classes of pathogens, including LPS, allergen, and crystalline silica particles.

Our experiments revealed that both PEGylated derivatives TMX-302 and TMX-306 presented a marginal pro-inflammatory response, yielding a minimal production of inflammatory cytokines in human PBMCs, no mo-DC maturation or B cell

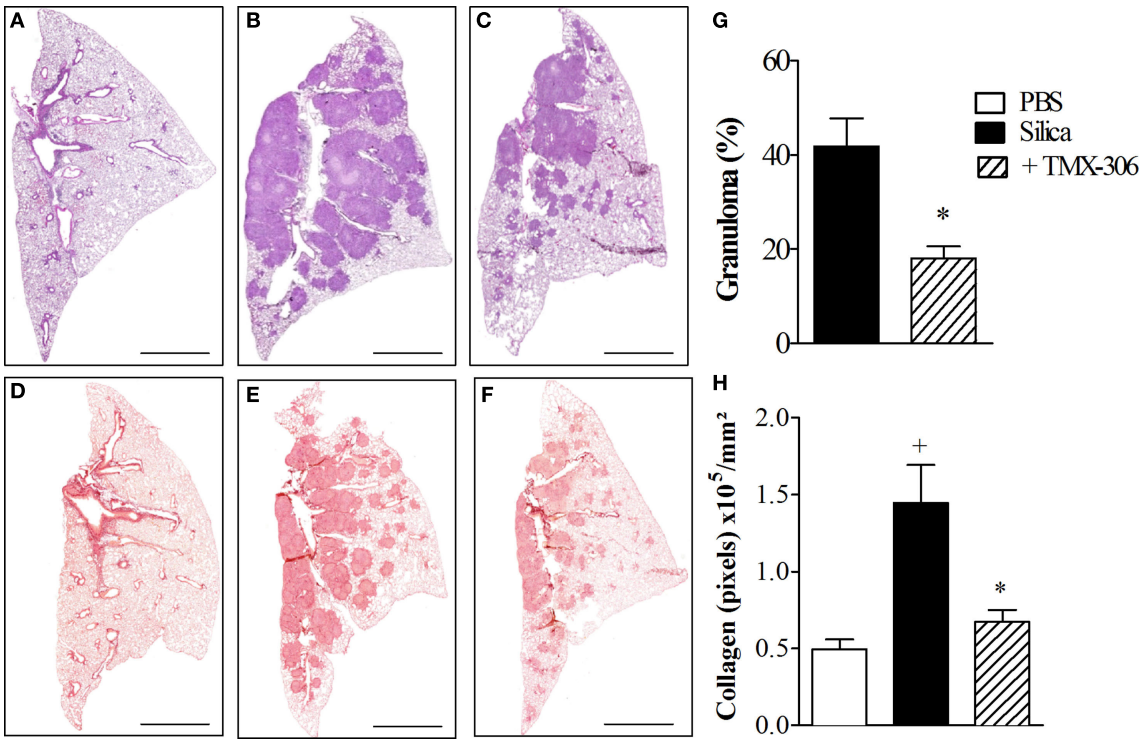

FIGURE 9 | Effect of intranasal treatment with TMX-306 on granuloma formation (upper panels) and collagen deposition (lower panels) in silica-challenged mice. Histological sections of mouse lungs on day 30 after silica challenge **(B,E)** and treated with TMX-306 (70 nmoles/mouse, intranasal) **(C,F)**. Animals instilled with PBS were used as controls **(A,D)**. Animals were treated with TMX-306 (70 nmoles/mouse, intranasal) days 15, 20, and 25 post-silica. Morphometric analyzes are seen in **(G)** granuloma area and **(H)** collagen deposition. Slides were stained with H&E (upper panels) and Picrosirius red (lower panels). Scale bars, 200 μm. Values represent mean ± SEM from at least five animals. $^+P < 0.05$ as compared to PBS-challenged group; $^*P < 0.05$ as compared to silica-challenged group.

proliferative responses, differently from the TLR7 full agonist used for comparison (TMX-202). In *in vivo* settings, subcutaneous pre-treatment with TMX-302 prevented LPS- and allergen-induced lung inflammation and AHR, while its topical administration failed to prevent allergen-induced AHR, and caused itself neutrophil infiltration, in parallel with cytokine and chemokine generation. Administered topically, TMX-306 prevented allergen-induced asthma changes, but did not modify them as given therapeutically. In contrast, in the silicosis model, interventional treatment with TMX-306 significantly reduced the pulmonary fibrogranulomatous response following crystallized silica particle inhalation in mice. Altogether, these studies highlight the putative value of TMX-306 in drug development for silicosis.

As candidates to anti-inflammatory therapy, TLR7 ligands should ideally be able to push the innate system to a state of tolerance, with minimal pro-inflammatory effects. Actually, the safe therapeutic use of TLR7 agonists has been proved to be a difficult task because of the cytokine release syndrome and pharmacokinetic limitations (16). Prior investigations have demonstrated that the conjugation of these ligands to polysaccharides, serum albumin or PEG widely improved their pharmacokinetics and pharmacodynamics properties (30, 39, 40). Accordingly, in our experiments assessing cytokine production by human PBMCs *in vitro*, the PEGylated compounds TMX-302 and TMX-306, at 1 μM, were clearly less active than the reference compound TMX-202, producing marginal amounts of IL-1β, IL-6, IL-12p70, IL-8, or TNF. TMX-302 and TMX-306 at 10 μM promoted only IL-6 and IL-8 release from human PBMCs, but failed to induce pro-inflammatory cytokine release by mo-DC and their maturation as well as B lymphocyte proliferation, which are hallmarks of TLR7 activation (41), supporting the interpretation that the two PEGylated 1V136 derivatives are indeed suitable molecules for further *in vivo* investigations.

The immune-regulatory effects of TLR ligands, in more ample sense, are heterogeneous and complex. For instance, inhalation of the TLR4 agonist LPS exacerbates silica-induced fibrogranulomatous pulmonary dysfunction in mice (42), but can attenuate ongoing asthmatic changes following long-term exposure of mice to allergen challenge (43). While investigating the therapeutic potential of TMX-302 and TMX-306, we have explored well-established murine models of acute lung injury (ALI) (44), asthma (4, 33, 34), and silicosis (1, 35). ALI is a severe clinical problem associated with elevated rates of morbidity and mortality worldwide (45). Triggered by LPS, a component of the cell wall of

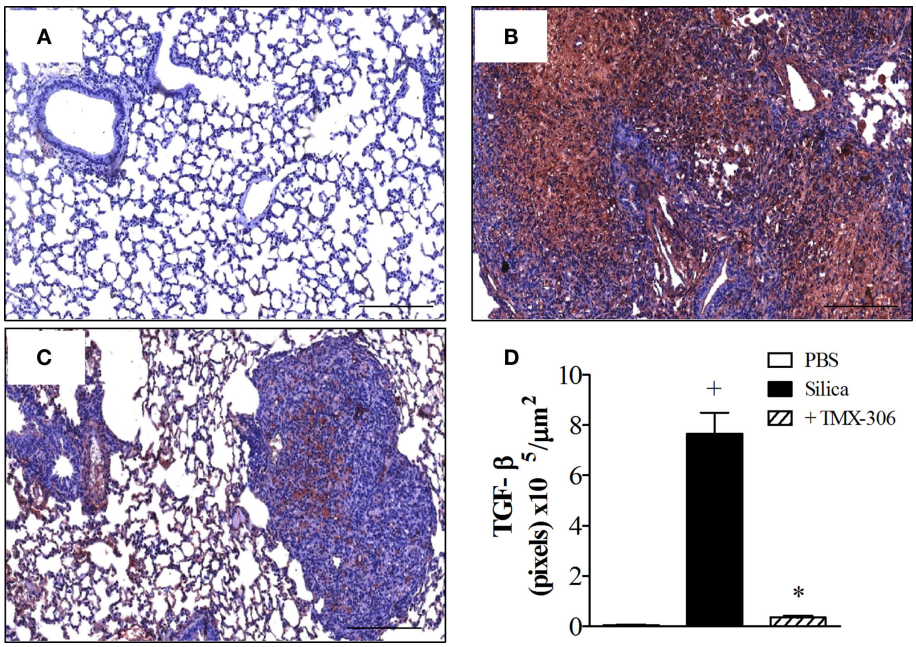

FIGURE 10 | Effect of intranasal treatment with TMX-306 on TGF-β production in the lung tissue of silica-challenged mice. Samples were analyzed in animals instilled with PBS **(A)**, silica (10 mg/mouse) **(B)**, and silica treated with TMX-306 (70 nmoles/mouse, intranasal) **(C)** 30 days after silica challenge. Treatment with TMX-306 was performed at days 15, 20, and 25 post-silica. Quantitative analyses are seen in **(D)**. Values represent mean ± SEM from at least six animals. $^{+}P < 0.05$ as compared to PBS-challenged group; $^{*}P < 0.05$ as compared to silica-challenged group.

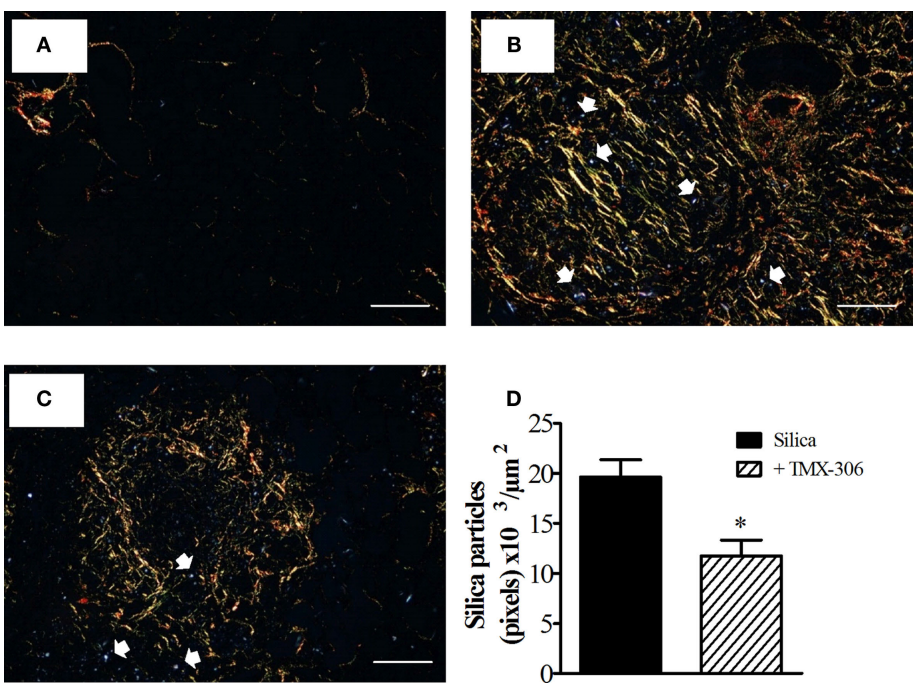

FIGURE 11 | TMX-306 reduces silica particles in lung tissue. Quantitative evaluation of silica particles was assessed in animals instilled with PBS **(A)**, silica (10 mg/mouse) **(B)**, and silica treated with TMX-306 (70 nmoles/mouse, intranasal) **(C)**, 30 days after silica challenge. Quantitative analyses are seen in **(D)**. Picrosirius red-stained sections were evaluated by light and polarized microscopy, respectively. Arrows indicate silica particles. Scale bar, 200 μm. Values represent mean ± SEM from at least seven animals. $^{*}P < 0.05$ as compared to silica-challenged group.

gram-negative bacteria, ALI is marked by pulmonary neutrophilic leukocyte infiltration, disruption of the endothelial and alveolar epithelial barrier, lung edema, and severe hypoxemia (45). We found that under conditions of intranasal instillation of LPS, the systemic pre-treatment with TMX-302 (500 nmoles/mouse), given subcutaneously 24 and 1 h before provocation, clearly inhibited the lung inflammatory changes, including the massive leukocyte accumulation in the bronchoalveolar space and lung parenchyma, plasma leakage and AHR-noted 24 h postchallenge. The protective effect of TMX-302 concerning leukocyte changes and respiratory function might be explained by the blockade of pro-inflammatory cytokine production, including TNF-α, IL-6 and MIP-1α, as attested by measurements done in lung tissue samples. Recent studies emphasize the involvement of the adapter molecule MyD88 in the LPS-TLR4 signaling pathway followed by activation of NF-kappa B in ALI (46, 47), though this is still a debatable issue (48). However, since MyD88 is a pivotal adapter to all TLRs, except TLR3, the possibility does exist that TMX-302 is acting here through induction of a tolerogenic mechanism accounted for by induction of cross-desensitization between TLR7 and TLR4 signaling pathways.

Differently from TLR4, TLR7 selectively detects viral RNA, leading to activation of T-helper cell (Th1) immune response and viral clearance. Several pieces of evidence suggest that TLR activation are protective against T-helper cell (Th2)-mediated diseases, such as asthma, possibly by interfering with the Th1 versus Th2 immune balance (15, 16, 23). Additionally, activation of TLR7 expressed on $CD4^+$ T cells and airway nerves can lead to anergy (49) and respiratory smooth muscle relaxation (24), respectively. TLR7 has also raised interest in asthma because respiratory viruses are a major cause of exacerbations. Notably, virus clearance depends on TLR-mediated Th1 response, which is down-regulated in the asthma Th2 microenvironment (50). We observed here that the systemic pre-treatment with TMX-302, given subcutaneously, prevented allergen-induced eosinophilic inflammatory infiltration and AHR in a short-term murine model of asthma. Nevertheless, the mucosal administration of TMX-302 (65 nmoles/mouse, intranasal instillation), 24 and 1 h before provocation, failed to prevent allergen-induced AHR though inhibiting the accumulation of eosinophils in the bronchoalveolar space and tissue samples. Actually, TMX-302 itself induced a significant increase in the levels of peribronchial neutrophils, in parallel with significant increase in the lung tissue production of MIP-1α, MIP-2, and TNFα, suggesting that caution in its use is required. We then decided to assess the effect of the analogue TMX-306, which is a molecular simplification of TMX-302 resulting from deletion of the triazol ring. It is relevant to mention that the intraperitoneal injection of 200 nmoles TMX-306 did not cause statistically significant changes in the number of monocytes, macrophages, neutrophils, T cells, or B cells in the bone marrow, spleen, and blood circulation in mice (Figure S1 in Supplementary Material). TMX-306 (500 nmoles/kg, subcutaneous) inhibited LPS-induced AHR as well as neutrophilic infiltration in samples of bronchoalvelar lavage (data not shown). Because the lungs provide a suitable route for aerosol delivery, we also tested the prophylactic treatment with aerosolized TMX-306 (6 mg/mL), which turned out to be effective in this model, preventing both eosinophilic infiltration and AHR triggered by allergen challenge. However, using a long-term model of asthma, TMX-306 (70 nmoles/mouse, intranasal) failed to modify the ongoing pathological changes triggered by allergen provocation, whereas the glucocorticoid agent dexamethasone was shown to be clearly active. These findings might suggest that, despite inhibiting LPS- and allergen-induced lung inflammation and AHR as given prophylactically, TMX-306 would not be as effective in modifying already established asthmatic changes following therapeutic administration.

In this study, we also investigated whether or not the pharmacological modulation of TLR7 with TMX-306 could be used to reduce silicosis. Remarkably contrasting with the lack of efficacy of the interventional TMX-306 treatment on experimental asthma, the therapeutic intranasal administration of this compound clearly attenuated lung inflammation, granuloma formation, fibrosis, and the functional respiratory changes noted in response to silica particles. Current thinking is that the pathogenesis of silicosis is largely attributed to the direct damage by silica particles to alternatively activated alveolar macrophages and DCs, engaged in the recognition, uptake, and clearance of silica particles and other environmental particulate matters that traffic in the lung (14, 51). When this barrier is broken, free silica crystals accumulate in the interstitial space and are taken up by M1 macrophages, which play a crucial role in promoting a state of pulmonary inflammation that evolves to granuloma formation and overlaps with fibrogenic areas in humans and animal models (14). Indeed, stronger lung inflammatory and fibrotic responses were noted in mice genetically deficient in macrophage receptors with collagenous structure (MARCO), a scavenger receptor deeply involved in the sense and uptake of crystalline silica by alveolar macrophages (52). This result gives support to the interpretation that M2 alveolar macrophages account for by the clearance while M1 interstitial macrophages drive the silica-induced inflammatory response (14). Remarkably, scavenger receptor class A type I/II (CD204) null mice fail to develop fibrosis following silica inhalation, in spite of keeping inflammation, suggesting that the CD204 are crucial to the development of fibrosis and resolution of inflammation (12).

In our experimental conditions, mice exposed to a single intranasal instillation of 10 mg of crystalized silica particles reacted with a progressive lung granulomatous response, which reach about 40% of the lung area 30 days postchallenge, as evidenced by scanned histopathological images of lung sections. In parallel, we found a marked increase in the levels of collagen fiber deposition, evidenced by Picrosirius red staining, which appeared densely distributed throughout areas occupied by granuloma, as previously reported (1, 35). These changes were clearly reversed following intranasal instillation of TMX-306, given at days 15, 20, and 25 post-silica. Furthermore, the extension of lung area occupied by granuloma appeared reduced in about 60% whereas a reduction of 95% was noted in the amount of deposited collagen. TMX-306 also almost abolished the levels of the pro-fibrotic TGF-β generated in response to silica exposure. Crystalline silica particles diffract light and appear as bright bluish specks against the dark tissue background, and can be seen under light microscope equipped with polarizing filters

(53). Using this technique, we could detect reduction of about 40% in the number of crystals of silica dispersed in the lung interstitial space, strongly suggesting that TMX-306, by reducing areas of granuloma and fibrosis, is probably favoring silica particle mobility and clearance from the lung through lymphatic draining. In fact, prior investigations have demonstrated that silica particles can be drained by the lymphatic system to the lymph nodes, particularly under conditions of effective anti-silicosis therapy (1).

Apart from the distinct impact on the silica-induced fibrotic response, our findings are very much in line with those ones reported by Re and collaborators (54). These authors found a significant reduction of lung inflammation and granuloma formation in MyD88-KO mice after silica, giving support to the interpretation that MyD88-related innate immunity is crucial in silicosis. In addition, like ours, their results showed a robust reduction in the fibrotic response to silica in granuloma areas, with the difference that increased levels of silica-induced collagen deposition were detected throughout the lung parenchymal area (54), suggesting that inflammatory and fibrotic responses to silica can be uncoupled, which did not happen in our experimental conditions.

We cannot exclude the possibility that the PEGylated compounds are simply less potent than the full agonist TMX-202, without any necessary impact on their efficacy on the TLR7. In addition, contrary to TMX-202, the intravenous administration of PEGylated analogues such as TMX-302 and others (200 nmoles/kg) failed to alter the systemic baseline levels of pro-inflammatory cytokines such as TNF-α and IL-6 (data not shown). Further studies and more accurate toxicological investigations should be carried out on candidate compounds such as TMX-302 and TMX-306.

In conclusion, these findings provide a comprehensive comparison of the anti-inflammatory effectiveness of two PEGylated TLR7 partial agonists, concerning distinct lung pathological conditions and several routes of administration. The results suggest that the putative clinical application of TMX-302 in lung disorders should be examined with caution because of its direct pro-inflammatory effects. Moreover, in this context, TMX-306 seems to be comparatively more effective and safer, deserving further investigations in drug development particularly for silicosis.

AUTHOR CONTRIBUTIONS

TF, LM, RB, AA, AF, MB, and VC – acquisition and analysis of data, illustration, revision for intellectual content and final approval. MU – contributions to design of the work, acquisition and analysis of data, drafting of the work, and supervision and final approval; RM – contributions to design of the work, drafting of the manuscript, revising it critically for important intellectual content, supervision, and final approval. AB – revising it critically for important intellectual content, supervision, and final approval. PS – contributions to design of the work, illustration, critical revision, supervision, and final approval; MM – design of the work, illustration, drafting of the manuscript, supervision, and final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00095

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